ACTA PHYSIOLOGICA SCANDINAVICA

SUPPLEMENTUM 321

FROM THE INSTITUTE OF NEUROPHYSIOLOGY
HAELSEN INSTITUTE UNIVERSITY OF COPENHAGEN, DENMARK

INTRA AND EXTRACELLULAR POTENTIAL FIELDS ACTIVE NERVE AND MUSCLE FIBRES

A physico-mathematical analysis of different models

BY

POUL ROSENFALCK

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Denne afhandling er af det matematisk naturvidenskabelige fakultet ved Københavns universitet antaget til offentlig at forsvares for den filosofiske doktorgrad

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CONTENTS

page

DUCTION 9

rimental studies of the spread of action ntials in volume conductors 10

tionship between intracellularly and extracellularly rded action potentials 11

lanations of the spread and shape of action potentials 11

e of the present study 14

I THE MATHEMATICAL THEORY

ion 1 The Maxwell field equations within conducting media 15

ion 2 The variation of the action potential within a fibre 19

ion 3 The density of the membrane current 22

ion 4 The core conductor model 25

ion 5 The extracellular action potentials in an unlimited conductor 28

ion 6 A fibre surrounded by a thin layer of conductor 35

ion 7 The theory of Lorente de Nó 42

ion 8 The radial decline of the extracellular action potential 47

tion 9 A fibre surrounded by an anisotropic conductor 50

tion 10 The simulated intracellular action potentials 58

A. The muscle action potential 59

B The nerve action potential 62

tion 11 The potentials produced by dipole and tripole generators 65

Page

PART 2 QUANTITATIVE TESTS AND APPLICATION OF THE MATHEMATICAL THEORY

Section 1 The intracellular action potentials 69
A The simulated muscle fibre 70
B The simulated nerve fibre 74

Section 2 The density of the membrane current 78

Section 3 The surface action potential 83

Section 4 The extracellular action potentials in an unlimited conductor 85
A The simulated muscle fibre 85
B The simulated nerve fibre 88
C Extracellular action potentials calculated from measured intracellular potentials 89
I The relation of the extracellular action potentials to the thickness of the fibre 98
II Comparison between the experimental and the calculated radial decline of the action potential 102
a The frog muscle fibre 102
b Human muscle fibres 104

PART 3 DISCUSSION

Section 1 Introduction 113

Section 2 The variation of the action potential within a fibre the membrane current and the core conductor model 115

Section 3 The extracellular action potentials in an unlimited conductor 117
A. The shape of the action potential 117
B The relation of peak to peak voltage to fibre thickness 120
C The radial decline of the action potential 122
I Frog muscle fibres 122
II Human muscle 123
a The generator of high voltage spike potentials 124
b Application 129

page

ion 4 The muscle fibre in air 130

ion 5 Conclusions future work 132

TDIX 1 Comparison between the potential generated by a point source and a linearly extended source 135

TDIX 2 The potential produced by a circular ring of charges of constant density 139

\RY 143

tÉ 147

)WLEDGEMENTS 151

tENCES 153

)F SYMBOLS 162

OF SUBJECTS 166

INTRODUCTION

This study presents a mathematical analysis of the spread of action currents within nerve and muscle fibres and through the volume conductor imbedding the fibres. The notion "action current" was introduced by Hermann (1879) at a time when it was firmly established that the activity of nerve and muscle is accompanied by transient electric currents in the surrounding tissue or fluid. These currents generate a potential field in the surroundings of the active cells. Numerous studies have been devoted to this subject and many attempts made to localize the generators of the action currents and to account for the electrical properties of the nerve and muscle fibres in terms of mathematical or physical models.

In a review of the early history of "animal electricity" Du Bois-Reymond (1848, 1849, 1884) referred to a German mathematician Christian August Hansen as the first to suggest that electricity was the active principle of nervous activity ("Novi prefectus in historia electricitatis", Leipzig 1743). In 1786 Galvani performed his famous experiments on the legs of frogs and attributed his findings to animal electricity stored in the muscles and flowing through the nerves (cf. Luigi Galvani, "De viribus Electricitatis in motu musculari Commentarius", Bologna 1791, English translation Green 1953). This interpretation was rejected by Volta in 1792 who believed the electricity to be "metallic or artificial" and to result from contact between different conductors. Fifty years elapsed before it was demonstrated that a tetanic contraction initiated by repetitive electrical stimulation of a muscle was accompanied by a significant reduction in the demarcation potential, to use modern terminology (Du Bois-Reymond 1849). Bernstein (1871) invented the recording technique with a sensitivity and speed which made it possible to measure the potential change accompanying a twitch contraction.

EXPERIMENTAL STUDIES OF THE SPREAD OF ACTION POTENTIALS IN VOLUME CONDUCTORS When vacuum tube amplifiers and cathode ray tubes were introduced to physiology about fifty years ago by Forbes Adrian Gasser and Erlanger it became possible to reproduce action potentials faithfully and to study their spread (cf Erlanger and Gasser 1937) Craib and Canfield (1927) and Craib (1928) initiated such work with heart and skeletal muscle immersed in salin They demonstrated that the action potential lead off from the volume conductor was triphasic while the potential recorded in air was monophasic Subsequent workers analyzed the distribution of extracellular action potentials in different preparations Lorente de Nó (1947a) used frog s sciatic nerve in situ and in a two dimensional conductor Tasaki and Tasaki (1950) recorded action potentials at different distance from a toad motor nerv fibre immersed in a thin layer of Ringer s solution Håkansson (1957a) compared the potentials from frog single muscle fibres in Ringer s solution and in air and Krnjević and Miledi (1958) studied action potentials of single fibres from rat diaphragm in Ringer Locke solution.

These studies had the limitation that the biological generator was removed from its natural environment. To interpret complex action potential recorded in situ the spread f the action potential under physiol gical conditions must be known to thi purpose small bundles and single fibre of human muscl were studied by Buchthal Guld and Rosenfalck (1957a) Rosenfalck and Buchthal (1962) Buchthal and Rosenfalck (1966) and Ekstedt (1964); single spinal ganglionic cells of frog by Svaetichin (1951) singl motoneurones of cat by Fatt (1957) Freygang and Frank (1958) and Nelson and Frank (1964) th Mauthner cell of the goldfish by Furshpan and Furukawa (1962) and pyramidal cells in the cortex of cat by Rosenthal Woodbury and Patton (1966) Krakau (1958) described the volume conduction of the electroretinogram of the rabbit in the eye ball and extraocularly Recent studies on th surface electrocardiogram notably by Frank and by Nelson are summarized in the report of a conference on the electrophysiology of the heart (Hecht 1957)

RELATIONSHIP BETWEEN INTRACELLULARLY AND EXTRACELLULARLY RECORDED ACTION POTENTIALS The difference in shape and amplitude between muscle or nerve action potentials recorded in air and in Ringer s solution reflects the difference between intracellular and extracellular action potentials single frog muscle fibres were investigated by Håkansson (1957a) Werman (1960 1963) and Murakami Watanabe and Tomita (1961) cardiac muscle fibres of the rabbit by Vaughan Williams (1959); giant axons of the crayfish by Murakami Watanabe and Tomita (1961) and spinal motoneurones by Fatt (1957) Freygang and Frank (1959) and Terzuolo and Araki (1961)

EXPLANATION OF THE SPREAD AND SHAPE OF ACTION POTENTIALS One of the prime questions of electrophysiology was whether the site and size of the electromotive forces of nerve and muscle could be derived from the extracellular current field. More than hundred years ago Helmholtz (1853) treated this problem assuming the electromotive forces to be constant. Under these condition the extracellular field does not specify the distribution of electromotive forces unequivocally Infinitely many distributions of fictive generators can be derived which produce the same potential field extracellularly According to Helmholtz (1853) the potential field satisfy Laplace s equation. Later investigators (Craib 1928 Wilson McLeod and Barker 1933 Benoit 1937 etc) assume that the equation is valid also for the potential field produced by action currents On this basis Craib (1928) explained the triphasic shape of the muscle action potential in terms of two dipole generators of opposite direction both propagating along the fibres Dipole and multipole generators were introduced to account for the spread of cardiac action currents through the body (Wilson McLeod and Barker 1933 Hecht 1957 Yeh Martin and Beaumont 1958 Yeh 1961) and a linear distribution of sources and sinks described the shape and radial decline of the action potential of a bullfrog sciatic nerve (Lorente de Nó 1947a)

In this classical account of extracellular potential fields of active nerve Lorente de Nó derived an expression for the extracellular

action potential in terms of the second derivative of the surface action potential in air or oil and the distance from the axis of the nerve. Tasaki (1953) accounted for the radial decline of the action potential around a node of Ranvier by considering the node a point sink of current, i.e. the amplitude decreases in inverse proportion to the distance in a three-dimensional conductor and linearly with the logarithm of the distance in a two-dimensional external medium.

Qualitative accounts of the triphasic shape of extracellular action potentials in terms of the theory of local circuits have been given by Bishop (1937), Offner (1954) and Mauro (1960). Bishop (1937) objected to a dipole model whose axis coincided with the axis of the muscle or nerve. In view of the existence of polarized surface membranes he found it more correct to consider the dipoles as oriented transversely to the surface of the fibres.

In an attempt to apply the mathematical model developed by Lorente de Nó (1947a) to the extracellular action potentials of single muscle fibres I used the intracellular action potential as a basis for the calculations (Rosenfalck 1957). Thereby conclusions could be drawn regarding the radial decline of action potentials and its dependence on the diameter of the generator and the area of depolarization (Buchthal, Guld and Rosenfalck 1957a). Krakau (1957, 1959) used Lorente de Nó's model and Fourier transforms and found good agreement between the theoretical radial decline in amplitude of different Fourier components of an action potential and the decline calculated from the action potentials recorded at different distances from a muscle fibre of frog (Håkansson 1957a).

Plonsey (1964) reformulated the theory of Lorente de Nó in greater detail than did my note of 1957. His calculations were based on quantities available from in situ measurements: the density of the membrane current and the action potential recorded just outside the generator. An alternative formulation (Plonsey 1965) involving integration over a solid angle was based on the intracellular action potential and the extracellular surface action potential, the difference between the internal and external conductivity being taken into account (see also Geselowitz 1967). Recently Clark and Plonsey (1966) presented rigorous mathematical expressions for the extracellular

action potentials of a cylindrical generator simulating an unmyelinated nerve fibre this formulation was used to evaluate the core conductor model of nerve and muscle fibres (Hermann 1872 1879) These models are discussed on p 25 and 115

In a short note G selowitz (1966) derived an elaborate expression for the membrane current of a cylindrical fibre in terms of the trans membrane potential using the model developed by Clark and Plonsey A simpler expression might have been obtained if the intracellular ac tion potential had been used instead of the transmembrane potential (p 23) Geselowitz (1967) suggested a more general derivation of the multipole components of the model of the heart than that of Yeh Martinek and Beaumont (1958)

The first ttempt to interpret the spread of the action potentials around cell bodies was made by Lorente de Nó (1947b) he analyzed qualitatively the spread f action currents in pools of motoneurones in the cat and the rabbit in terms of the activity of the individual cells and their anatomical arrangement. A emi quantitative treat ment of the potential field around a single neurone in the cortex of cat arrived at a potential which declined inversely with the distance from the center of the cell body (Tasaki Polley and Orrego 1954) Th field of potential around the motoneurone obeyed this relation to good approxim tion (Fatt 1957) Rall (1962) calculated the distribution of the extracellular potential around a dendritic neurone model consisting of a spherical soma (active) and seven cylindrical dendrites (passive) He showed that th shape of the action potential the de crement of its amplitude with radial distance and the increase of its spike latency with distance could be predicted to good approxi mation. The voltage versus distance curves of action potentials' from pyramidal neurone in the cortex of cat were accounted for by a dipole mod l by Rosenthal Woodbury and Patton (1966)

Krakau (1958) described the volume conduction of the electro-retinogram of the rabbit in the eye and extraocularly with the eye immersed in NaCl solution in terms of an electric double layer of source and sinks in the wall of th eye the moment of the double layer increasing towards the posterior pole of the eye

AIMS OF THE PRESENT STUDY: The main object of this study was to account for the extracellular action potentials recorded from isolated frog muscle fibres in Ringer s solution and in air (Håkanson 1956 1957a) in terms of the intracellular action potential. To this purpose action potentials of a cylindrical fibre were treated mathematically to determine (i) the relationship betw n the intracellular and the extracellular action potentials (ii) the duration amplitude shape and radial decline of the extracellular action potential and (iii) the relationship betwee the thickness of the fibre and the amplitude of the extracellular action potential. The calculati ns were based on the intracellular action potential just beneath the surface of the fibre I considered the case of an unlimited extracellular fluid and that of a thin layer of fluid the latter being treated as equivalent to the linear core conductor model An attempt will be made in thi study to take into account anisotropy of the volume conductor surrounding the fibre when the extracellular action potentials are calculated.

The results derived from the linear core conductor model and from the three-dimensional model will be compared to the results of Clark and Plonsey (1966) and will amplify them The validity of the approximation Lorente de Nó (1947a) made u e of to derive the external action potentials will be examined

My calculations will be based p rtly on simulated intracellular action potentials i.e analytical expre sions which reproduce the appearance of action potential of nerve and mu cle fibres to good approximation partly on measured muscle action potentials The question as to whether spike potentials of high voltage in normal and denervated human muscle arise from singl fibre or from a small group of fibres (Buchthal Guld and Rosenfalck 1957a Krnjević and Mil di 1958 Rosenfalck and Buchthal 1962 Ekstedt 1964 Buchthal and Rosenfalck 1966) will be dealt with

The paper is divided int three parts Part 1 contains the formal mathematical deduction Part 2 contains the numerical analysis of the mathematical model in terms of simulated and recorded intracellular action potentials The results and their relation to the findings of others are discussed in Part 3

PART 1

THE MATHEMATICAL THEORY

Section 1

The Maxwell field equations within conducting media

The electromagnetic field in a conducting medium such as muscle or nerve tissue is governed by Maxwell s equations:

(1) $\operatorname{curl}\vec{H} \quad \frac{4\pi}{c}\,\vec{i} + \frac{1}{c}\,\dot{\vec{D}}$

(2) $\operatorname{curl}\vec{E} \quad -\frac{1}{c}\,\dot{\vec{B}}$

(3) $\operatorname{div}\vec{D} \quad 4\pi\rho$

(4) $\operatorname{div}\vec{B} \quad 0$

$\vec{E}$ is the strength of the electric field and $\vec{D}$ the electric displacement $\vec{H}$ the strength of the magnetic field and $\vec{B}$ the magnetic induction $\vec{i}$ the current density and ρ the charge density c is the velocity of light all measured in c. g. s. electrostatic units. These quantities are furthermore related by

(5) $\vec{i} \quad \sigma\,\vec{E}$

(6) $\vec{D} \quad \xi\,\vec{E}$

(7) $\vec{B} \quad \mu\,\vec{H}$

where σ is the conductivity and ξ the capacitivity both of which may be tensors. μ is the magnetic permeability. It is a prerequisite for (5) that no currents from external generators interfere. The

tissue in which the action currents flow is inhomogeneous hence ε σ and μ vary in space As a first approximation the tissue is assumed to be homogeneous and isotropic The significance of an isotropy is considered in Section 9

Combining (1) and (3) gives the equation of continuity:

(6) $$\mathrm{div}\vec{I} + \rho \quad 0$$

From (3) (5) (5) and (6) we obtain

$$\rho \quad \frac{4\pi\sigma}{\varepsilon} \rho$$

and hence

$$\rho \quad \rho_0(x\ y\ z) \exp\left(\frac{4\pi\sigma}{\varepsilon} t\right)$$

The capacitivity (dielectric constant) ε of muscle tissue at 1 10 kHz is about 10^5 times that of empty space (Spector 1956) This high value reflects mainly the capacity of the muscle fibre membranes (Schwan and Cole 1950) The capacitivity of the intracellular and extracellular fluids is of the same order as that of water i.e $\varepsilon \approx 10^2$ (electrostatic units)

The conductivity σ of the intracellular fluid of muscle is about 0 4 mho/m 3 6 10^9 e s.u (Katz 1948) Hence the time constant τ with which the charg density ρ decays is

$$\tau \quad \frac{\varepsilon}{4\pi\sigma} \approx 2\ 10^{-9} \text{ sec}$$

Therefore an accumulated charge disappears in a time which is negligible as compared with the duration of action potentials and we can assume

(8) $$\rho \quad 0$$

To solve the field equations (1) (4) the scalar (φ) and the vector potential ($\vec{A}$) are introduced

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(9) $\quad \rho \quad 0$

To solve the field equations (1) (4) the scalar (φ) and the vector potential ($\vec{A}$) are introduced

(10) $\vec{B}$ curl $\vec{A}$

(11) $\vec{E}$ grad φ $\frac{1}{c}$ $\vec{A}$

From (3) (6) (8) and (11) we obtain:

(12) $\Delta\varphi$ $\frac{1}{c}$ div $\vec{A}$

where Δ is the Laplacian operator The definitions (10) and (11) do not determine φ and $\vec{A}$ uniquely Therefore to simplify equation (12) the following condition is imposed on φ and $\vec{A}$:

(13) div $\vec{A}$ $\frac{\varepsilon\mu}{c}$ φ

Thereby (12) becomes

(14) $\Delta\varphi$ $\frac{\varepsilon\mu}{c^2}$ φ

The action potentials to be considered are propagated at constant velocity U along the fibre Let us introduce cylindrical coordinates (x θ):

(15) φ $\varphi(r$ $+U$ t $\theta)$

(16) $\vec{A}$ $\vec{A}(r$ x+U t $\theta)$

and therefore

(17) φ U $\frac{\partial\varphi}{\partial x}$

(18) φ U^2 $\frac{\partial^2\varphi}{\partial x^2}$

(19) $\vec{A}$ U $\frac{\partial\vec{A}}{\partial}$

Inserting (18) and (14) we get:

$$\Delta\varphi \quad \frac{\varepsilon\mu U^2}{c^2} \quad \frac{\partial^2\varphi}{\partial z^2} \tag{20}$$

In estimating the size of the right hand term we assume

$$\mu \approx 1 \quad \varepsilon \leq u$$

The conduction velocity U in striated mammalian and amphibian muscle fibres is of the order of 100-500 cm/sec (Hâkansson 1956 1957a b Buchthal and Rosenfalck 1958) and in unmyelinated giant nerve fibres the maximum velocity is 3500 cm/sec (Spector 1956) The maximum velocity of 3500 cm/sec gives:

$$\frac{\varepsilon\mu U^2}{c^2} \approx 1 \; 4 \; 10^{9}$$

Hence the right hand term of (20) can be disregarded so that the scalar potential φ can be assumed to satisfy Laplace s equation

$$\Delta\varphi \quad 0 \tag{21}$$

According to (11) the significance of the vector potential $\vec{A}$ as regards the strength of the electric field $\vec{E}$ can be estimated from the ratio

$$\alpha \quad \frac{1}{\omega} \left| \frac{\partial A_z}{\partial t} \right| / \left| \frac{\partial \varphi}{\partial z} \right|$$

From (17) and (19) we obtain.

$$\alpha \quad \frac{U^2}{c} \left| \frac{\partial A_z}{\partial z} \right| / |\varphi|$$

Replacing $\frac{\partial A_z}{\partial}$ by $\operatorname{div}\vec{A}$ α is according to (13) estimated from

$$\alpha \approx \frac{\varepsilon\mu U^2}{c^2} \quad 1 \; 4 \; 10^{9}$$

Thus the lectric field is characterized by the scalar potential φ the vector potential $\vec{A}$ being negligible

Section 2

The variation of the action potential within a fibre

The fibre is assumed to be an infinitely long circular cylinder of radius a. The electric potential φ_i inside the fibre is then determined by Laplace's equation (21). In view of the axial symmetry the equation can be written:

$$\frac{\partial^2 \varphi_i}{\partial r^2} + \frac{1}{r}\frac{\partial \varphi_i}{\partial r} + \frac{\partial^2 \varphi_i}{\partial z^2} = 0 \qquad (22)$$

Furthermore we assume that the action potential V_i is recorded from just under the surface membrane of the fibre. Considering V_i a function of z the boundary condition is

$$\varphi_i(a, z) = V_i(z) \qquad (23)$$

(22) and (23) uniquely determine $\varphi_i(r, z)$; this is an example of Dirichlet's problem of potential theory (Kellogg 1929). The solution could be obtained numerically by replacing the partial differential equation (22) by a set of finite difference equations solved by matrix methods (Karlqvist 1952).

Particular solutions of Laplace's equation (22) are found by separation of variables (e.g. Bland 1961). Formal solutions which satisfy (22) and the boundary condition (23) can be obtained by means of integral transforms (Tranter 1951). For the purposes of this study the Fourier transform was most appropriate. We therefore introduce

$$\Phi_i(r, p) = \int_{-\infty}^{\infty} \varphi_i(r, z) \exp(ipz)\,dz \qquad (24)$$

with the inversion:

$$\varphi_i(r, z) = \frac{1}{2\pi} \int_{-\infty}^{\infty} \Phi_i(r, p) \exp(-ipz)\,dp \qquad (25)$$

Transforming the individual terms of (22) we are lead to:

$$(26) \qquad \frac{\partial^2 \Phi_1}{\partial r^2} + \frac{1}{r}\frac{\partial \Phi_1}{\partial r} \quad p^2 \Phi_1 \quad 0$$

if the potential φ_1 is measured relative to the resting potential. Similarly the boundary condition (23) leads to:

$$(27) \qquad \Phi_1(a\ p) = \int_{-\infty}^{\infty} V_1(z)\ \exp(i\,p\,z)dz$$

The complete solution of (26) is:

$$(28) \qquad \Phi_1(r\ p) \quad A(p)\ I_o(r\,p) + B(p)\ K_o(r|p|)$$

where I_o and K_o are modified Bessel functions (Jahnke and Emde 1948 Carslaw and Jaeger 1959) Since $K_o(r|p|) \to \infty$ for $r \to 0$ the solutions which are physically possible have the form

$$(29) \qquad \Phi_1(r\ p) \quad A(p)\ I_o(r\,p)$$

Introducing (29) in (27) we obtain:

$$A(p) \quad \frac{1}{I_o(a\,p)} \int_{-\infty}^{\infty} V_1(z)\ \exp(i\,p\,z)dz$$

and hence

$$\Phi_1(r\ p) \quad \frac{I_o(r\,p)}{I_o(a\,p)} \int_{-\infty}^{\infty} V_1(z)\ \exp(i\,p\,z)dz$$

Inverting the transform we obtain

$$(30) \qquad \varphi_1(r\ z) \quad \frac{1}{2\pi} \int_{-\infty}^{\infty} \frac{I_o(r\,p)}{I_o(a\,p)}\ \exp(\ i\,p\,z)\ \left\{\int_{-\infty}^{\infty} V_1(u)\ \exp(i\,p\,u)du\right\} dp$$

which can be written as:

$$(31) \qquad \varphi_1(r\ z) \quad \frac{1}{\pi} \int_{0}^{\infty} \frac{I_o(r\,p)}{I_o(a\,p)}\ \left\{\int_{-\infty}^{\infty} V_1(u)\cos p(z\ u)du\right\} dp$$

or

$$(32) \qquad \varphi_1(r\ z) \quad \frac{1}{\pi} \int_0^{\infty} \frac{I_0(r\,p)}{I_0(a\,p)} \left\{ \int_0^{\infty} [V_1(z\ u) + V_1(z+u)] \cos up\, du \right\} dp$$

This solution was arrived at by purely formal deductions diare garding the additional conditions which have to be satisfied by V_1 and $\varphi_1(r\ z)$ How far these deductions are justified is evaluated by means of simulated intracellular action potentials (Part 2)

Section 3

The density of the membrane current

The radial current density $i_r(r\ x)$ is given by

$$i_r \quad \sigma_i \frac{\delta \varphi_i}{\delta r}$$

where σ_i is the conductivity of the interior of the fibre Using (30) and $I_o \quad I_1$ w obtain:

$$i_r(r\ ) \quad \frac{\sigma_i}{2\pi}\int_{\infty}^{\infty} \frac{p I_1(rp)}{I_o(ap)} \exp(\ ipx) \left\{ \int_{\infty}^{\infty} V_i(u)\ \exp(ipu)du \right\} dp$$

Therefore the density of the current i_m passing outwards through the membran is

$$(33) \quad i_m(x) \quad \frac{\sigma_i}{2\pi} \int_{\infty}^{\infty} \frac{p I_1(ap)}{I_o(ap)} \exp(\ ipx) \left\{ \int_{-\infty}^{\infty} V_i(u)\ \exp(ipu)du \right\} dp$$

This may also be written

$$(34) \quad i_m(\ ) \quad \frac{\sigma_i}{\pi} \int_{0}^{\infty} \frac{p I_1(ap)}{I_o(ap)} \left\{ \int_{0}^{\infty} [V_i(x\ u)+V_i(x+u)]\ \cos up\, du \right\} dp$$

since $p\ I_1(ap) \quad |p|\ I_1(a|p|)$

The current density i_x in the fibre direction is

$$i \quad \sigma_i \frac{\delta \varphi_i}{\delta}$$

or according to (30)

$$(35) \quad i\ (r\ ) \quad \frac{\sigma_i}{2\pi} \int_{\infty}^{\infty} \frac{ip I_o(rp)}{I_o(ap)} \exp(\ ip\ ) \left\{ \int_{\infty}^{\infty} V_i(u)\ \exp(ipu)du \right\} dp$$

Expressed in t rms of second derivatives the radial current density i_r is according to (22)

$$i_r \quad \sigma_i\ r \left\{ \frac{\delta^2 \varphi_i}{\delta r^2} + \frac{\delta^2 \varphi_i}{\delta x^2} \right\}$$

Then the density of the membrane current i_m is

$$(36) \qquad i_m \quad \sigma_i a \left\{ \left. \frac{\partial^2 \varphi_i}{\partial r^2} \right|_{r\ a} + \frac{d^2 V_i}{dx^2} \right\}$$

From (30) we obtain since $I_i \quad \frac{1}{2}(I_o + I_2)i$

$$\frac{\partial^2 \varphi_i}{\partial r^2} \quad \frac{1}{4\pi} \int_{-\infty}^{\infty} p^2 \frac{(I_o(rp)+I_2(rp))}{I_o(ap)} \exp(ipx)$$

$$\cdot \left\{ \int_{-\infty}^{\infty} V_i(u) \exp(ipu)du \right\} dp$$

and

$$(37) \qquad \frac{\partial^2 \varphi_i}{\partial x^2} \quad \frac{1}{2\pi} \int_{-\infty}^{\infty} \frac{p^2 I_o(rp)}{I_o(ap)} \exp(ipx)$$

$$\left\{ \int_{-\infty}^{\infty} V_i(u) \exp(ipu)du \right\} dp$$

Therefore:

$$(38) \qquad \left. \frac{\partial^2 \varphi_i}{\partial r^2} \right|_{r\ a} \quad \frac{1}{2} \frac{d^2 V_i}{dx^2} + \frac{1}{4\pi} \int_{-\infty}^{\infty} \frac{p^2 I_2(ap)}{I_o(ap)} \exp(ipx)$$

$$\left\{ \int_{-\infty}^{\infty} V_i(u) \exp(ipu)du \right\} dp$$

which in (36) gives

$$(38) \qquad i_m(x) \quad \frac{\sigma_i}{2} \frac{d^2 V_i}{dx^2} + \frac{\sigma_i a}{4\pi} \int_{-\infty}^{\infty} \frac{p^2 I_2(ap)}{I(ap)} \exp(ipx)$$

$$\left\{ \int_{-\infty}^{\infty} V_i(u) \exp(ipu)du \right\} dp$$

This can also be written:

$$(40)\quad i_m(x) \quad \frac{\sigma_i a}{2}\,\frac{d^2V_i}{dx^2} + \frac{\sigma_i a}{2\pi}\int_0^{\infty} \frac{p^2 I_2(ap)}{I_0(ap)} \left\{ \int_0^{\infty} [V_i(x\ u) + V_i(x+u)] \cos up\, du \right\} dp$$

since $I_2(ap) \quad I_2(a|p|)$

If the integral term of (39) and (40) can be neglected the density of the membrane current is proportional to the second derivative of the intracellular action potential. An evaluation of the integral term is given on p 78

Section 4

The core conductor model

To clarify the origin of the electrical phenomena of resting and of stimulated nerves and muscles detailed studies were made of the passive response of these tissues to electric currents which were too weak to act as a stimulus (e g Pflüger 1859) Such studies lead Matteucci (1863) and Hermann (1872) to analyse the electric properties of a model consisting of a metal wire covered by a polarizable surface layer Weber (1873) and Weinberg (1941) calculated the potential field generated by stationary currents flowing through this core conductor model. They assumed the core to be a perfect conductor and hence its potential was constant along the axis of the model. This implies that these analyses contribute little to the understanding of the potential fields in and around active nerve and muscle fibres surrounded by a large volume of conducting fluid

In Hermann s (1879) core conductor model (Fig 1) the interior of the fibre and the external medium are characterized by the resistance per unit length (r_i and r_e respectively) The properties of the polarized surface membrane of the fibre are disregarded in the following analysi of this model.

The following notation is used:

$V_e(\ )$ potential in external fluid

$V_i(x)$ intracellular potential

$i_e(x)$ total longitudinal current in external fluid

$i_i(\ )$ total longitudinal current in the fibre

$2\pi a\, i_m(\ )$ membrane current per cm length of fibre

r_e resistance of external fluid per cm length

r_i resistance of intracellular fluid per cm length.

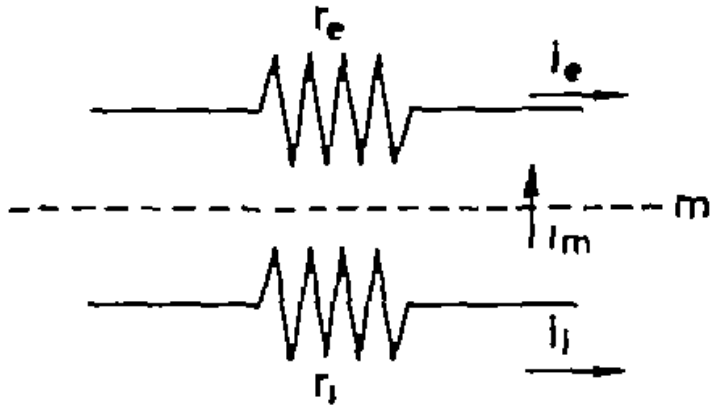


Fig 1 Element of the core conducto mod l
(Hermann 1879)
m surface membrane
r_i resistor representing 1 cm length of the intra ellular fluid (the core)
r resistor repre enting 1 cm length of the external conductor
i_f longitudinal current in the core
$_e$ longitudinal current in the external conductor
i_m density of the current leaving the core through the membrane

From Ohm law we get

(41) $\frac{dV}{dx}$ r_e $_i$

(42) $\frac{dV}{d}$ $_i$

Sin e no charge accumulat we furthermore have

(43) 2π $_m$ $\frac{d}{dx}$

(44) $i(\ )$ $_e(\ )$

As to the rel tion between V_i and V_e we get from (41) (42) and (44)

$$\frac{1}{r_e}\frac{dV_e}{d} + \frac{1}{r_i}\frac{dV_i}{dx} \quad 0$$

and hence

$$(45) \qquad V_e(x) \quad \frac{r_e}{r_i} \; V_i(x)$$

V_i being measured from the resting level
Let us consider the density of the membrane current i_m:
From (42) and (43) we obtain

$$2x \; i_m \quad \frac{1}{r_i}\frac{d^2V_i}{d^2}$$

Introducing the conductivity and radius of the fibre we have

$$(46) \qquad \frac{1}{r_i} \quad \sigma_i \; xa^2$$

and h nce

$$(47) \qquad i_m \quad \frac{\sigma_i a}{2}\frac{d^2V_i}{d^2}$$

Comparing thi expression with that derived for the three dimensional model (39) and (40) it is seen that the two models differ by the term

$$(48) \quad \frac{\sigma_i a}{4\pi}\int_{-\infty}^{\infty}\frac{p^2 I_2(ap)}{I_0(ap)}\exp(\;ip\;)\left\{\int_{-\infty}^{\infty} V_i(u)\exp(ipu)du\right\}dp$$

o in th real domain

$$(49) \quad \frac{\sigma_i a}{2\pi}\int_0^{\infty}\frac{p^2 I_2(ap)}{I_1(ap)}\left\{\int_0^{\infty}[V_i(\;\;u)+V_i(\;+u)]\;\cos up\,du\right\}dp$$

The density of th membrane current can also be related to the xtracellular potential V_e

From (47) (45) and (46) we obtain:

$$(50) \qquad i_m(\;) \quad \frac{1}{2\pi a\; r_e}\frac{d^2V_e}{dx^2}$$

Section 5

The extracellular action potentials in an unlimited conductor

Considering again the three dimensional model of a fibre we assume the external medium to extend infinitely in all directions As shown in Section 1 the potential φ_e in the external medium is determined by Laplace s equation

$$\frac{\partial^2 \varphi_e}{\partial r^2} + \frac{1}{r}\frac{\partial \varphi_e}{\partial r} + \frac{\partial^2 \varphi_e}{\partial z^2} = 0 \tag{51}$$

The boundary condition which φ_e has to s tisfy is that the current density through the fibre membrane is given by (33) (34) With

$$i_m = \sigma_e \left.\frac{\partial \varphi_e}{\partial r}\right|_{r=a} \tag{52}$$

where σ_e is the conductivity of the external medium (33) implies

$$\left.\frac{\partial \varphi_e}{\partial r}\right|_{r=a} = \frac{\sigma_i}{2\pi\sigma_e}\int_{-\infty}^{\infty} \frac{p I_1(ap)}{I_0(ap)} \exp(ipz) \left\{\int_{-\infty}^{\infty} V_1(u)\exp(ipu)du\right\} dp \tag{53}$$

Hence φ_e is a solution to the Neumann problem of potential theory (Kellogg 1929) Introducing the Fourier transform

$$\Phi_e(r,p) = \int_{-\infty}^{\infty} \varphi_e(r,z)\exp(ipz)dz \tag{54}$$

(51) leads to

$$\frac{\partial^2 \Phi_e}{\partial r^2} + \frac{1}{r}\frac{\partial \Phi_e}{\partial r} - p^2 \Phi_e = 0$$

which has the general solution

$$\Phi_e(r,p) = A(p) I_0(rp) + B(p) K_0(r|p|)$$

Since $I_o \to \infty$ for $r \to \infty$ we need only consider solutions of the form:

$$\Phi(r\ p) \quad B(p)\ K_o(r|p|)$$

which by inversion gives

$$(55) \qquad \varphi_e(r\ z) \quad \frac{1}{2\pi} \quad \int_{-\infty}^{\infty} B(p)\ K_o(r|p|)\ \exp(\ ipz)dp$$

With $K_o \quad K_1$ this implies:

$$(56) \qquad \left.\frac{\partial \varphi_e}{\partial r}\right|_{r=a} \quad \frac{1}{2\pi} \int_{-\infty}^{\infty} |p|\ B(p)\ K_1(a|p|)\ \exp(\ ipz)dp$$

Hence (55) is satisfied when

$$B(p) \quad \frac{\sigma_1}{\sigma_e}\ \frac{I_1(a|p|)}{I_o(ap)K_1(a|p|)} \quad \int_{-\infty}^{\infty} V_1(u)\ \exp(ipu)du$$

Inserting this in (55) we obtain

$$(57) \quad \varphi(r\ z) \quad \frac{\sigma_1}{2\pi\sigma_e} \int_{-\infty}^{\infty} \frac{I_1(a|p|)K_o(r|p|)}{I_o(ap)\ K_1(a|p|)}\ \exp(\ ipz)$$

$$\left\{ \int_{-\infty}^{\infty} V_1(u)\ \exp(ipu)du \right\} dp$$

An alternative form of this expression is

$$\varphi_e(r\ ) \quad \frac{\sigma_1}{\pi\sigma_e} \quad \int_0^{\infty} \frac{I_1(ap)\ K_o(rp)}{I_o(ap)\ K_1(ap)}$$

$$\left\{ \int_{-\infty}^{\infty} V_1(u)\cos p(\sigma-)du \right\} dp$$

and hence

$$(58) \quad \varphi_e(r\ z) \quad \frac{\sigma_1}{\pi\sigma_e} \quad \int_0^{\infty} \frac{I_1(ap)\ K_o(rp)}{I_o(ap)\ K_1(ap)}$$

$$\left\{ \int_0^{\infty} [V_1(\quad u) + V_1(z+u)]\cos pu\, du \right\} dp$$

The potential $\varphi_e(a\ z)$ just outside the fibre is

$$(59)\qquad \varphi_e(a\ z) \quad \frac{\sigma_i}{\pi\sigma_e} \int_0^{\infty} \frac{I_1(ap)\ K_0(ap)}{I_0(ap)\ K_1(ap)} \left\{ \int_{-\infty}^{\infty} V_1(u)\cos p(u\ z)du \right\} dp$$

The extracellular potential can also be expressed in terms of the density of the membrane current $i_m(z)$ From (52) and (56) we get

$$i_m(z) \quad \frac{\sigma_e}{2\pi} \int_{-\infty}^{\infty} |p|\ B(p)\ K_1(a|p|)\ \exp(\ ipz)dp$$

Considering this an inversion of a Fourier transform we obtain

$$B(p) \quad \sigma_e\ |p|\ K_1(a|p|) \int_{-\infty}^{\infty} i_m(s)\ \exp(ips)ds$$

which in (55) gives

$$\varphi_e(r\ z) \quad \frac{1}{2\pi\sigma_e} \int_{-\infty}^{\infty} \frac{K_0(r|p|)}{|p|\ K_1(a|p|)}\ \exp(\ ipz) \left\{ \int_{-\infty}^{\infty} i_m(s)\ \exp(ips)ds \right\} dp$$

Interchanging the order of integration we find:

$$\varphi_e(r\ z) \quad \frac{1}{2\pi\sigma_e} \int_{-\infty}^{\infty} i_m(s) \left\{ \int_{-\infty}^{\infty} \frac{K_0(r|p|)}{|p|\ K_1(a|p|)} \exp(ip(s\ z))dp \right\} ds$$

This can also be written as

$$(60)\qquad \varphi_e(r\ z) \quad \frac{1}{\pi\sigma_e} \int_{-\infty}^{\infty} i_m(s) \left\{ \int_0^{\infty} \frac{K_0(rp)}{pK_1(ap)}\ \cos p(s-z)dp \right\} ds$$

An approximate expression for the inner integral can be obtained from the series expansion of $K_1(ap)$ (Carslaw and Jaeger 1959 Appendix III) which gives:

$$ap\ K_1(ap) \to 1 \quad \text{for} \quad ap \to 0$$

From the tables of K_1 (e g Dwight 1958) it is seen that $ap\ K_1(ap)$ decreases with increasing values of ap However in Part 2 Section 4 it is shown that in the calculations of action potentials we need not consider values of ap exceeding 0 4 For ap 0 4 one has $ap\ K_1(ap)$ 0 874 and the following approximation is justified:

$$(61) \qquad \int_0^\infty \frac{K_0(rp)}{pK_1(ap)} \cos p(s-x)dp \approx a \int_0^\infty K_0(rp) \cos p(s-x)dp$$

$$\frac{a}{r} \int_0^\infty K_0(u) \cos(u \frac{s}{r})du$$

This integral is evaluated in Watson (1966 p 388):

$$\int_0^\infty K_0(u) \cos(u \frac{s}{r})du \quad \frac{\pi}{2\sqrt{1 + (\frac{x}{r})^2}}$$

which by insertion in (61) gives

$$\int_0^\infty \frac{K_0(rp)}{pK_1(ap)} \cos p(s-x)dp \approx \frac{a\pi}{2\sqrt{r^2 + (s-x)^2}}$$

and hence (60) may be approximated by

$$(62) \qquad \varphi_e(r\ s) \approx \frac{a}{2\sigma} \int_{-\infty}^{\infty} \frac{i_m(s)}{\sqrt{r^2 + (s-x)^2}} ds$$

An alternative formulation of (60) can be obtained by using

$$(63) \qquad \frac{1}{ap} \quad I_0(ap)\ K_1(ap) + K_0(ap)\ I_1(ap)$$

(Carslaw and Jaeger 1959 Appendix III) From (63) we get.

$$\frac{1}{p\,K_1(ap)} \quad a\left\{I_o(ap)+K_o(ap)\,\frac{I_1(ap)}{K_1(ap)}\right\}$$

Introducing this in (60) we get

$$\varphi_e(r\ z) \quad \frac{a}{\pi\sigma_e} \int_{-\infty}^{\infty} i_m(s)\left\{\int_0^{\infty} K_o(rp)\left[I_o(ap)+K_o(ap)\,\frac{I_1(ap)}{K_1(ap)}\right]\right.$$
$$\left.\cos p(s\ z)dp\right\} ds$$

For small values of ap the term $K_o(ap)\,I_1(ap)/K_1(ap)$ is small as compared to $I_o(ap)$ Thus for ap 0 4 $K_o\,I_1/K_1$ amounts to 10 per cent of $I_o(ap)$ This leads to the approximate expression for $\varphi_e(r\ z)$

$$(64) \quad \varphi_e(r\ z) \approx \frac{a}{\pi\sigma_e} \int_{-\infty}^{\infty} i_m(\ ) \left\{\int_0^{\infty} K_o(rp)\ I_o(ap)\right.$$
$$\left.\cos p(s-z)dp\right\} ds$$

The inner integral is given in Watson (1966 p 389)

$$(65) \quad \int_0^{\infty} K_o(rp)\ I_o(ap)\cos p(s-z)dp$$
$$\int_0^{\frac{\pi}{2}} \frac{d\theta}{\sqrt{(s-z)^2+(s+r)^2 \quad 4ar\sin^2\theta}}$$
$$\frac{1}{2}\int_0^{\pi} \frac{du}{\sqrt{(s\ z)^2+s^2+r^2 \quad 2ar\cos u}}$$

where $u \quad \pi \quad 2\theta$

Introducing (65) in (64) we get

$$(66) \quad \varphi_e(r\ z) \approx \frac{1}{4\pi\sigma_e} \int_{-\infty}^{\infty}\int_{\pi}^{\pi} \frac{i_m(s)\ a\,du\,ds}{\sqrt{(s-z)^2+s^2+r^2 \quad 2ar\cos u}}$$

Thus whereas the first approximation (62) was a line integral the present approximation is a surface integral The denominator represents the distance R between (r z) and the point (s s) located

on the surface of the fibre and in plane t an angle u from the plane through (r z) and the fibre axi Thus

$$\varphi_e(r\ z) \approx \frac{1}{4\pi\sigma_e} \int_S \frac{i_m(s)}{R}\, dS \tag{67}$$

where S is the surface of the fibre and dS an element of its area

Integrating (66) after u one obtains

$$\varphi_e(r\ z) \approx \frac{s}{\pi\sigma_e} \int_{-\infty}^{\infty} \frac{i_m(s)}{\sqrt{(z\ s)^2+(r+s)^2}}\, K\left(\sqrt{\frac{4sr}{(z\ s)^2+(s+r)^2}}\right) ds \tag{68}$$

where K denotes the complete elliptic integral of the first kind The integrand of (68) and (62) are nearly the same for most values of r and z s th deviations occurring near the singularity of (68) i.e r s s z (Table 1)

Table 1

Percentage difference between the integrands of (62) and (68) at various axial and radial positions

axial position	radial position		
z s	r=a	r 3a	r 7a
0	(100)	2.9	0 4
2a	7	0 1	0 2
4a	2 5	0 8	0
6a	1 4	0 7	0 3
8a	0 8	0 6	0
10a	0 6	0 6	0
12a	0 4	0 4	0
14	0 4	0	0
16a	0	0	0

The xtracellular potential can be expressed in still more general terms than in (58) and (60) Considering the region of space out xld r b where b ≥ a and assuming the radial gradient of the potential to be known at r b we obtain in analogy to (60):

$$(69) \qquad \varphi_e(r\ z) \quad \frac{1}{\pi} \int_{-\infty}^{\infty} \left.\frac{\partial \varphi_e(r\ s)}{\partial r}\right|_{r=b} \left\{ \int_0^{\infty} \frac{K_0(r\,p)}{p\,K_1(b\,p)} \cos p(s \quad) dp \right\} ds$$

Section 6

A fibre surrounded by a thin layer of conductor

In this Section the external conductor is assumed to be limited to a circular cylinder of radius b which is only slightly larger than the radius a of the fibre. The action potential in the external fluid is then determined by (51) and (53) together with the additional condition

$$\left.\frac{\partial \varphi_e}{\partial r}\right|_{r\,b} \qquad 0 \tag{70}$$

The general solution of (51) is

$$\varphi_e(r\ z) \quad \frac{1}{2\pi} \int_{-\infty}^{\infty} \left\{ (A(p)\ I_0(rp) + B(p)\ K_0(r|p|) \right\} \exp(\ ipz)dp$$

With $I_0 \quad I_1$ and $K_0 \quad K_1$ we have

$$\frac{\partial \varphi_e}{\partial r} \quad \frac{1}{2\pi} \int_{-\infty}^{\infty} \left\{ p\,A(p) I_1(rp) \quad |p| B(p) K_1(r|p|) \right\} \exp(\ ipz)dp$$

The boundary condition (70) is fulfilled if:

$$p\,A(p)\ I_1(bp) \quad |p|\ B(p)\ K_1(b|p|) \tag{71}$$

Similarly the condition (53) is satisfied if

$$A(p)\ I_1(ap) \quad \frac{|p|}{p}\ B(p)\ K_1(a|p|) \tag{72}$$

$$\frac{\sigma_i}{\sigma_e} \quad \frac{I_1(ap)}{I_0(ap)} \int_{-\infty}^{\infty} V_i(u) \exp(ipu)du$$

From (71) and (72) we obtain

$$A(p) \quad \frac{\sigma_i}{\sigma_e} \quad \frac{\dfrac{I_1(ap)}{I_0(ap)}\ K_1(b|p|) \int_{-\infty}^{\infty} V_i(u) \exp(ipu)du}{I_1(\ p)\ K_1(b|p|) \quad K_1(a|p|)\ I_1(bp)}$$

$$B(p) \quad \frac{\sigma_i}{\sigma_e} \frac{p}{|p|} \frac{I_1(ap)}{I_0(ap)} \frac{I_1(bp)}{I_1(ap)\, K_1(b|p|) - K_1(a|p|)\, I_1(bp)} \int_{-\infty}^{\infty} V_1(u) \exp(ipu)du$$

and hence

$$(73) \quad \varphi_e(r\ ) \frac{\sigma_i}{2\pi\sigma_e} \int_{-\infty}^{\infty} \frac{K_1(b|p|)\, I_0(rp) + \frac{|p|}{p} I_1(bp)\, K_0(r|p|)}{I_1(ap)\, K_1(b|p|) - K_1(a|p|) I_1(bp)} \frac{I_1(ap)}{I_0(ap)} \exp(ipz) \left\{ \int_{-\infty}^{\infty} V_1(u) \exp(ipu)du \right\} dp$$

Let us consider the potential on the surface of the thin conducting layer $\varphi_e(b\ z)$

Using $I_0(rp) \quad I_0(-rp) \quad I_1(ap) \quad I_1(-ap)$ and

$$K_1(b|p|)\, I_0(b|p|) + I_1(b|p|)\, K_0(b|p|) \quad \frac{1}{b|p|}$$

(Carslaw and Jaeger 1959 Appendix III) we get

$$(74) \quad \varphi_e(b\ z) \frac{\sigma_i}{2\pi\sigma_e b} \int_{-\infty}^{\infty} \frac{1}{I_1(ap)\, K_1(b|p|) - K_1(a|p|)\, I_1(bp)} \frac{I_1(ap)}{|p|\, I_0(ap)} \exp(ipz) \left\{ \int_{-\infty}^{\infty} V_1(u) \exp(ipu)du \right\} dp$$

An alternative expression is obtained by using the symmetry properties of I_0 and I_1

$$\varphi_e(b\ ) \frac{\sigma_i}{\pi\sigma_e b} \int_{0}^{\infty} \frac{1}{I_1(ap)\, K_1(bp) - K_1(ap)\, I_1(bp)} \frac{I_1(ap)}{p\, I_0(ap)} \left\{ \int_{-\infty}^{\infty} V_1(u) \cos p(u\ z)du \right\} dp$$

which can be written as

(75) $$\varphi_e(b\ z) \quad \frac{\sigma_i}{\pi \sigma_e b} \int_0^\infty I_1(ap)\ K_1(bp)^{1}\ K_1(ap)\ I_1(bp)$$

$$\frac{I_1(ap)}{pI_0(ap)} \left\{ \int_0^\infty [V_i(\ \ u) + V_i(z+u)] \cos pu\, du \right\} dp$$

With $b-a \ll a$ we can derive an approximate and simpler expression for $\varphi_e(b\ )$ Using the Taylor s series of $K_1(bp)$ and $I_1(bp)$ and the relations connecting I_1 and K_1 we get

(76) $$I_1(ap)\ K_1(bp) \quad K_1(\ p)\ I_1(bp)$$

$$\approx I_1(ap)\ \{K_1(ap) + (b-a)\cdot p\ K_1'(ap)\}$$

$$K_1(ap)\ \{I_1(\ p) + (b-a)\ p\ I_1(ap)\}$$

$$(b-a)\ p\ \{I_1(ap)\ K_1'(ap) \quad K_1(ap) I_1'(ap)\}$$

$$(b\ a)\ p\ (\ \frac{1}{ap}) \qquad \frac{b-a}{a}$$

A still bette approxim tion i

(77) $$I_1(ap)\ K_1(bp) \quad K_1(\ p)\ I_1(bp)$$

$$\approx (b-a)p\ \{I_1(\ p)\ K_1(\ p) \quad K_1(ap)\ I_1'(ap)\}$$

$$+ \tfrac{1}{2}(b-a)^2 p^2\ \{I_1(ap)\ K_1(ap) \quad K_1(ap)\ I_1(ap)\}$$

$$\frac{b-a}{a} + \tfrac{1}{2}(b-a)^2 p^2\ \frac{1}{a}\frac{d}{dp}$$

$$\{I_1(\ p)\ K_1(ap) - K_1(ap)\ I_1(ap)\}$$

$$\frac{b-a}{} + \frac{(b\ a)^2}{2}\ p^2\ \frac{d}{dp}(\ \frac{1}{ap})$$

$$\frac{b-a}{} + \frac{(b\ a)^2}{2a^2}$$

$$B(p) \quad \frac{\sigma_i}{\sigma_e} \frac{\frac{p}{|p|} \frac{I_1(ap)}{I_0(ap)} I_1(bp)}{I_1(ap)\, K_1(b|p|) - K_1(a|p|)\, I_1(bp)} \int_{-\infty}^{\infty} V_1(u)\, e$$

and hence

$$(73) \quad \varphi_e(r\ z) \quad \frac{\sigma_i}{2\pi\sigma_e} \int_{-\infty}^{\infty} \frac{K_1(b|p|)\, I_0(rp) + \frac{|p|}{p} I_1(bp)\, K_0(}{I_1(ap)\, K_1(b|p|) - K_1(a|p|) I_1(bp)} -$$

$$\frac{I_1(ap)}{I_0(ap)} \exp(ipz) \left\{ \int_{-\infty}^{\infty} V_1(u) \exp(ipu)du \right\} dp$$

Let us consider the potential on the surface of the thin conducting layer $\varphi_e(b\ z)$

Using $I_0(rp) \quad I_0(rp) \quad I_1(ap) \quad I_1(ap)$ and

$$K_1(b|p|)\, I_0(b|p|) + I_1(b|p|)\, K_0(b|p|) \quad \frac{1}{b|p|}$$

(Carslaw and Jaeger 1959 Appendix III) we get

$$(74) \quad \varphi_e(b\ z) \quad \frac{\sigma_i}{2\pi\sigma_e b} \int_{-\infty}^{\infty} I_1(ap)\, K_1(b|p|) \frac{1}{} K_1(a|p|)\, I_1(bp)$$

$$\frac{I_1(ap)}{|p|\, I_0(ap)} \exp(ipz) \left\{ \int_{-\infty}^{\infty} V_1(u) \exp(ipu)du \right\} dp$$

An alternative expression is obtained by using the symmetry properties of I_0 and I_1

$$\varphi_e(b\ z) \quad \frac{\sigma_i}{\pi\sigma_e b} \int_{0}^{\infty} I_1(ap)\, K_1(bp) \frac{1}{} K_1(ap)\, I_1(bp)$$

$$\frac{I_1(ap)}{p\, I_0(ap)} \left\{ \int_{-\infty}^{\infty} V_1(u) \cos p(u\ z)du \right\} dp$$

which can be written as

(75) $$\varphi_e(b\ z)\quad \frac{\sigma_l}{\pi\sigma_e b}\quad \int_0^{\infty} I_1(ap)\ K_1(bp)^{1}\ K_1(ap)\ I_1(bp)$$

$$\frac{I_1(ap)}{pI_0(ap)}\ \left\{\int_0^{\infty}[V_1(z\ u)+V_1(z+u)]\cos pu\,du\right\}\,dp$$

With b-a a w can derive an approximate and simpler expression for $\varphi_e(b\ z)$ Using th Taylo a series of $K_1(bp)$ and $I_1(bp)$ and the relations connecting I_1 and K_1 we get

(76) $$I_1(ap)\ K_1(bp)\quad K_1(ap)\ I_1(bp)$$

$$\approx I_1(ap)\ \{K_1(ap)+(b-a)\ p\ K_1'(ap)\}$$

$$K_1(ap)\ \{I_1(ap)+(b-a)\ p\ I_1'(ap)\}$$

$$(b\ \ )\ p\ \{I_1(ap)\ K_1(\ p)\quad K_1(ap)I_1(ap)\}$$

$$(b-a)\ p\ (\ \ \frac{1}{ap})\qquad \frac{b-a}{a}$$

A still bett approximation s

(77) $$I_1(\ p)\ K_1(bp)\quad K_1(ap)\ I_1(bp)$$

$$\approx (b\ a)p\ \{I_1(ap)\ K_1(ap)\quad K_1(ap)\ I_1(ap)\ \}$$

$$+\tfrac{1}{2}(b\ a)^2p^2\ \{I_1(ap)\ K_1''(\ p)\quad K_1(ap)\ I_1(ap)\}$$

$$\frac{b-a}{a}+\tfrac{1}{2}(b-\ \ )^2p^2\ \ \frac{1}{a}\ \frac{d}{dp}$$

$$\{I_1(\ p)\ K_1(ap)\ K_1(ap)\ I_1(ap)\}$$

$$\frac{b-a}{}+\frac{(b-\ \ )^2}{2a}\ \ p\ \ \frac{d}{dp}\ (\ \ \frac{1}{ap})$$

$$\frac{b-a}{a}+\frac{(b-a)^2}{2a^2}$$

Taking the third derivatives into account we get

(77) $$I_1(ap)\,K_1(bp) - K_1(ap)\,I_1(bp) \approx \frac{b-a}{a} + \frac{(b-a)^2}{2a^2} - \frac{(b-a)^3}{6a^3}\{3+(ap)^2\}$$

Considering p values for which $a|p| < 0.4$ (p. 83) $(ap)^2$ can be neglected in (78) and

$$I_1(ap)\,K_1(bp) - K_1(ap)\,I_1(bp) \approx \frac{b-a}{a} + \frac{(b-a)^2}{2a^2} - \frac{(b-a)^3}{2a^3}$$

Introducing the first approximation (78) in (74) and (75) we get

$$\varphi_e(b,x) \approx \frac{\sigma_i a}{2\pi\sigma_e b(b-a)} \int_{-\infty}^{\infty} \frac{I_1(ap)}{pI_0(ap)} \exp(ipx) \left\{\int_{-\infty}^{\infty} V_1(u)\exp(ipu)du\right\} dp$$

This expression can be reformulated using:

(79) $$I_1(ap) - \frac{ap}{4}[I_0(ap) - I_2(ap)]$$

and Fourier's integral formula

(80) $$V_1(x) - \frac{1}{2\pi}\int_{-\infty}^{\infty} \exp(ipx)\left\{\int_{-\infty}^{\infty} V_1(u)\exp(ipu)du\right\} dp$$

We get.

(81) $$\varphi_e(b,x) \approx \frac{\sigma_i a^2}{2\sigma_e b(b-a)}\left[V_1(x) - \frac{1}{2\pi}\int_{-\infty}^{\infty} \frac{I_2(ap)}{I_0(ap)} \exp(ipx) \left\{\int_{-\infty}^{\infty} V_1(u)\exp(ipu)du\right\} dp\right]$$

where V_i is measured from the resting level. The proportionality factor in (81) represents the ratio between the external (r_e) and intracellular longitudinal resistance (r_i) since for b-a $\ll$ a:

(82) $$r_e \quad \frac{1}{\sigma_e \, 2\pi b(b-a)}$$

(83) $$r_i \quad \frac{1}{\sigma_i \, \pi a^2}$$

and hence

$$\frac{r_e}{r_i} \quad \frac{\sigma_i a^2}{2\sigma_e b(b-\ )}$$

Therefore the first term in (81) represents the extracellular potential expected from the linear core conductor model (45) in Part 2 Section 3 it is shown that the second term can be neglected.

The second derivative of the surface action potential $\varphi_e(b,z)$ given in (74) is

$$\frac{\partial^2 \varphi_e(b,\ )}{\partial z^2} \quad \frac{\sigma_i}{2\pi \ \sigma_e b} \int_{-\infty}^{\infty} |p| \, \frac{I_1(\ p)}{I_0(\ p)} \exp(\ ipz)$$

$$I_1(ap)\, K_1(b|p|)\ K_1(\ |p|)\, I_1(\ ap)$$

$$\left\{ \int_{-\infty}^{\infty} V_1(u) \exp(ipu)du \right\} dp$$

Using the approximation (78) we get

(84) $$\frac{\partial^2 \varphi_e(b,\ )}{\partial z^2} \approx \frac{\sigma a}{2\pi\sigma\ b(b-\ )} \int_{-\infty}^{\infty} p I_1(ap) \, \frac{}{I_0(ap)} \exp(\ ipz)$$

$$\left\{ \int_{-\infty}^{\infty} V_1(u) \exp(ipu)du \right\}$$

Comparing this expression with that derived for the membrane current density $i_m(\ )$ (33) we obtain

$$\frac{\partial^2 \varphi_e(b\ x)}{\partial x^2} \approx \frac{a}{\sigma_e b(b-a)}\ i_m(x)$$

or

$$(85)\qquad i_m(x) \approx \frac{\sigma_e b(b-a)}{a}\ \frac{\partial^2 \varphi_e(b\ x)}{\partial x^2}$$

Introducing the longitudinal extracellular resistance r_e given by (82) this can be written:

$$(86)\qquad i_m(x) \approx \frac{1}{2\pi a\ r_e}\ \frac{\partial^2 \varphi_e(b\ x)}{\partial x^2}$$

which represents the density of the membrane current as expected from the core conductor model (50)

The variation in potential through the thin conducting layer $\varphi_e(a\ x)\ \ \varphi_e(b\ )$ can be estimated by using a Taylor expansion of $\varphi_e(a\ x)$:

$$\varphi_e(a\ x)\ \ \varphi_e(b\ ) + (a\ b)\left.\frac{\partial \varphi_e}{\partial r}\right|_{r=b} + \frac{(a\ b)^2}{2}\left.\frac{\partial^2 \varphi_e}{\partial r^2}\right|_{r\ b} +$$

Using (70) and Laplace s equation (51) we get the following approxi mation

$$\varphi_e(a\ x)\ \ \varphi_e(b\ x) \approx \frac{(b\ x)^2}{2}\ \frac{\partial^2 \varphi_e(b\ x)}{\partial x^2}$$

Introducing (84) and (79) we obtain

$$(87)\qquad \varphi_e(a\ x)\ \ \varphi_e(b\ x) \approx \frac{\sigma_1 a^2(b\ a)}{4\pi\sigma_e b}\int_0^{\infty} p^2\left(1\ \ \frac{I_2(ap)}{I_0(ap)}\right)$$

$$\left\{\int_0^{\infty} [V_1(\ \ u) + V_1(\ +u)]\cos pu\, du\right\} dp$$

From (80) follow that:

$$\frac{d^2V_1}{dx^2} \quad \frac{1}{\pi}\int_0^{\infty} p^2 \left\{\int_0^{\infty} [V_1(x\ u)+V_1(x+u)]\cos up\,du\right\} dp$$

Therefore (87) can be written

$$(88) \quad \varphi_e(a\ x)\ \varphi_e(b\ x) \approx \frac{\sigma_1 a^2(b-a)}{4\sigma_e b}$$

$$\left\{\frac{d^2V_1}{dx^2} + \frac{1}{\pi}\int_0^{\infty} \frac{p^2 I_2(ap)}{I_o(ap)} \left\{\int_0^{\infty} [V_1(x\ u)+V_1(x+u)]\right.\right.$$

$$\left.\left.\cos pu\,du\right\} dp\right\}$$

In Part 2 Section 2 it is shown that the integral term can be neglected.

Se tion 7

The theory of Lorente de Nó

Lorente de Nó (1947a) analyzed mathematically th spread in volume conductors of action currents from nerves (recently revi wed by Plonsey 1964) The analysis was based on theorems of potential theory (cf Kellogg 1829) as derived by Helmholtz (1853) Lorente de Nó showed that the nerve action potential in a volume conductor having th same conductivity as the nerve can be calculated from the surf ce action potential of a nerve surrounded by oil or air He assumed that the internal electromotive forces f the nerve fibre do not depend on the resistance of th external conductor More specifically Lorente de Nó found that the action potential could be considered to arise from an electric double l yer lo ated just outside the nerve with a moment of $\frac{1}{4\pi}$ times the surface action potential V_e in oil or air

$$(89) \qquad \varphi(P) \quad \frac{1}{4\pi} \int_S V_e(P) \frac{\partial \frac{1}{R}}{\partial n} \, dS$$

where th integration extends over the surface S of the nerve; dS denotes a surface element $\frac{\partial}{\partial n}$ the normal d rivative on S and R ip the distanc between the point P and P Considering the nerve to be a right circular cylinder Lorente de Nó transformed the surface integral (89) to one more suited for num rical evaluation:

$$(90) \qquad \varphi_e(r\ z) \quad \frac{1}{4\pi} \int_\tau \frac{\partial^2 V_e(\ )}{\partial\ ^2} \frac{d\tau}{R}$$

wh re τ is the volum of the nerve and $d\tau$ a volume element. His calculations were based on d ta from the alpha fibres of bullfrog cistic nerves They lead to triphasic action potential and account ed for the decline in amplitud with increasing distance from the nerve

Lorent d Nó (1947a) also expre sed the action potentials in terms of th current passing th surface of the nerve This was obtained by using the relation (50) between the density of the mem

brane current $i_m(z)$ and the surface action potential $V_e(z)$ as derived from the core conductor model

$$(91) \qquad \varphi_e(r\ z) \quad \frac{r_e a}{2} \int_{\tau} \frac{i_m(\ )}{R}\ d\tau$$

Lorente de Nó repl ced the volume integrals (90) and (91) by line integrals e g

$$(92) \qquad \varphi_e(r\ z) \quad \frac{\pi r_e a^3}{2} \int_{-\infty}^{\infty} \frac{i_m(s)}{R}\ ds$$

where R is the distance between (r z) and the line element ds on the axis of the nerve

Lorente de Nó derived th line int grals by considering the potential at distances large as compared to the radius of the nerve The line integrals can be obtained from the volume integrals without this restriction. Thus (91) can be written:

$$\varphi_e(r\ z) \quad \frac{r_e a}{2} \int_{-\infty}^{\infty} ds \int_0^a dr_1 \int_0^{2\pi} r_1 d\theta\ \frac{i_m(s)}{R}$$

where

$$R^2 \quad (\ \ s)^2 + \ ^2 + r_1^{\ 2} \quad 2rr_1 \cos\theta$$

Introducing the expansion (M rse and F shbach H 1953)

$$\frac{1}{R} \quad \sum_{m=0}^{\infty} \varepsilon_m \cos m\theta \quad \int_0^{\infty} J_m(kr)J_m(kr_1) \exp(-k|\ \ s|)dk$$

where

$$\varepsilon_m \quad \begin{cases} 1 & \text{for } m = 0 \\ 2 & \text{for } m > 0 \end{cases}$$

we get by integrating after θ

$$\varphi_e(\ \ ) \quad \pi r_e a \int_{-\infty}^{\infty} ds \int_0^a dr_1 \int_0^{\infty} dk\ r_1\ i_m(\ )$$

$$J_o(kr) J_o(kr_1) \exp(-k|\ \ s|)$$

Integration after r_1 (cf Carslaw and Jaeger 1959) leads to

$$(93)\quad \varphi_e(r\ \ ) \quad x r_e a^2 \int_{-\infty}^{\infty} i_m(s) \left\{ \int_0^{\infty} J_1(ka)\, J_o(kr)\, \exp(\ k|\, z-s|\ )/k\, dk \right\} ds$$

An approximate expression can be found by assuming

$$J_1(ka) \approx \frac{ka}{2}$$

This approximation i valid for small valu of ka the deviation is 3 % for ka 0 5 8 % for ka 0 8 and 17 % for ka 1 2 Hence as long as the xponential term of (93) does not decline too slowly with increasing k i e a long as $|z\ s| \gtrsim a$

$$\int_0^{\infty} J_1(ka) J_o(kr)\, \exp(\ k|\quad\ |) \frac{dk}{k}$$

$$\approx \frac{a}{2} \int_0^{\infty} J_o(kr)\, \exp(\ k|\quad\ |)\, dk$$

$$\frac{a}{2\sqrt{(z\ s)^2 + r^2}}$$

according to th tabl s of Laplace transforms (e g McLachlan and Humbert 1950) Introducing this evaluation in (93) we get

$$(94)\quad \varphi\ (r\ \ ) \approx \frac{x r_e a^3}{2} \int_{-\infty}^{\infty} \frac{i_m(s)}{\sqrt{(z\ \ )^2 + r^2}}\, ds$$

Thus the lin integral (92) obtained by Lorente d Nó (1947a) by concentrating the fictive source and sinks of the action potential on the axis of the nerve (94) is identical with (62) which was d rived from the rigorous model in Section 5 if the ext rnal con ductor act s if ts cros se tion were equal to that of the nerve Thi implies

$$r_e \quad \frac{1}{\sigma_e\ \pi^{\ 2}}$$

An evaluation of the error introduced by replacing the volume integral of (91) by the line integral of (94) is given in Appendix 2 The calculations indicate that the error is small even for points (r z) near the surface of the nerve

The action potentials as given by the approximation (94) satisfy Laplace's equation Let us consider to what extent (94) gives the *correct density of the current which passes the surface of the nerve* The current density $i_{m\,1}(z)$ corresponding to (94) is

$$i_{m\,1}(z) \quad \sigma_e \frac{\partial \varphi_e}{\partial r}\Big]_{r\,a} \quad \frac{\pi \sigma_e r_e a^4}{2} \int_{-\infty}^{\infty} \frac{i_m(s)}{\{(\quad s)^2 + a^2\}^{3/2}}\, ds$$

Assuming r_e given by (95) we get

$$(96) \qquad i_{m\,1}(z) \quad \frac{a^2}{2} \int_{-\infty}^{\infty} \frac{i_m(\ )}{\{(z\ s)^2 + a^2\}^{3/2}}\, ds$$

This integral can be transformed so that the difference between $i_{m\,1}(z)$ and $i_m(z)$ is easier to assess To this end we u

$$(97) \qquad \frac{a^2}{2} \int_{-\infty}^{\infty} \frac{i_m(\ )}{\{(z-s)^2 + a^2\}^{3/2}}\, d \quad i_m(z)$$

as is shown by introducing the variabl u $1 + \dfrac{a^2}{(z\ s)^2}$

On account of the rapid increase of the denominator with $|z\ s|$ the major contribution to the integral (97) comes from values of s which deviate only slightly from z i.e 90 per cent from z 2a ≤ s ≤ +2a and 98 per cent from z 3a ≤ s ≤ z+3a.

From (96) and (97) it follows that

$$(98) \quad i_{m\,1}(\ ) \quad i_m(\ ) + \frac{a^2}{2} \int_{-\infty}^{\infty} \frac{i_m(s)\ i_m(\ )}{\{(z\ )^2 + a^2\}^{3/2}}\, ds$$

where the integral represents the difference between the density of the *current* $i_m(\ )$ *which passes the surface of* the nerve and the

current density $i_{m\ 1}(x)$ as calculated from the approximation to the external field given by (84) and (85) Because of the rapid increase of the denominator with $|x\ s|$ the integral of (98) can be neglected if $i_m(s)$ varies little in the range $x\ 2a < s < x+2a$ Under this condition the approximation (94) gives the correct current density at the surface of the nerve (see Section 4 of Part 2)

Section 8

The radial decline of the extracellular action potential

According to (57) the potential $\varphi_e(r\ z)$ can formally be given as

$$(99)\qquad \varphi_e(r\ z) \qquad \int_{-\infty}^{\infty} C(p)\ K_o(r|p|)\ \exp(\ ip\ )dp$$

where

$$C(p) \qquad \frac{\sigma_i}{2\pi\sigma_e}\ \frac{I_1(a|p|)}{I_o(ap)\ K_1(a|p|)}\ \int_{-\infty}^{\infty} V_1(u)\ \exp(ipu)du$$

To evaluate the significance of (99) time is introduced as a variable in addition to the space variables For this purpose z is replaced by $z + U\ t$ where U is the conduction velocity of the action potential (cf p 17) Thus

$$\varphi_e(r\quad t) \qquad \int_{\infty}^{\infty} C(p)\ \exp(\ ip\ )\ K_o(r|p|)\ \exp(\ ipUt)dp$$

Introducing

$$\omega \quad p\ U$$

we get

$$(100)\quad \varphi_e(r\quad t) \qquad \frac{1}{U}\ \int_{-\infty}^{\infty} C(\tfrac{\omega}{U})\ \exp(\ i\omega\ \tfrac{z}{U})\ K_o(\tfrac{|\omega|}{U})\ \exp(\ i\omega t)d\omega$$

(100) represent the Fourier expansion of the action potential. The different frequency components of the action potential differ with respect to their radial decline the highest frequencies being attenuated close to the fibre (Fig 2) In Table 2 are given the distances at which the amplitudes of components of various frequencies have declined to one tenth of its value at the surface of the fibre For frequencies of 4000 5000 Hz the distance is 8–10 times shorter than for frequencies of 250 300 Hz To further

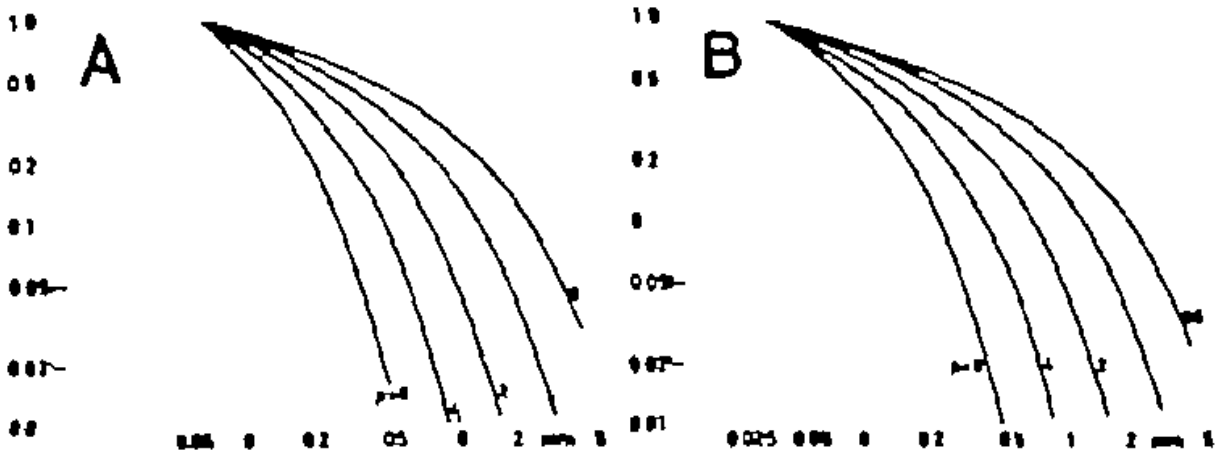


Fig 2 Radial decline in amplitude of different Fourier components of an action potential calculated for a fibre in an unlimited isotropic conductor The curves represent $K_0(rp)/K_0(ap)$ for different values of p; r is the radial distance from the axis of the fibre and a the radius of the fibre

A a 0 05 mm
conduction velocity (U) 3 m/sec
Fourier frequencies 240 3800 Hz

B a 0 025 mm
U 4 m/sec
Fourier frequencies 320 5100 Hz

characterize the radial declin the slope of the curves of Fig 2 is given in Table 2 For high frequency components the slope doubles within few radii from the fibre

With K_0 K_1 we obtain from (100)

$$(100a) \qquad \frac{\delta \varphi_e}{\delta r} \frac{1}{U^2} \int_{-\infty}^{\infty} C(\frac{\omega}{U}) \; |\omega| \exp(i\omega x/U) \; K_1(r|\omega|/U) \exp(i\omega t) d\omega$$

This expression can be used to estim te the radial variation of the gradient $\frac{\delta \varphi_e}{\delta r}$ and hence of the current flowing radially in the volume conductor

Table 2

Radial decline of different Fourier components of the action potential.

frequency (Hz)	distance with decline to 1/10 (mm)			slope of curves of Fig 2	
	isotropic	anisotropic A)	anisotropic B++)	at surface	4 8 radii from fibre
radius 0 05 mm					
3600	0 25	0 18	0 15	1 0	2 7
1900	0 42	0 31	0 23	0 7	1 3
950	0 70	0 52	0 38	0 5	1 0
475	1 2	0 90	0 63	0 4	0 7
240	2 1	1 62	1 12	0 3	0 5
radius 0 025 mm					
5100	0 21	0 15	0 12	0 65	1 6
2550	0 35	0 25	0 19	0 5	1 0
1275	0 61	0 43	0 32	0 4	0 7
640	1 07	0 77	0 55	0 3	0 5
320	1 89	1 37	0 95	0 3	0 3

) $\frac{\sigma_r}{\sigma} \quad \frac{4}{x^2}$

) $\frac{\sigma}{\sigma_x} \quad \frac{1}{5}$

Section 9

A fibre surrounded by an anisotropic conductor

In the preceding sections the medium surrounding the fibre was assumed to be homogeneous and isotropic In fact in muscle nerve and brain conditions are more complicated The membrane impedance of inactive fibres and cells is so high that the action currents of an active fibre or cell passes mainly through the extracellular space (cf Buchthal Guld and Rosenfalck 1955b) As a result the conductivity of whole muscle and of white and grey matter of the brain is only about 1/10 of that of Ringer s solution (Burger and van Dongen 1960 61 Buchthal and A Rosenfalck 1966 Geddes and Baker 1967) Furthermore the tight parallel arrangement of the muscle and nerve fibres cause a significant difference in conduction properties in the direction of th fibres and perpendicular to it. Hermann (1879) found the transverse resistance of frog nerves to be 5 times the longitudinal resistance a similar difference was deduced for skeletal muscle (Geddes and Baker 1967) Considering the fibre to have a quadratic cross section and the extracellular space to be small the effective conductivity in a direction perpendicular to the fibres is half that in the direction of the fibres (Buchthal Guld and Ro enfalck 1955b) A larger anisotropy is indicated by a model f closely packed circular fibres *)

An estimate of the anisotropy can be obtained from the following considerations Let β denote the ratio between the distance through which a current flows to traverse the muscle and th short st distance

Then we have

$$\frac{1}{\beta} \quad \frac{1}{x} \quad \int_{\frac{x}{2}}^{\frac{x}{2}} \cos\theta d\theta \quad \frac{2}{x}$$

assuming the path of the current to consist of randomly orient ed elements. Let α denote the fraction of the muscle which is extracellular space If σ denotes the conductivity of the extra

*) Calculations performed by Professor Lottrup Knudsen in personal discussions concerning the range of conduction velocities in a muscle (Buchthal Guld and Rosenfalck 1955b)

cellular fluid and if $\alpha \ll 1$ the effective conductivity of the muscle in the transverse direction is:

$$\sigma_r \quad c \; \frac{\alpha}{\beta} \; \frac{1}{\beta}$$

and in the longitudinal direction

$$\sigma_x \quad \sigma \; \alpha$$

and hence

$$(101) \qquad \frac{\sigma_r}{\sigma_x} \quad \frac{1}{\beta^2} \quad \frac{4}{2} \approx 0\ 4$$

The anisotropy of the external medium can be taken into account by assuming that th fibre is surrounded by a homogeneou medium of different conductiviti s in the fibre direction and perpendicularly to it This approximation is justified when the distance is large as comp red to the di meter of the fibre

We therefore have the following conductivity tensor:

$$(102) \qquad \sigma \quad \begin{Bmatrix} \sigma_r & 0 & 0 \\ 0 & \sigma_r & 0 \\ 0 & 0 & \sigma_x \end{Bmatrix}$$

Simila ly the capacitivity is represented by a tensor:

$$(103) \qquad \varepsilon \quad \begin{Bmatrix} \varepsilon_r & 0 & 0 \\ 0 & \varepsilon_r & 0 \\ 0 & 0 & \varepsilon_x \end{Bmatrix}$$

The electromagnetic field is determined by equations (1) (8) of Section 1 together with (102) and (103) Let us consider the elec tric displacement $\vec{D}$ as consisting of two parts

$$(104) \qquad \vec{D} \quad \vec{D}_1 + \vec{D}_2$$

where

(105) $\vec{D}_1 \quad (D_x \quad D_y, \quad 0)$

and

(106) $\vec{D}_2 = (0 \quad 0 \quad D_z)$

Similarly the charge density ρ is resolved in two parts

(107) $\rho \quad \rho_1 + \rho_2$

defined by:

(108) $\mathrm{div}\,\vec{D}_1 \quad 4\pi\rho_1$

and

(109) $\mathrm{div}\,\vec{D}_2 \quad 4\pi\rho_2$

From (3) (5) (6) (8) (102) and (103) we obtain:

$$\rho \quad \frac{\sigma_r}{\varepsilon_r}\left(\frac{\delta D_x}{\delta x} + \frac{\delta D_y}{\delta y}\right) + \frac{\sigma_z}{\varepsilon_z} \quad \frac{\delta D_z}{\delta z}$$

and hence according to (104) (109)

$$\rho_1 \quad \rho_2 \quad \frac{\sigma_r}{\varepsilon_r}\ \mathrm{div}\,\vec{D}_1 + \frac{\sigma_z}{\varepsilon_z}\ \mathrm{div}\,\vec{D}_2$$

$$\frac{4\pi\sigma_r}{\varepsilon_r} \quad \rho_1 + \frac{4\pi\sigma_z}{\varepsilon_z} \quad \rho_2$$

A solution of this equation is

$$\rho_1 \quad \rho_1(x\ y\ z)\ \exp(\ 4\pi\sigma_r/\varepsilon_r\ t)$$

$$\rho_2 \quad \rho_2(x\ y\ z)\ \exp(\ 4\pi\sigma/\varepsilon_z\ t)$$

and hence

$$\rho \quad \rho_1 \exp(4\pi\sigma_r/\varepsilon_r \, t) + \rho_2 \exp(4\pi\sigma_z/\varepsilon_z \, t)$$

In Section 1 the time constants of this expression were estimated to be $2\ 10^{-9}$ sec; hence ρ can be assumed to be zero as in the isotropic case

Introducing the scalar and vector potentials as defined by (10) and (11) we find from (5) (6) and (102):

$$\mathrm{div}(g \ \{ \ \mathrm{grad}\, \varphi \quad \frac{1}{c} \frac{\partial \vec{A}}{\partial t} \ \}) \quad 0$$

i.e

$$(110) \quad \sigma_r \ \frac{\partial^2 \varphi}{\partial x^2} + \sigma_r \ \frac{\partial^2 \varphi}{\partial y^2} + \sigma_z \ \frac{\partial^2 \varphi}{\partial z^2} \quad \frac{1}{c} \ \mathrm{div}(g \ \vec{A})$$

If we introduce the condition

$$\mathrm{div}(g \ \vec{A}) \quad \frac{\sigma_z \ \varepsilon_z \mu}{c} \quad \varphi$$

(110) becomes

$$(111) \qquad \frac{\sigma_r}{\sigma_z} \left(\frac{\partial^2 \varphi}{\partial x^2} + \frac{\partial^2 \varphi}{\partial y^2}\right) + \frac{\partial^2 \varphi}{\partial z^2} \quad \frac{\varepsilon \ \mu}{c^2} \quad \varphi$$

For an isotropic medium the right hand term of (111) could be neglected this holds also in the anisotropic case and hence:

$$(112) \qquad \frac{\sigma_r}{\sigma_z} \left(\frac{\partial^2 \varphi}{\partial x^2} + \frac{\partial^2 \varphi}{\partial y^2}\right) + \frac{\partial^2 \varphi}{\partial z^2} \quad 0$$

To simplify this equation a new coordinate system is introduced

$$(113) \qquad \begin{cases} x_1 & x\sqrt{\sigma / \sigma_r} \\ y_1 & y\sqrt{\sigma_z / \sigma_r} \\ z_1 & z \end{cases}$$

Thereby (112) is transformed to:

$$\frac{\partial^2 \varphi}{\partial x_1^2} + \frac{\partial^2 \varphi}{\partial y_1^2} + \frac{\partial^2 \varphi}{\partial z_1^2} = 0 \tag{114}$$

Thus the transformation (113) formally changes (112) to the equation of the isotropic case. Denoting the potential corresponding to anisotropic conditions by φ_{an} and that of the isotropic case by φ_{iso} it follows from (114) that:

$$\varphi_{an}(x\ y\ z) = k\ \varphi_{iso}(x_1\ y_1\ z_1)$$

where x_1 y_1 z_1 are given by (113) k is a proportionality factor which is determined by σ_r and σ and the conductivity σ_e of the isotropic conductor. Introducing (113) we get

$$\varphi_{an}(x\ y\ z) = k\ \varphi_{iso}\left(x\sqrt{\frac{\sigma_x}{\sigma_r}}\ \ y\sqrt{\frac{\sigma_x}{\sigma_r}}\ \ z\right) \tag{115}$$

Considering fields which are radially symmetrical around the Z-axis the distance from this axis is related to x and y by:

$$r = \sqrt{x^2 + y^2}$$

Hence

$$\varphi_{an}(r\ z) = k\ \varphi_{iso}\left(r\sqrt{\frac{\sigma_x}{\sigma_r}}\ \ z\right) \tag{116}$$

To determine the proportionality factor k the density of the membrane current $i_m(z)$ is assumed to be independent of whether the external medium is isotropic or not. Therefore

$$\sigma_e \left.\frac{\partial \varphi_{iso}}{\partial r}\right|_{r=a} = \sigma_r \left.\frac{\partial \varphi_{an}}{\partial r}\right|_{r=a}$$

Using (115) we obtain.

$$k \quad \frac{\sigma_e}{\sqrt{\sigma_r \sigma_z}} \quad \frac{\left.\frac{\partial \varphi_{iso}}{\partial r}\right|_{r=a}}{\left.\frac{\partial \varphi_{iso}}{\partial r}\right|_{r=a\sqrt{\sigma_z/\sigma_r}}}$$

and hence (116) becomes

$$(117) \quad \varphi_{an}(r\ z) \quad \frac{\sigma_e}{\sqrt{\sigma_r \sigma_z}} \quad \frac{\left.\frac{\partial \varphi_{iso}}{\partial r}\right|_{r=a}}{\left.\frac{\partial \varphi_{iso}}{\partial r}\right|_{r=a\sqrt{\sigma_z/\sigma_r}}} \quad \varphi_{iso}\left(r\sqrt{\frac{\sigma_z}{\sigma_r}}\ z\right)$$

As to the relative decline of the potential in the radial direction we get from (116)

$$(118) \quad \frac{\varphi_{an}(r\ z)}{\varphi_{an}(\ z)} \quad \frac{\varphi_{iso}\left(r\sqrt{\frac{\sigma_z}{\sigma_r}}\ z\right)}{\varphi_{iso}\left(a\sqrt{\frac{\sigma_z}{\sigma_r}}\ z\right)}$$

According to the estimate (101)

$$\sqrt{\frac{\sigma_z}{\sigma_r}} \quad \frac{x}{2} \quad 1\ 87$$

so that (118) becomes:

$$(119) \quad \frac{\varphi_{an}(r\ z)}{\varphi_{an}(a\ z)} \quad \frac{\varphi_{iso}\left(\frac{xr}{2}\ z\right)}{\varphi_{iso}\left(\frac{xa}{2}\ z\right)}$$

From (118) it follows that the anisotropy of the external conductor causes a radial attenuation of the action potential which is larger than that corresponding to isotropic conditions.

Introducing the decline calculated for isotropic conditions (*Fig* 2) into (119) $\sigma_r/\sigma \quad 4/x^2$ brings about a decline to one tenth within a distance which is about 26 per cent smaller than for an isotropic medium (Table 2) Similarly (118) gives that the distance over which the amplitude decline to one tenth i 40 50 per cent smaller than for an isotropic medium if the anisotropy has the degree indicated by H rmann s (1879) findings ($\sigma_r/\sigma \quad 1/5$) (Table 2)

As to the absolute values of the potential at the surface of the fibre (r a) we get from (117) and (101)

$$\varphi_{an}(a\ z) \quad \frac{\sigma_e}{\sqrt{\sigma_r \sigma_z}} \quad \frac{\left.\frac{\delta \varphi_{iso}}{\delta r}\right|_{r=a}}{\left.\frac{\delta \varphi_{iso}}{\delta r}\right|_{r\ \frac{ax}{2}}} \quad \varphi_{iso}\left(\frac{ax}{2}\quad z\right)$$

and hence

$$(120) \quad \varphi_{an}(a, z) \quad \frac{\sigma_e}{\sqrt{\sigma_r \sigma_z}} \quad \frac{\left.\frac{\delta \varphi_{iso}}{\delta r}\right|_{r\ a}}{\left.\frac{\delta \varphi_{iso}}{\delta r}\right|_{r\ \frac{ax}{2}}} \quad \frac{\varphi_{iso}\left(\frac{ax}{2}\quad z\right)}{\varphi_{iso}(a\ z)} \quad \varphi_{iso}(z\ z)$$

As mentioned above this relation is valid for all value of the conductivity of the isotropic volume conductor (σ_e) To obtain an isotropic case which is a first approximation to the actual conditions we may either assume

$$\sigma_e \quad \sqrt{\sigma_r\ \sigma_z}$$

or

$$\sigma_e \quad \frac{\sigma_r + \sigma_z}{2}$$

so that

$$(121) \qquad \frac{\sigma_e}{\sqrt{\sigma_r \sigma_z}} \quad 1 \quad \text{or} \quad \frac{1}{x} + \frac{x}{4} \quad 1\ 10$$

The ratio between the radial gradients of (120) can be estimated by using (100a) and a table of K_1 For the Fourier components with frequencies of 1000 2000 Hz and with the values of the fibre radius and conduction velocity given in Section 8 we obtain

$$(122) \qquad \frac{\left.\frac{\delta \varphi_{iso}}{\delta r}\right|_{r\ a}}{\left.\frac{\delta \varphi_{iso}}{\delta r}\right|_{r\ \frac{ax}{2}}} \approx 1\ 6$$

From the curves of Fig 2 we get the following estimate:

$$(123) \qquad \frac{\varphi_{iso}(\frac{\pi a}{2} \quad x)}{\varphi_{iso}(a \quad)} \approx 0\ 75 \quad \text{or} \quad 0\ 83$$

Combining (120) (123) we finally obtain:

$$(124) \qquad \varphi_{an}(a\ x) \approx 1\ 05\ 1\ 6\ 0\ 75\varphi_{iso}(a\ x) \quad 1\ 26\varphi_{iso}(a\ x)$$

or

$$(125) \qquad \varphi_{an}(a\ x) \approx 1\ 05\ 1\ 6\ 0\ 83\varphi_{iso}(a\ x) \quad 1\ 38\varphi_{iso}(a\ x)$$

Thus taking the anisotropy into account we obtain for $\sigma_r/\sigma_z \quad 4/\pi^2$ an action potential amplitude which is 25-40 per cent higher just outside the fibre than under isotropic conditions if the conductivity σ_e in the isotropic case is given by (121) Similarly assuming $\sigma_r/\sigma_z \quad 1/5$ (117) shows that the amplitude just outside the fibre is about 50 per cent higher than in case of isotropy

Inserting (121) and (122) in (117) we obtain the following expression for the amplitude-distance relationship ($\sigma_r/\sigma_z \quad 4/\pi^2$):

$$\varphi_{an}(r\ x) \approx 1\ 68\ \varphi_{iso}(\frac{\pi r}{2} \quad x)$$

and for $\sigma_r/\sigma_z \quad 1/5$ the relation is

$$\varphi_{an}(r\ x) \approx 3\ \varphi_{iso}(r\sqrt{5} \quad x)$$

Section 10

The simulated intracellular action potentials

The preceding sections contain mathematical expressions to calculate the intracellular and the extracellular action potentials from the action potential measured just inside the membrane of the fibre

The applicability of the theory is tested by its ability to account for the extracellular action potentials observed experimentally The mathematical model can also be tested by means of a simulated intracellular action potential. Since some integrations can be performed analytically a *mathematical expression for the boundary* values simplifies the calculations

The simulated action potentials were chosen so as to approximate the intracellular action potentials of nerve and muscle fibres and to be mathematically simple without regard to membrane theories for the conduction of action potentials along excitable fibres as e g the Hodgkin Huxley equations

In preliminary calculations expressions of this type were used.

$$G(z) \begin{cases} Az^2 \exp(\lambda z) \quad B & \text{for} \quad z \geq 0 \\ B & \text{for} \quad < 0 \end{cases}$$

where z U t U being the conduction velocity A, B and λ are parameters They were adjusted to give reasonable fit with experimental resting and action potentials in nerve and muscle The factor exp(λ) was chosen since the later part of the phase of repolarization in a muscle fibre declines nearly exponentially Multiplying exp(λ z) by z^2 gave a simulation of both the phase of depolarization and of the initial phase of repolarization. In the calculations to be described z^2 was replaced by z^3 to avoid the discontinuity of the second derivative G (z) at 0

A The muscle action potential

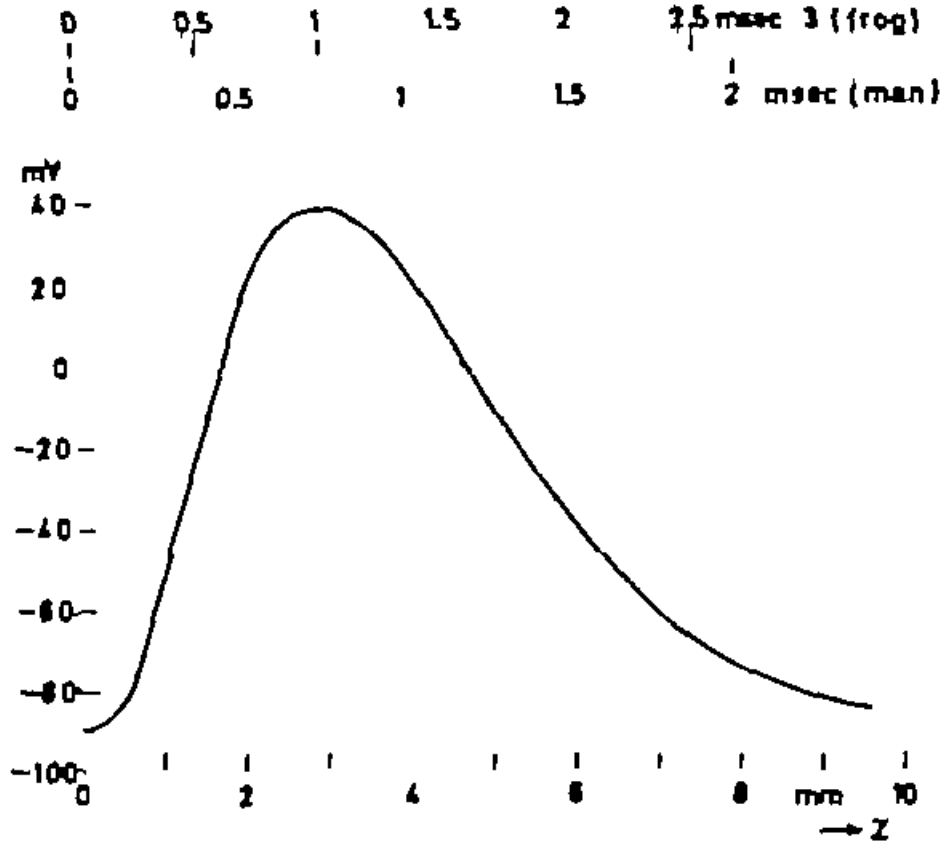


Fig 3 Simulated intracellular action potential of a muscle fibre

$$G(\ ) \begin{cases} 96\ ^{3}\exp(\ x) - 90 & \text{for} \quad x \geq 0 \\ -90 & \text{for} \quad x < 0 \end{cases}$$

x distance along the fibre in mm (abscissa below)
G potential in mV (ordinate)
Upper scale time in msec when the conduction velocity is 3 m/sec (frog) and 4 m/sec (man)

To simulate the intracellular action potential of a muscle fibre the following function was chosen.

$$(126) \qquad G(\ ) \begin{cases} 96\ ^{3}\exp(\ x) - 90 & \text{for} \quad x \geq 0 \\ -90 & \text{for} \quad x < 0 \end{cases}$$

where x is measured in mm and G in mV (Fig 3) From (126) it follows that

(127) $G(x) \quad 96x^2(3-x)\exp(-x) \quad \text{for} \quad x \geq 0$

and hence

$$G(x) \quad 0 \qquad \textit{for} \quad x \quad 0 \quad \text{and} \quad 3 \ \textit{mm}$$

i.e the action potential G(x) has its maximum at x 3 mm. With a conduction velocity of 3 m/sec (frog) or 4 m/sec (man) this corresponds to a duration of 1 or 0 75 msec for the phase of depolarization The maximum deflection is 129 mV

Regarding the second derivative we get from (127)

(128) $G''(x) \quad 96x(6-6x+x^2)\exp(-x) \quad \textit{for} \quad x \geq 0$

and therefore

$$G''(x) \quad 0 \quad \text{for} \quad x \quad 0 \quad \text{and} \quad x \quad 3 \pm \sqrt{3} \ \text{mm}$$

The corresponding maximum and minimum slopes of G are

$$G'_{max} \quad 75 \text{ mV/mm} \quad \text{at} \quad G \quad 35 \text{ mV}$$

and

$$G'_{min} \quad 33 \text{ mV/mm} \quad \text{at} \quad G \quad 0\ 4 \text{ mV}$$

With the conduction velocities of 3 and 4 m/sec this means that

$$G'_{max} \quad 225 \quad \text{or} \quad 300 \text{ V/sec}$$

and

$$G'_{min} \quad 98 \quad \text{or} \quad 131 \text{ V/sec}$$

Further differentiation of (128) gives:

(129) $G'''(x) \quad 96(6-18x+9x^2-x^3)\exp(-x) \quad \text{for} \quad x \geq 0$

From (129) it follows that

$$G''(z) = 0 \quad \text{for} \quad z = 0.42 \;\; 2.29 \;\; \text{and} \;\; 6.29 \text{ mm}$$

The corresponding maxima and minima of G are:

$G_{max\ 1}$ 97 mV/mm^2

G_{min} 66 mV/mm^2

$G_{max\ 2}$ 9 mV/mm^2

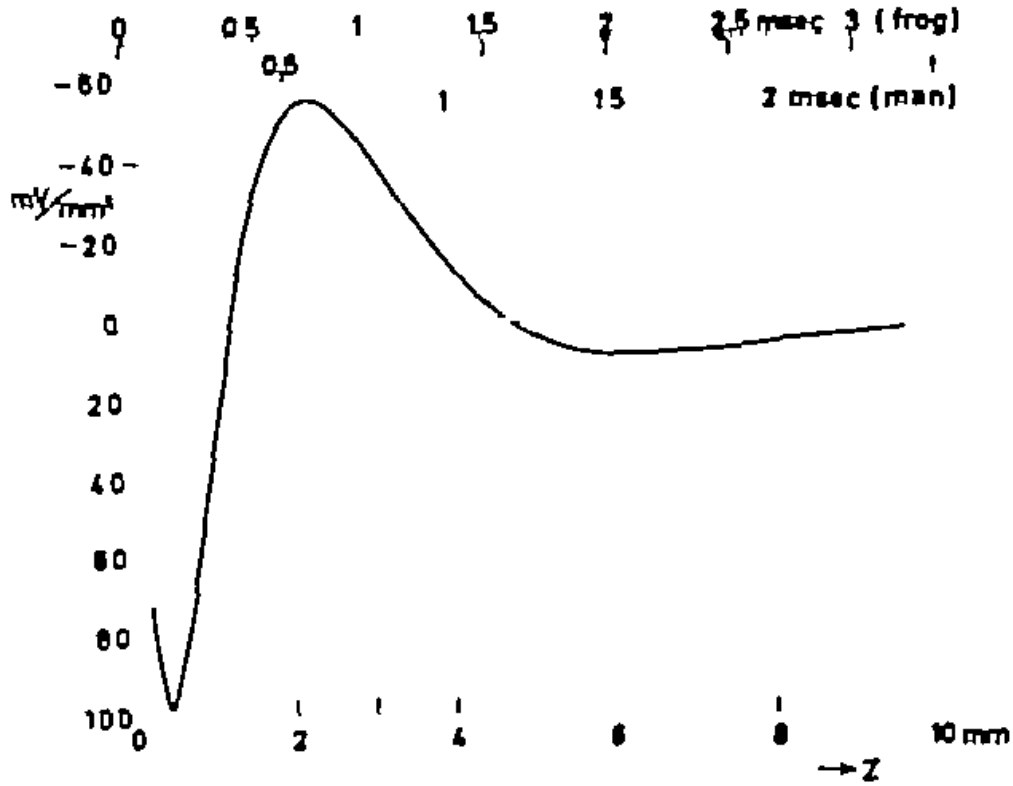


Fig 4. Second derivative of a simulated intracellular action potential of a musol fibre (see Fig 3)
Abscissae below distance along the fibre in mm (z); above: time in msec for a conduction velocity of 3 m/sec (frog 20 C) and 4 m/sec (man 36.5 C)

or in other units

$$G_{max\ 1} \quad 8\ 7\ 10^5 \text{ or } 15\ 8\ 10^5 \text{ V/sec}^2$$
$$G_{min} \quad 5\ 0\ 10^5 \text{ or } 8\ 9\ 10^5 \text{ V/sec}^2$$
$$G_{max\ 2} \quad 0\ 8\ 10^5 \text{ or } 1\ 4\ 10^5 \text{ V/sec}^2$$

Section 3 and 5 have shown that the density of the membrane current and the extracellular action potentials are closely related to the second derivative of the intracellular action potential Fig 4 gives the second derivative G (x) of the simulated intracellular action potential G is triphasic the duration of the initial positive phase being 0 42 or 0 32 msec of the negative phase 1 15 or 0 87 msec depending on the conduction velocity assumed

These data show that G(x) is a satisfactory representation of the intracellular muscle action potential

B The nerve action potential

An approximation to the intracellular action potential of a giant unmyelinated nerve fibre is given by

$$(130) \qquad G(x) = \begin{cases} 0\ 179\ ^3\ (20-x)\ \exp(\ 0\ 3\ x) \quad 60 & \text{for } \geq 0 \\ 60 & \text{for } x \quad 0 \end{cases}$$

where x is measured in mm and G in mV (Fig 5) Th slope of G is given by

$$(131) \qquad G(x) \quad 0\ 179x^2\ (60\ 10x+0\ 3x^2)\ \exp(\ 0\ 3\ x) \text{ for } x \geq 0$$

Hence the maximum and minimum of G occur at 7 85 mm and 25 5 mm The maximum deflection is 100 mV and the positive afterpotential is 7 8 mV Assuming a conducti n velocity of 20 m/sec the phase of depolarization lasts 0 4 msec and the spike 1 msec The maximum rate of rise is 430 V/se and of decline 240 V/sec occurring at 16 mV and at +2 mV respectively The second derivative of G obtained from (131) i

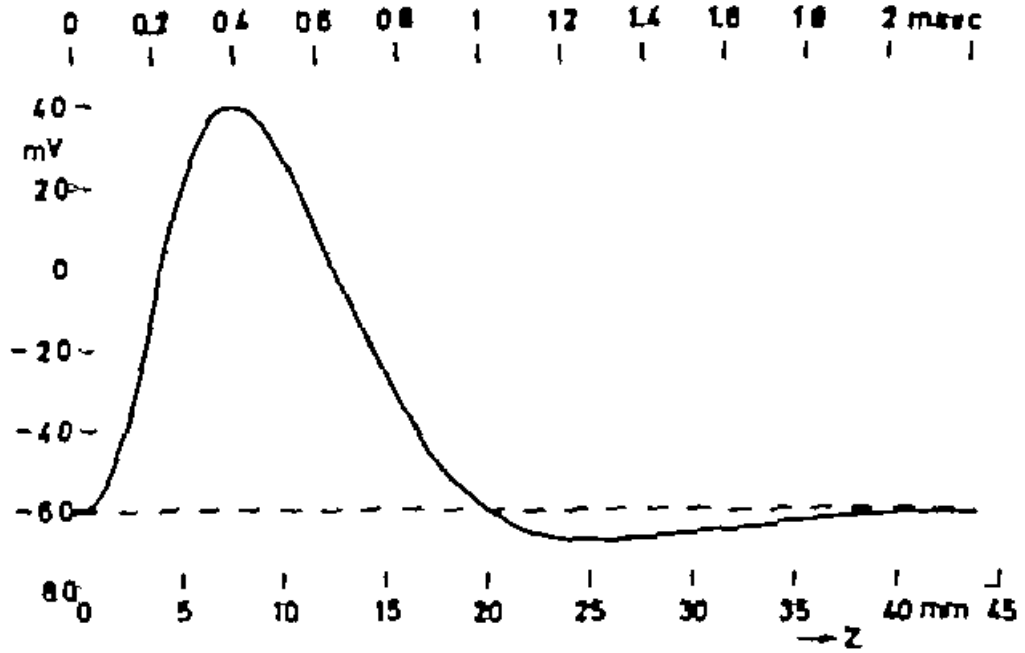


Fig 5 Simulated intracellular action potential of a non medullated quid giant nerve fibre

$$G(x) \begin{cases} 0.179\,x^3(20-x)\exp(-0.3x)-60 & \text{for } x \geq 0 \\ -60 & \text{for } x < 0 \end{cases}$$

x distance along the fibre in mm (abscissa below);
G potential in mV (ordinate)
Upper abscissa time in msec conduction velocity 20 m/sec 18 C

(132) $G'(x) = 0.179\,\{120 - 48x + 4.2x^2 - 0.09x^3\}\exp(-0.3x)$ for $x \geq 0$

and is plotted in Fig 6

Further differentiation leads to

(133) $G''(x) = 0.179\,\{120 - 152x + 27x^2 - 1.62x^3 + 0.027x^4\}\exp(-0.3x)$

and:

$$(134)\quad G^{zz}(z) \;=\; 0.179\,\{\,1.68+93.6z-12.96z^2+0.594z^3-0.0081z^4\}\,\exp(-0.3z)$$

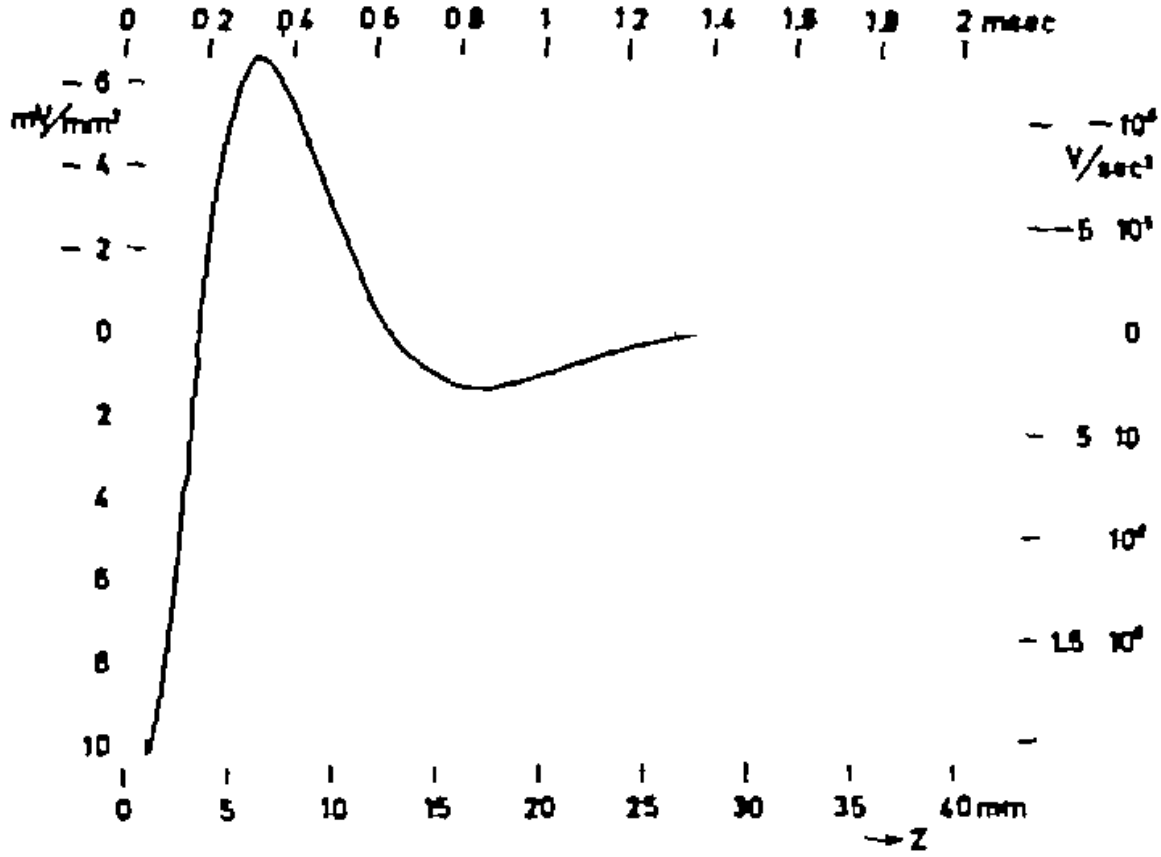


Fig 6 Second derivative of a simulated action potential of a non-medullated giant nerve fibre (see Fig 5) Abscissae below distance along the fibre in mm (z); above: time in msec conduction velocity 20 m/sec. Ordinates: second derivative left spatial in mV/mm^2 right temporal in V/sec^2

Section 11

The potentials produced by dipole and tripole generators

The simplest model used to interpret extracellular action potentials of muscle is the dipole concept applied to cardiac as well as to skeletal muscle (Wilson McLeod and Barker 1933 Hecht 1957 Craib and Canfield 1927) To account for the triphasic shape of the action potentials of skeletal muscle Craib (1928) considered two dipoles directed oppositely i.e a tripole

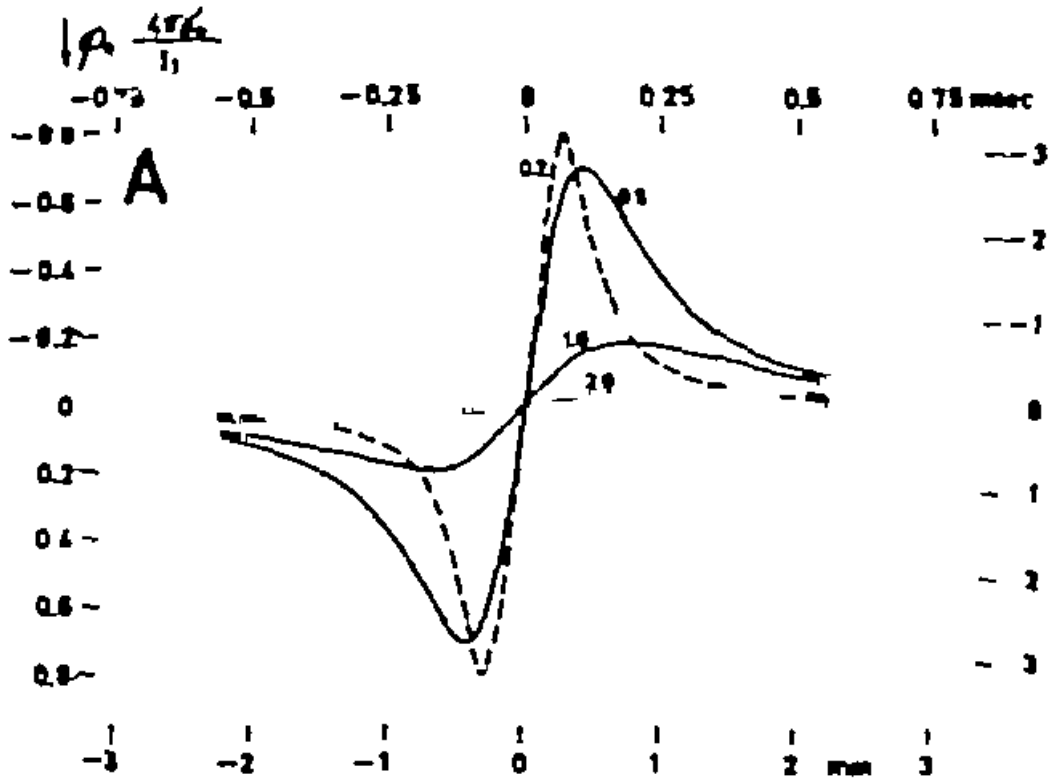
Let us consider point sources and sinks of current in an infinite isotropic and homogeneous medium of conductivity σ_e The arguments used in Section 1 to derive Laplace s equ tion for the scalar potential do not hold at the sources and sinks since the *equation of continuity (8) is not fulfilled at these points* At all other points Laplace equation still has to be satisfied. A current source of strength I_1 produces the spherically symmetric potential field.

$$\varphi_e(r) \quad \frac{I_1}{4\pi\sigma_e r}$$

where r is the distance from the source The potential produced by dipole generator consisting of a source I_1 and a sink I_1 a distance 2 apart is

$$\varphi_e(r \quad) \quad \frac{I_1}{4\pi\sigma_e}\left[\frac{1}{\sqrt{(z+a)^2+r^2}} \quad \frac{1}{\sqrt{(z \quad a)^2+r^2}}\right]$$

if th source i situated in (o a) and the sink in (o a) Examples of the action potentials of dipole generator with the poles 0 5 mm apart are given in Fig 7 A. These calculated action potentials are similar to th extracellular action potentials of single frog muscl fibres in Ringer solution (Håkanasson 1957a) except that the radial decline of the calculated ction potentials i somewhat f ster (Fig 7 B) Thus in the experiments the peak to peak amplitude 2 mm from the fibre axis was 4 per cent of that measured at a distance of 0 2 mm; the corresponding calculated amplitude was 1 6 pe cent.



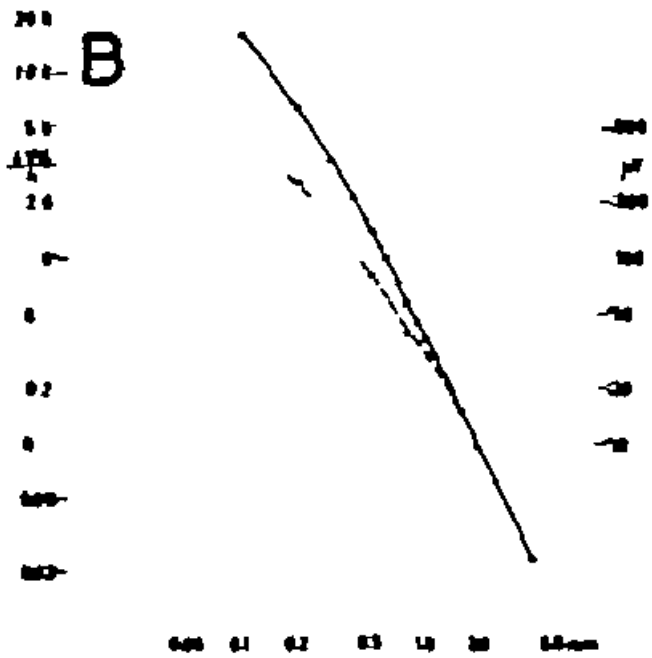


Fig 7

Let us next consider a tripole generator consisting of two sources I_1 and I_3 and a sink I_2 situated on a common axis so that I_1 is at (0 s) I_2 at (0 z) and I_3 at (0 u)

Let us assume that

$$I_1 \quad \alpha \; I_2$$

$$I_3 \quad (1 \quad \alpha)\, I_2$$

The potential produced by the tripole in the infinite conductor then is

$$\varphi_e(r\ z) \quad \frac{I_2}{4\pi\sigma_e}\left[\frac{\alpha}{\sqrt{(z+s)^2+r^2}} \quad \frac{1}{\sqrt{(z\ s)^2+r^2}} + \frac{1\ \alpha}{\sqrt{(z\ u)^2+r^2}}\right]$$

An example of a tripole action potential is given in Fig 8 The positions and strengths of sink and sources were chosen on the basis of the experimental intracellul r action potentials (p 88) The calculated action potential was triphasic the amplitude of the t rminal positive phase being 15 per c nt of the peak to-peak amplitude

Fig 7

Action potential and their radial decline calculated for a dipole generator moving t constant speed in the axial direction.
A. Action potentials t different distances (given in mm on the curve) from the axis of the dipole generator A current source of strength I_1 and sink I_1 0 5 mm apart were surrounded by a medium of conductivity σ_e

Ordinate potential in unit of $I_1/4\pi\sigma_e$ th scale to the right refers to the broken curve
Ab ci sa below distance along the axi of the dipole (in mm) above tim n msec conduction velocity 4 m/sec

B Peak to-peak amplitude of th action potentials as a function of the distan from the axis of th dipol generator (●——●) as compared with the relation for a frog muscle fibre (●– –○) (radiu 41μ Håkansson (1957a))

Ordinate: left amplitude of dipole action potential in units $I_1/4\pi\sigma_e$; right amplitude of frog action pot ntial in μV
Abscissa distance from the axis of the dipole or the muscle fibre

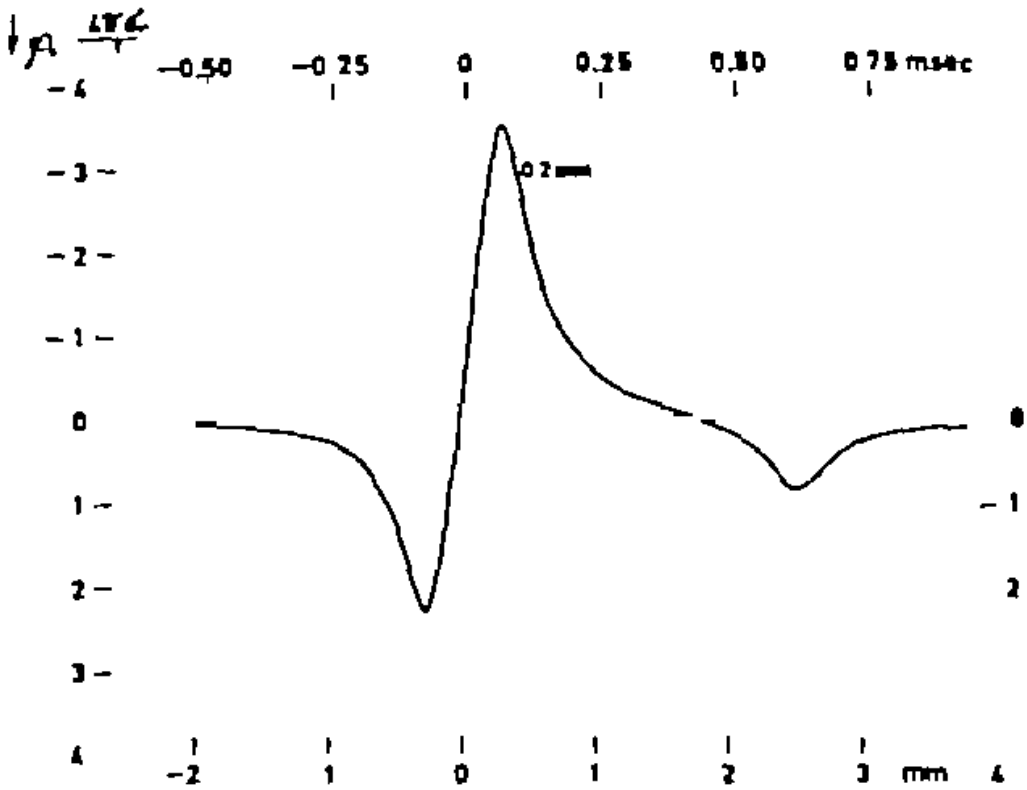


Fig 8 Action potential 0 2 mm from the axis of a tripole generator. A current source of strength 0 8 I_2 a sink I_2 0 5 mm from it and a source 0 2 I_2 2 25 mm further away; surrounded by a medium of conductivity σ_e

Ordinate potential in units of $I_2/4\pi\sigma_e$

Abscissa, below: distance along the axis of the tripole (in mm)

above time in msec conduction velocity 4 m/sec

PART *

QUANTITATIVE TESTS AND APPLICATION OF THE MATHEMATICAL THEORY

Section 1

The intracellular action potentials

The radial variation of the action potential within a nerve or muscle fibre is usually assumed to be negligible (e g Taylor 1963) That this is justified can be shown by the following considerations and calculation

From Hâkansson s (1957) measurements on single frog muscle fibres the radial gradient of the extracellular potential field is at most 3 mV/mm. With a specifi resistance of 87 Ω cm in the Ringer solution and 230 Ω cm in the sarcoplasm the radial variation inside the fibre amounts to at most 3 $\frac{230}{87}$ 8 mV/mm In a fibre 100 μ in diameter this corresponds to a radial variation in potential of no more than 8 0 05 0 4 mV Although the radial variation is negligibl the second derivative of the potential in the radial direction cannot be disregarded. The second derivative may be s great as 8/0 05 160 mV/mm^2 f the same ord r as th second deri vative in th fibre direction In the muscle fibres considered in my calculati ns it was at most 700 mV/mm^2 (p 92)

A more exact expression f the variation of the action potential within a fibre can be obtained from (30)

$$\varphi_1(\quad) \quad \frac{1}{2}\int_{-\infty}^{\infty} \frac{I_0(rp)}{I'(ap)} \exp(\,ip\,) \left\{ \int_{-\infty}^{\infty} V_1(u)\, \exp(ipu)du \right\} dp$$

wh re φ_1 and V_1 are m asured from th resting leval To analyse this potential the simulated ction potentials (p 58) were used

A. The simulated muscle fibre

Following (126) we put

$$V_1(u) \begin{cases} 96u^3\exp(-u) & \text{for } u \geq 0 \\ 0 & \text{for } u < 0 \end{cases} \tag{135}$$

Inserting (135) in (30) integration gives:

$$\varphi_1(r,x) \frac{288}{\pi} \int_{-\infty}^{\infty} \frac{I_o(rp)}{I_o(ap)} \frac{\exp(-ipx)}{(ip-1)^4}\, dp \tag{136}$$

I_o is a symmetric function and (136) is transformed to

$$\varphi_1(r,x) \frac{288}{\pi} \int_0^{\infty} \frac{I_o(rp)}{I_o(-p)} \left\{ \frac{\exp(-ipx)}{(ip-1)^4} + \frac{\exp(ip)}{(ip+1)^4} \right\} dp$$

from which is found

$$\varphi_1(r,x) \frac{576}{\pi} \int_0^{\infty} \frac{I_o(rp)}{I_o(ap)} \frac{(p^4-6p^2+1)\cos p}{(1+p^2)^2} \frac{(4p^3-4p)\sin px}{} dp \tag{137}$$

Let us introduce a phase angle $\theta(p)$ to reformulate (137) in a more convenient way:

$$\cos\theta(p) \frac{p^4-6p^2+1}{(1+p^2)^2} \tag{138}$$

$$\sin\theta(p) \frac{4(p^3-p)}{(1+p^2)^2} \tag{139}$$

i.e.

$$\theta(p) \tan^{-1} \frac{4(p^3-p)}{p^4-6p^2+1} \tag{140}$$

(137) can now be written

$$(141)\quad \varphi_1(r\ z)\quad \frac{576}{\pi}\quad \int_0^{\infty} \frac{I_o(rp)}{I_o(ap)}\ \frac{\cos(pz+\theta(p))}{(1+p^2)^2}\ dp$$

With $I_o(o)$ 1 the potential on the axis of the fibre is

$$\varphi_1(o\ z)\quad \frac{576}{\pi}\quad \int_0^{\infty} \frac{1}{I_o(ap)}\ \frac{\cos(pz+\theta(p))}{(1+p^2)^2}\ dp$$

Just inside the surface of the fibre the potential is:

$$(142)\quad \varphi_1(a\ z)\quad \frac{576}{\pi}\quad \int_0^{\infty} \frac{\cos(pz+\theta(p))}{(1+p^2)^2}\ dp$$

This results in a total radial variation of the potential of

$$(143)\quad \varphi_1(a\ )-\varphi_1(o\ z)\quad \frac{576}{\pi}\quad \int_0^{\infty} \left\{1\quad \frac{1}{I_o(ap)}\right\} \frac{\cos(pz+\theta)}{(1+p^2)^2}\ dp$$

$\theta(p)$ defined by (138) (140) is a monotonically decreasing function and approach s an asymptotic valu as $p \to \infty$ (Fig 8) Thus $\theta(p)$ has a negative slope which goes towards zero

$$\frac{d\theta}{dp}\quad \frac{4}{1+p^2}$$

θ varies only slightly fo $p > 4$ mm ^{1}

The integral (143) can be evaluated numerically Values of p for which $ap > 0\ 5$ hardly contribute to it Introducing

$$1\quad \frac{1}{I_o(\ p)} \approx \frac{(ap)^2}{4}$$

(Carslaw & Jaeger 1959) in (143) a simple approximate expression is derived

$$(144)\quad \varphi_1(a\ z)\ \varphi_1(o\ z) \approx \frac{144\,a^2}{\pi}\quad \int_0^{\infty} \frac{p^2\cos(p\ +\theta)}{(1+p^2)^2}\ dp$$

From (142) follows that

density of the longitudinal current decreases insignificantly (probably less than 1 per cent) towards the axis of the fibre (Table 4)

Table 4

p (mm $^{-1}$)	0	5	10	15
$1/I_0(0\ 05\ p)$	1	0 9845	0 9403	0 8729

B The simulated nerve fibre

In accordance with (130) we put

$$V_1(u) \begin{cases} 0\ 179u^3(20\ u)\exp(-0\ 3u) & \text{for } u \geq 0 \\ 0 & \text{for } u < 0 \end{cases} \tag{147}$$

Inserting this in the expression for the intracellular action potential (30) integration after u give

$$\varphi_1(r\ z)\ \frac{10\ 74}{\pi} \int_{-\infty}^{\infty} \frac{I_0(rp)}{I_0(ap)}\ \frac{ip\ 0\ 5}{(ip-0\ 3)^5}\ \exp(-ipz)dp$$

The symmetry of I_0 gives

$$\varphi_1(r\ z)\ \frac{10\ 74}{\pi} \int_0^{\infty} \frac{I_0(rp)}{I_0(ap)} \left\{ \frac{ip-0\ 5}{(ip-0\ 3)^5}\ \exp(-ipz) + \frac{ip+0\ 5}{(ip+0\ 3)^5}\ \exp(ipz) \right\} dp \tag{148}$$

which can be written as

$$\varphi_1(r\ z)\ \frac{21\ 48}{\pi} \int_0^{\infty} \frac{I_0(rp)}{I_0(ap)}\ \frac{1}{(0\ 09+p^2)^5} [(p^5\ 0\ 15p^4\ 0\ 0945p^2+0\ 001215)\cos pz + (p^5+0\ 15p^3\ 0\ 01782p)\sin pz]\ dp$$

To simplify this expression the phase angles $\theta_1(p)$ and $\theta_2(p)$ are introduced

$$(149)\qquad \begin{cases} \cos\theta_1(p) & \dfrac{0.5}{\sqrt{0.25+p^2}} \\ \sin\theta_1(p) & \dfrac{p}{\sqrt{0.25+p^2}} \end{cases}$$

i.e.

$$(150)\qquad \theta_1(p) \quad \tan^{-1} 2p$$

Similarly

$$(151)\qquad \begin{cases} \cos\theta_2(p) & \dfrac{0.3}{\sqrt{0.09+p^2}} \\ \sin\theta_2(p) & \dfrac{p}{\sqrt{0.09+p^2}} \end{cases}$$

i.e.

$$(152)\qquad \theta_2(p) \quad \tan^{-1}\frac{p}{0.3}$$

With

$$(153)\qquad \theta(p) \quad 5\theta_2 \quad \theta_1$$

(148) can be written as

$$(154)\quad \varphi_1(r,x) \quad \frac{21.48}{x}\int_0^{\infty}\frac{I_0(rp)}{I_0(ap)}\,\frac{(0.25+p^2)^{1/2}}{(0.09+p^2)^{5/2}}\,\cos(px-\theta(p))\,dp$$

$\theta(p)$ increases monotonically with p but changes only little for $p > 2\ \mathrm{mm}^{-1}$ (Fig. 10). For unmyelinated nerve fibres the radius a can be as great as 0.2–0.35 mm (Spector 1956). However values of $p < 1\ \mathrm{mm}^{-1}$ contribute insignificantly to the integral (154)

density of the longitudinal current decreases insignificantly (probably le s than 1 per cent) towards the axis of the fibre (Table 4)

Table 4

p (mm ^{1})	0	5	10	15
$1/I_0(0\ 05\ p)$	1	0 9845	0 9403	0 8729

B The simulated nerve fibre

In accordance with (130) we put

$$(147) \qquad V_1(u) \begin{cases} 0\ 17\,u^3(20\ u)\exp(\ 0\ 3u) & \text{for } u \geq 0 \\ 0 \quad \text{for} \quad u < 0 \end{cases}$$

Inserting this in the expression for the intracellular action potential (30) integration after u gives

$$\varphi_1(r\ z)\ \frac{10\ 74}{\pi}\int_{-\infty}^{\infty}\frac{I_0(rp)}{I_0(ap)}\ \frac{ip-0\ 5}{(ip-0\ 3)^5}\ \exp(\ ipz)dp$$

Th symmetry of I_0 gives

$$(148) \quad \varphi_1(r\ z)\ \frac{10\ 74}{\pi}\int_{0}^{\infty}\frac{I_0(rp)}{I_0(ap)}\left\{\frac{ip\ 0\ 5}{(ip-0\ 3)^5}\ \exp(\ ip\ )\right.$$

$$\left. +\ \frac{ip+0\ 5}{(ip+0\ 3)^5}\ \exp(ipz)\right\}dp$$

which can be written as

$$\varphi_1(r\ )\ \frac{21\ 48}{\pi}\int_{0}^{\infty}\frac{I_0(rp)}{I_0(ap)}\ \frac{1}{(0\ 09+p^2)^5}$$

$$[(p^6\ 0\ 15p^4\ 0\ 0945p^2+0\ 001215)\cos pz$$

$$(p^5+0\ 18p^3\ 0\ 01782p)\sin pz]\,dp$$

Therefore we can assume $a_p < 0.4$ and the radial variation of the action potential within the nerve fibre is approximated by (146) The radial difference varies between 0.31 mV and +0.20 mV (Table 5)

As in the muscle fibre expression (148) for the intracellular action potentials shows that the longitudinal current density $\sigma_i \frac{\partial \varphi_i}{\partial x}$ varies only little over the cross section of the nerve fibre

Section 2

The density of the membrane current

General expressions for the density of the membrane current are given by (33) (34) (38) and (40) e g

$$(39)\quad i_m(z) \quad \frac{\sigma_i a}{2} \frac{d^2 V_i}{dz^2} + \frac{\sigma_i a}{4\pi} \int_{-\infty}^{\infty} \frac{p^2 I_2(ap)}{I_0(ap)} \exp(ipz) \left\{ \int_{-\infty}^{\infty} V_i(u) \exp(ipu)du \right\} dp$$

A. The "muscle fibre

Using the simulated muscle action potential (126) th procedure described in Section 1 gives

$$(155)\quad i_m(\ ) \quad \frac{\sigma_i a}{2} \frac{d^2 V_i}{dz^2} + \frac{288\sigma_i a}{\pi} \int_0^{\infty} \frac{p^2 I_2(\ p)}{I_0(ap)} \frac{\cos(p\ +\theta(p))}{(1+p^2)^2} dp$$

According to (145)

$$(156)\qquad \frac{d^2 V_i}{dz^2} \quad \frac{576}{\pi} \int_0^{\infty} \frac{p^2 \cos(p\ +\theta)}{(1+p^2)^2} dp$$

and (155) an be rewritten s

$$(157)\quad i_m(z) \quad \frac{288\sigma_i a}{\pi} \int_0^{\infty} \left\{ 1 \quad \frac{I_2(ap)}{I_0(ap)} \right\} \frac{p^2 \cos(p\ +\ )}{(1+p^2)^2} dp$$

Hence the influence of the second term in (155) is determined by $\frac{I_2(ap)}{I_0(ap)}$

(157) shows that p-value above 10 mm ^{1} contribute only litl to the membrane current and that the econd term in (155) only a few per cent of the first term (Table 6) A better estimate is obtained by means of the following pproximations which are valid for small values of ap

Table 6

p (mm^{-1})	$\frac{I_2(0\ 05\ p)}{I_o(0\ 05\ p)}$
0	0
4	0 005
6	0 011
8	0 020
10	0 030
16	0 072
20	0 107
24	0 145

(158) $$I_o(ap) \approx 1 + \frac{a^2}{4} p^2$$

(159) $$I_2(ap) \approx \frac{a^2}{8} p^2$$

Therefore

(160) $$\frac{2\pi\sigma_1 a}{\kappa} \int_0^\infty \frac{p^2 I_2(ap)}{I_o(ap)} \frac{\cos(px+\theta)}{(1+p^2)^2} dp$$

$$\approx \frac{3\pi\sigma_1 a^3}{\kappa} \int_0^\infty \frac{p^4 \cos(px+\theta)}{(1+p^2)^2} dp$$

According to (156)

$$\frac{d^4 V_1}{dx^4} \frac{576}{\kappa} \int_0^\infty \frac{p^4 \cos(px+\theta)}{(1+p^2)^2} dp$$

Hence (160) can be expre sed as

$$\frac{2\pi\sigma_1 a}{\kappa} \int_0^\infty \frac{p^2 I_2(ap)}{I_o(ap)} \frac{\cos(p\ +\theta)}{(1+p^2)^2} dp \approx \frac{\sigma_1 a^3}{16} \frac{d^4 V_1}{dx^4}$$

Table 7

The ratio α between the second and the first term of (155)

z(mm)	α
0 4	2 3 10 ^{3}
1 2	4 0 10 ^{3}
2 2	0 5 10 ^{3}
4 8	0 1 10 ^{3}
6 4	0 1 10 ^{3}
8 2	0 1 10 ^{3}

The ratio α between the second and the first t rm of (155) is therefore

$$\alpha \approx \frac{a^2}{8} \frac{\frac{d^4V_i}{dx^4}}{\frac{d^2V_i}{dx^2}} \tag{161}$$

Using (135) and differentiating we get

$$\approx \frac{a^2}{8} \quad 3 \quad \frac{12z^2 + 36z \quad 24}{3 \quad 6z^2 + 6z}$$

With 0 05 mm $|\alpha|$ is less than 4 10 ^{3} (Table 7) i e the econd term in (155) is n gligible so that

$$i_m(z) \approx \frac{\sigma_i a}{2} \frac{d^2V_i}{dx^2} \tag{162}$$

(162) is the membrane current derived from th core conductor model (cf (47))

Introducing the temporal derivative in stead of the spatial and the conduction velocity U of the action potential (162) becomes

$$(163) \qquad i_m(t) \approx \frac{\sigma_i a}{2U^2} \frac{d^2V_i}{dt^2}$$

B The nerve fibre

Similar considerations can be applied to the artificial nerve action potential (130) Inserting (130) in (39) gives:

$$(164) \quad i_m(z) \quad \frac{\sigma_i a}{2} \frac{d^2V_i}{dz^2} + \frac{10\ 74\sigma_i a}{\pi} \int_0^{\infty} \frac{I_2(ap)}{I_o(ap)} \frac{p^2\ (0\ 25+p^2)^{1/2}}{(0\ 09+p^2)^{5/2}} \cos(pz-\theta)\,dp$$

Using (154) this can be transformed to

$$(165) \quad i_m(\ ) \quad \frac{10\ 74\sigma_i a}{\pi} \int_0^{\infty} \left\{1 \quad \frac{I_2(ap)}{I_o(ap)}\right\} \frac{p^2\ (0\ 25+p^2)^{1/2}}{(0\ 09+p^2)^{5/2}} \cos(pz\ \theta)\,dp$$

Thus also in the nerve fibre the second term of (164) is determined by $I_2(ap)/I_o(ap)$ The integrand of (165) declines so rapidly that p-value above 3 mm ^{1} contribute insignificantly and the main contribution is due to p-values which are smaller than 1 mm ^{1}

This implies that for non-myelinated giant axons the second term of (164) amounts to only a few per cent of the first term (Table 8)

Table 8

$p(mm^{-1})$	$\frac{I_2(0\ 35\ p)}{I_o(0\ 35\ p)}$	$\frac{I_2(0\ 2p)}{I_o(0\ 2p)}$
0	0	0
1	0 016	0 005
2	0 057	0 020
3	0 116	0 043
4	0 185	0 072
6	0 320	0 145

Section 3

The surface action potential

The approximate expression (81) for the action potential recorded on the surface of a thin layer of conducting fluid around a fibre contained as the first term the extracellular potential calculated from the linear core-conductor model (45) (81) can with (82) and (83) be written:

$$(166)\quad \varphi_e(b\ z) \approx \frac{r_e}{r_i}\ V_i(z) + \frac{r_e}{2\pi r_i} \int_{-\infty}^{\infty} \frac{I_2(ap)}{I_o(ap)} \exp(\ ipz) \left\{ \int_{-\infty}^{\infty} V_i(u)\ \exp(ipu)du \right\} dp$$

Inserting (80) in (166) allows an estimate of the second term of (166)

$$(167)\quad \varphi_e(b\ \ ) \approx \frac{r_e}{2\pi r_i} \int_{-\infty}^{\infty} \left[1 \quad \frac{I_2(ap)}{I_o(ap)} \right] \exp(\ ipz) \left\{ \int_{-\infty}^{\infty} V_i(u)\ \exp(ipu)du \right\} dp$$

A. The muscle fibre

Introducing the simulated intracellular muscle action potential (126) in (167) we obtain (cf Section 1):

$$(168)\quad \varphi_e(b\ \ ) \approx \frac{575\, r_e}{\pi r_i} \int_0^{\infty} \left\{ 1 \quad \frac{I_2(ap)}{I_o(ap)} \right\} \frac{\cos(px + \theta(p))}{(1+p^2)^4}\, dp$$

where $\theta(p)$ is given by (140) $I_2(ap)/I_o(ap)$ increases slowly with p and i only 0 011 if p 6 mm^{-1} and a 0 05 mm (Table 6) The integrand of (168) declines so rapidly with increasing p that p-value exceeding e g 6 mm^{-1} can be disregarded. Therefore the second term of (166) which corresponds to the I_2/I_o term of (168) is less than 1 per cent of the first term of (166) Hence:

$$(169) \qquad \varphi_e(b\ x) \approx \frac{r_o}{r_i}\ V_i(x)$$

$V_i(\ )$ being measured from the resting level.

β The nerve fibre

Use of the simulated intracellular nerve action potential (130) leads to the same result as obtained with the simulated muscle action potential Thus (167) gives:

$$(170) \quad \varphi_e(b\ x) \approx \frac{21\ 48\, r_o}{\pi r_i} \int_0^\infty \left\{1 - \frac{I_2(\pi p)}{I_0(\pi p)}\right\} \frac{(0\ 25+p^2)^{1/2}}{(0\ 09+p^2)^{5/2}} \cos(p\pi\ \theta(p))\, dp$$

where $\theta(p)$ is given by (153) Inspection of the integrand shows that p-values above 2 mm^{-1} only contribute little to the integral and the second term of (168) is at most a few per cent of the first (Table 8) Hence (169) expresses the surface action potential to good approximation.

Section 4

The extracellular action potentials in an unlimited conductor

A. The simulated muscle fibre

Inserting the intracellular muscle action potential (126) in the expression (57) we get.

$$(171)\quad \varphi_e(r\ z) \quad \frac{288\sigma_i}{\pi\sigma_e} \int_{-\infty}^{\infty} \frac{I_1(a|p|)K_0(r|p|)}{I_0(ap)\ K_1(a|p|)} \frac{\exp(\ ipz)}{(ip\quad 1)^4}\ dp$$

As in the case of the intracellular potential (cf p 70) (171) is transformed to:

$$(172)\quad \varphi_e(r\ z) \quad \frac{576\sigma_i}{\pi\sigma_e} \int_0^{\infty} \frac{I_1(ap)K_0(rp)}{I_0(ap)K_1(ap)} \frac{\cos(pz + \theta(p))}{(1+p^2)^2}\ dp$$

where $\theta(p)$ is defined by (138) (140) I_1 can be expressed in terms of I_0 and I_2 (Carslaw & Jaeger 1959)

$$(173)\quad \varphi_e(r\ \ ) \quad \frac{288\sigma_i a}{\pi\sigma_e} \int_0^{\infty} \left\{1 \quad \frac{I_2(ap)}{I_0(ap)}\right\} \frac{K_0(rp)}{K_1(ap)} \frac{p}{(1+p^2)^2}$$

$$\cos(pz + \theta)dp$$

With a 0 05 mm the integrand of (173) declines rapidly and the contribution from p-value above 8 mm $^{-1}$ (i.e ap > 0 4) to the potential is negligible as assumed in deriving the line integral expression (62) for the extracellular action potentials in terms of the density of the membrane current $i_m(z)$ distributed as sources and sinks along th axis of the fibre:

$$(63)\qquad \varphi_e(r\ z) \approx \frac{a}{2\sigma_e} \int_{-\infty}^{\infty} \frac{i_m(s)}{\sqrt{r^2 + (z\ s)^2}}\ ds$$

The assumption that p-values above 8 mm $^{-1}$ can be neglected lead also to the expression (66) in which the virtual sources and sinks of the potential were located at the surf ce of the fibre.

$$(169) \qquad \varphi_e(b\ z) \approx \frac{r_e}{r_i}\ V_i(z)$$

$V_i(z)$ being measured from the resting level.

B. The nerve fibre

Use of the simulated intracellular nerve action potential (130) leads to the same result as obtained with the simulated muscle action potential. Thus (167) gives

$$(170) \qquad \varphi_e(b\ z) \approx \frac{21\ 48\ r_e}{\pi r_i} \int_0^\infty \left\{1 - \frac{I_2(ap)}{I_0(ap)}\right\} \frac{(0\ 25+p^2)^{1/2}}{(0\ 09+p^2)^{5/2}} \cos(pz\ \theta(p))\,dp$$

where $\theta(p)$ is given by (153). Inspection of the integrand shows that p-values above 2 mm^{-1} only contribute little to the integral and the second term of (166) is at most a few per cent of the first (Table 8). Hence (169) expresses the surface action potential to good approximation.

Table 8

Lorente de Nó type of approximation (173) for the extra cellul r action potentials tested by means of the simulated intrac llular muscle action potential (126)

distance along fibre( )	$\frac{d^2V_i}{dz^2}$	A(z) $\frac{d^2V_i}{dz^2}$)
mm	mV/mm^2	mV/mm^2
0 2	0	0 450
0 1	0	1 415
0	0	8 606
0 1	46 99	0 070
0 2	76 08	3 046
0 4	96 78	2 408
0 8	63 49	0 169
1 2	8 33	0 442
1 6	32. 25	0 605
2 0	51 97	0 472
2 8	48 38	0 112
3 6	24 83	0 037
4 4	5 39	0 068
5 2	5 07	0 049
6 0	5 51	0 017

) A( ) defined by (179)

To compare $_{1m\ 1}(z)$ and $\frac{\sigma_i a}{2}\ \frac{d^2V_i}{dz^2}$ the following integral wa cal culated for the simulated intracellular muscle action potential (126) nd its second derivative (126):

$$(178) \qquad A(z) \quad \frac{a^2}{2} \int_{-\infty}^{\infty} \frac{\frac{d^2V_i}{ds^2}}{\{(\ \ s)^2+a^2\}^{3/2}}\ ds$$

assuming $\underline{a}$ 0 05 mm. A(z) deviated only slightly from $\frac{d^2V_1}{dz^2}$ (Table 9) Thus the initial positive peak and the negative peak of A(z) were 2 5 and 0 9 per cent lower than those of $\frac{d^2V_1}{dz^2}$ and the initial positive phase of A(z) was only a small fraction of a millimetre wider than that of $\frac{d^2V_1}{dz^2}$

Hence it is justified to apply Lorente de Nó-type approximations in the calculations of extracellular action potentials of muscle fibres.

B The simulated nerve fibre potential

Introducing (130) in (57) we get

$$(180)\quad \varphi_e(r\ z) \quad \frac{21\ 48\sigma_i}{\pi\sigma_e} \int_{-\infty}^{\infty} \frac{I_1(a|p|)K_0(r|p|)}{I_0(ap)\ K_1(a|p|)} \exp(\ ipz) \frac{ip\quad 0\ 5}{(ip\quad 0\ 3)^5}\ dp$$

In analogy to (135) this can be transformed to

$$(181)\quad \varphi_e(r\ z) \quad \frac{42\ 96\sigma_i}{\pi\sigma_e} \int_0^{\infty} \frac{I_1(ap)K_0(rp)}{I_0(ap)K_1(ap)} \frac{(0\ 25 + p^2)^{1/2}}{(0\ 09 + p^2)^{5/2}} \cos(pz\quad \theta)\,dp$$

Introducing I_2 in place of I_1 we get

$$(182)\quad \varphi_e(r\ z) \quad \frac{21\ 48\sigma_i a}{\pi\sigma_e} \int_0^{\infty} \left\{1\quad \frac{I_2(ap)}{I_0(ap)}\right\} \frac{K_0(rp)}{K_1(ap)} \frac{p(0\ 25 + p^2)^{1/2}}{(0\ 09 + p^2)^{5/2}} \cos(pz\quad \theta)\,dp$$

The integrand of (182) declines rapidly and p-values > 2 mm (i.e. $ap > 0.7$ and $ap > 0\ 4$ for a 0 35 mm and 0 2 mm) give an

insignificant contribution. Hence the line and surface integrals (174) and (175) are valid approximations in the case of nerve fibres with a 0 2 mm as well as for the muscle fibre They are probably also applicable to thicker nerve fibres since ap K_1(ap) 0 735 at ap 0 7 i.e not markedly different from 1 (cf p 31)

C Extracellular action potentials calculated from measured intracellular potentials

To study the properties of the extracellular action potential as given by the approximate solution (174) I have used measured intracellular action potentials in frog (Buchthal and Engbæk unpublished) rat (Mu choll 1957 Fig 5b) and human muscle fibres (Lædin unpublished) (Fig 11) The rat muscle fibre potential was recorded far from the end plate of a fibre in an isolated diaphragm kept in Tyrode-solution at 21 C The resting potential was 90 mV the overshoot during the action potential 33 mV The conduction velocity not availabl for thi particular fibre was assumed to be 2 m/sec This was the average propagation velocity along the fibres of the diaphragm which varied little from fibre to fibre (Muscholl 1957) Th action potentials in the frog were derived from single fibres of isolated sartorius muscles kept in Ringer's solution at 25 C and 18 C The resting potentials were 75 and 73 mV; the overshoot during the action potential was 23 mV and 38 mV respectively and th conduction velocity was 3 3 m/sec and 1 8 m/sec Although an overshoot of 23 mV might indicate that th fibre has not been in the best physiological state (see p 98) calculations were made also on this fibre since the conduction velocity was normal and the rate of rise was only slightly reduced (Buchthal and Engbæk 1963) The action potential from man was recorded in excised intercostal muscl of a 45 year old man. The tissue was kept in Tyrode s solution containing 5 4 mM potassium (37 C) A 20 MΩ micro el ctrode was used fo the recording The peak deflection was 97 mV and the maximum rate of rise 420 V/sec. The resting potential most probably was 75–80 mV (Lædin 1958) The second derivative of the action potential was measured by means of two

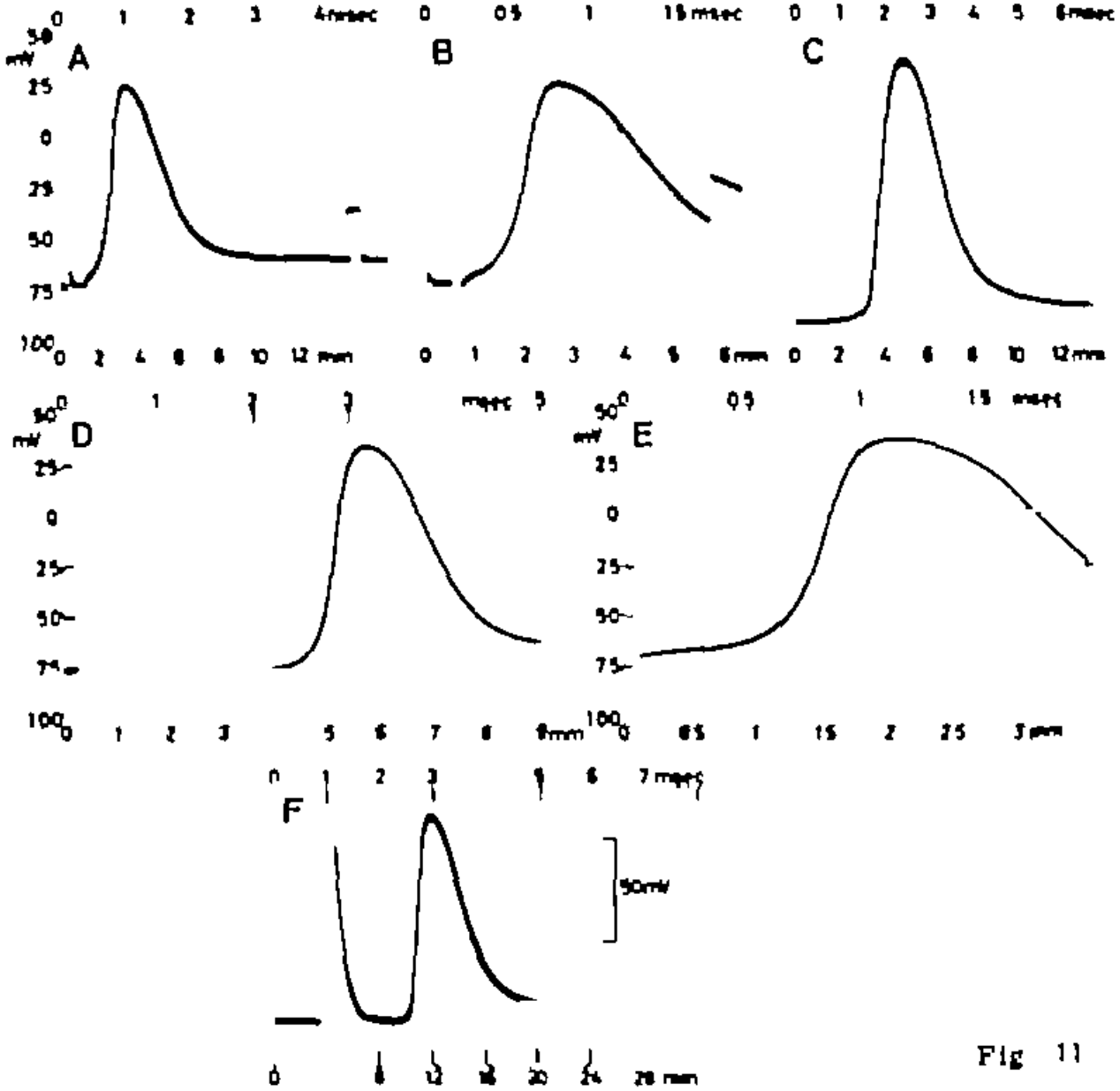


Fig 11

RC units with a time constant of 20 μsec Th conduction velocity was not determined A value of 4 m/sec was assumed in the calculations (Buchthal Guld and Rosenfalck 1955a)

The second derivative of the intracellular muscle action potentials was determin d graphically on photographic enlargements (10×) (Fig 12) The temporal and spatial derivatives are related by (18) Th se distributions of sources and sinks show that the dipole and the tripole model (p 65 Figs 7 and 8) represent approximation to the Lorente d Nó type of model.

The radii of the fibres were not known However Muscholl (1957) found th t the amplitude and the time course of the intracellular action potential were independent of the fibre thickne s and we have chosen a representative value for the radius of 0 05 mm for the mu cle fibres of the frog of 0 02 mm for the fibre of the rat muscle and of 0 028 mm for the human muscle fibre

Fig 11 Intracellular ction potential from fibres of frog at and human muscl

A B (25 C) D E (18 C) two fibres each studied at two tim bases frog s m sartorius in Ringer' solution (experiments by Buchthal and Engbek)

C from a muscle fibre in an isolated rat diaphragm in Tyrode solution at 21 C (Fig 6b in M scholl (1957))

F from a fibre in excised int rcostal muscle of a 45 year old man (Tyrode solution 37 C) (Ludin unpublished)

Ordinate intracellular potential in mV;
Abscissa below distance along the fibre in mm; above time in msec conduction velocity f 3 3 m/sec (A B) and 1 8 m/sec (D E) (frog) 2 m/se (C rat) and 4 m/sec (F man)

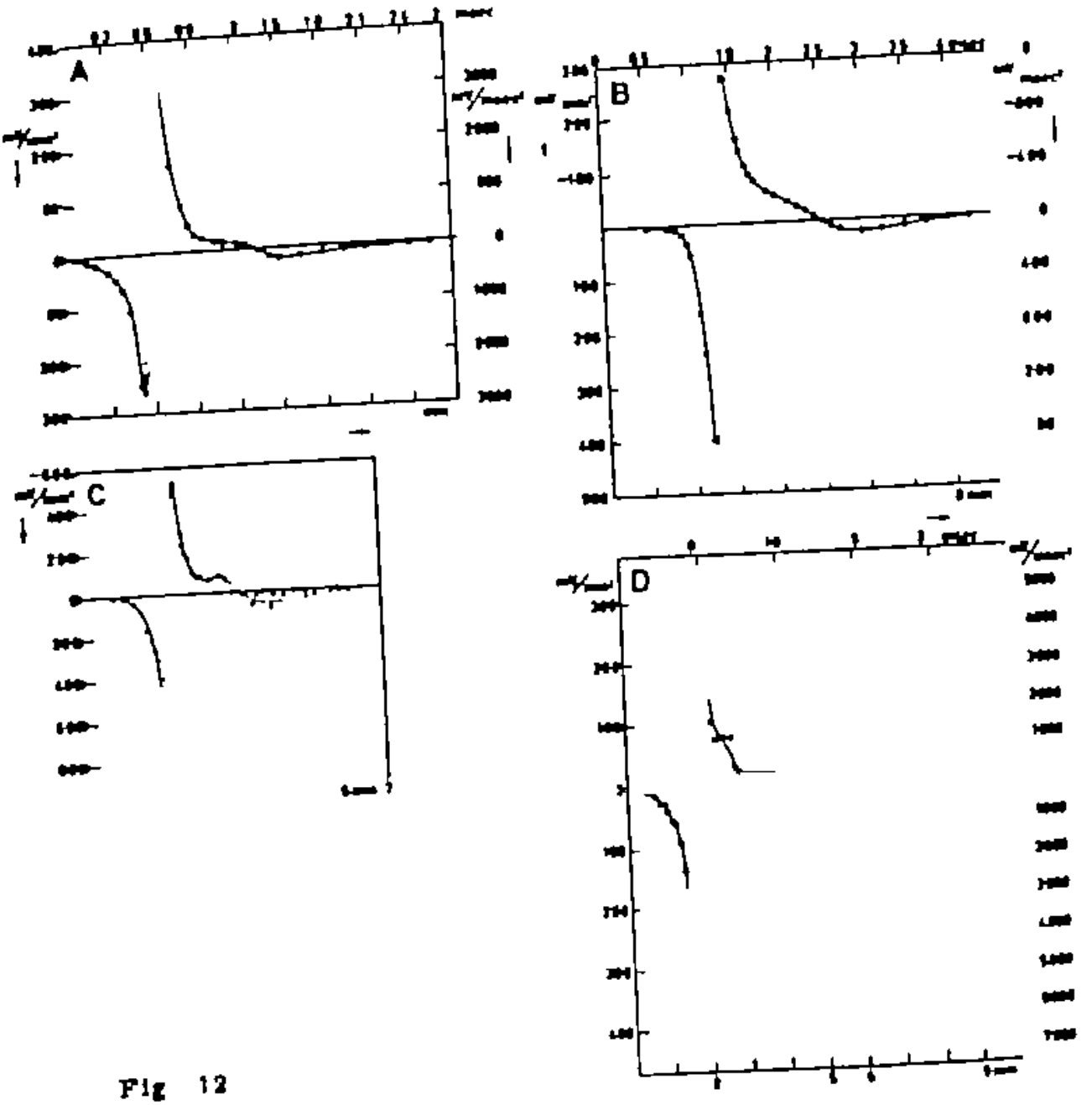


Fig 12

As to the ratio σ_i/σ_e between the conductivities of the axoplasm and the external medium Fatt (1964) found the same conductivity of the axoplasm in fibres of the rat diaphragm and in frog muscle From the values given by Fatt (1964) for rat muscle and by Katz (1948) for frog muscle I assumed that

$$\frac{1}{\sigma_i} \quad 330\ \Omega\ \text{cm}$$

The conductivity of the extracellular fluid was assumed to be identical with that of Ringer's or Ringer Locke solution (Katz 1948 Fatt 1964)

$$\frac{1}{\sigma_e} \quad \begin{cases} 77\ \Omega\ \text{cm (frog 25 C)} \\ 66\ \Omega\ \text{cm (rat 21 C)} \end{cases}$$

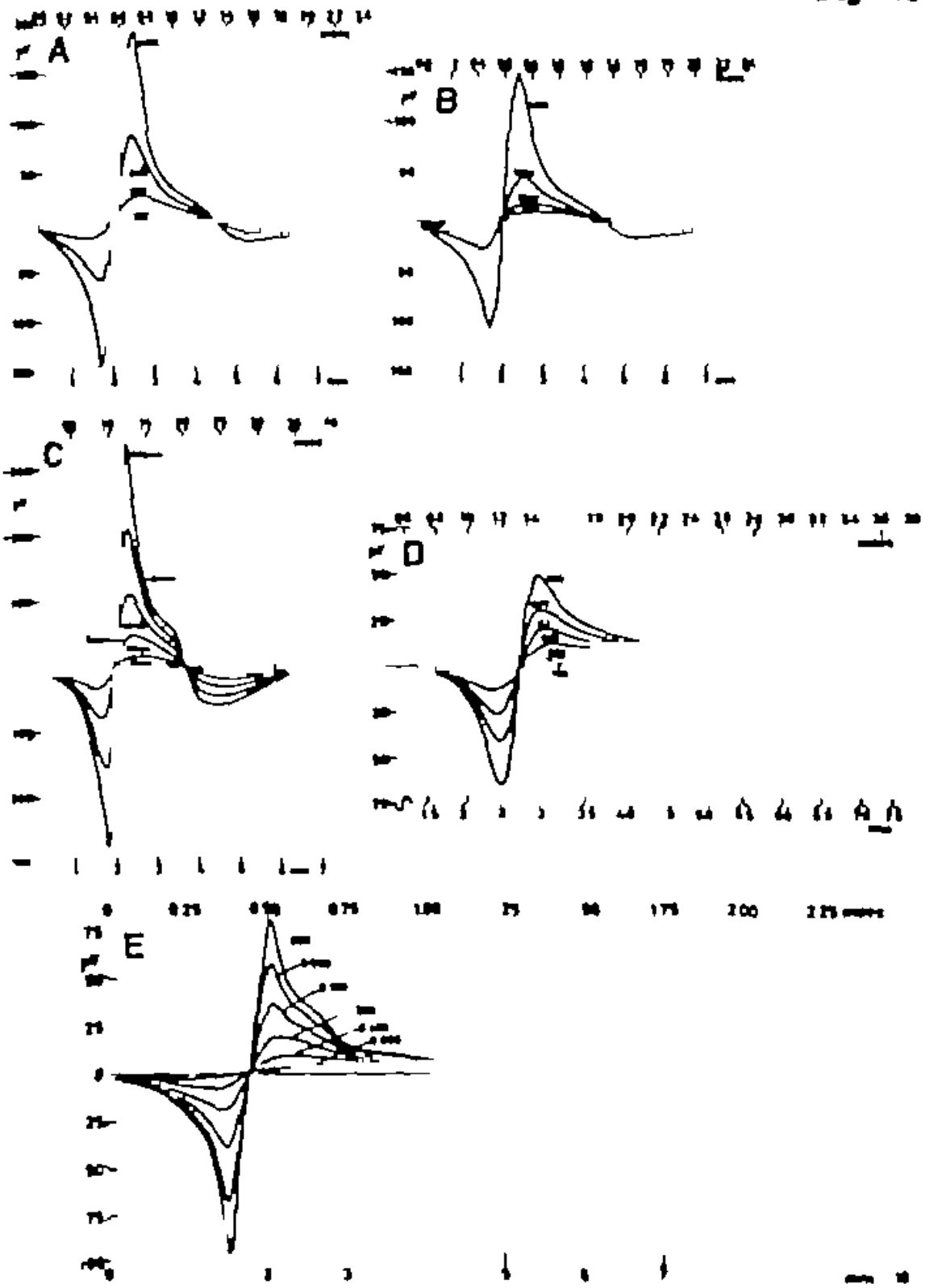
Th refore we assume

$$\frac{\sigma_i}{\sigma_e} \quad \frac{1}{3}$$

The integration of (174) was performed numerically at a number of distance r from the axis of the fibre and for different values of z using a step length of 0 167 mm and 0 05 mm for the fibres of frog muscle and 0 1 mm for the fibre of rat and of human muscle

Fig 12 Second derivative of the intracellular muscle action potential illustrated in Fig 11
A, C frog muscle fibres
B rat muscle fibre
D human muscle fibre
Ordinates: second derivative left spatial in mV/mm^2; right temporal in $mV/msec^2$ The conduction velocity was 3 3 m/sec (A) and 1 8 m/sec (C) (frog) 2 m/sec (B) (rat) and 4 m/sec (D) (man)

Fig 13



When necessary correction was made for the error introduced by replacing the continuous distribution of (174) with a discrete series of sources and sinks (Appendix 1)

The calculated action potentials (Fig 13) are triphasic in shape the two first phases are similar in size and near the fibre the amplitude of the low terminal phase amounts to only 5-10 per cent of the peak to-peak amplitude The action potential declines rapidly as a function of the distance from the axis of the muscle fibre and the relative decline of the terminal phase is smaller than that of the main phases (Fig 14 cf Fig 2 and Table 2) At a distance (r 0 5 mm) at which the peak to-peak amplitude has declined to about 10 per cent of its maximum value the terminal phase has declined to 20-25 per cent of its value just outside the fibre

Another feature of the radial change of the action potential field is the displacement of the peaks with increasing distance resulting in a doubling of the separation between the two main peaks within 0 3 0 5 mm for the frog muscle fibres and 0 3 mm for the rat muscle fibre (Fig 14D) The increase was demonstrated in

Fig 13 Calculated action potentials at different distances from a muscle fibre Obtained from the intracellular action potentials of Fig 11 on the basis of a Lorente de Nó type approximation for the description of the extracellular field (174)

σ_i/σ_e 1/3

A, B C: frog muscle fibres radius 0 05 mm.
D: rat muscle fibre radius 0 02 mm
E human muscle fibre radius 0 025 mm
Ordinate potential in μV_i
Abscissae: below: distance along the fibre (in mm)
above: time in msec conduction velocity 3 3 m/sec (A, B) and 1 8 m/sec (C) (frog) 2 m/sec (D) (rat) and 4 m/sec (E) (man)

Part 2

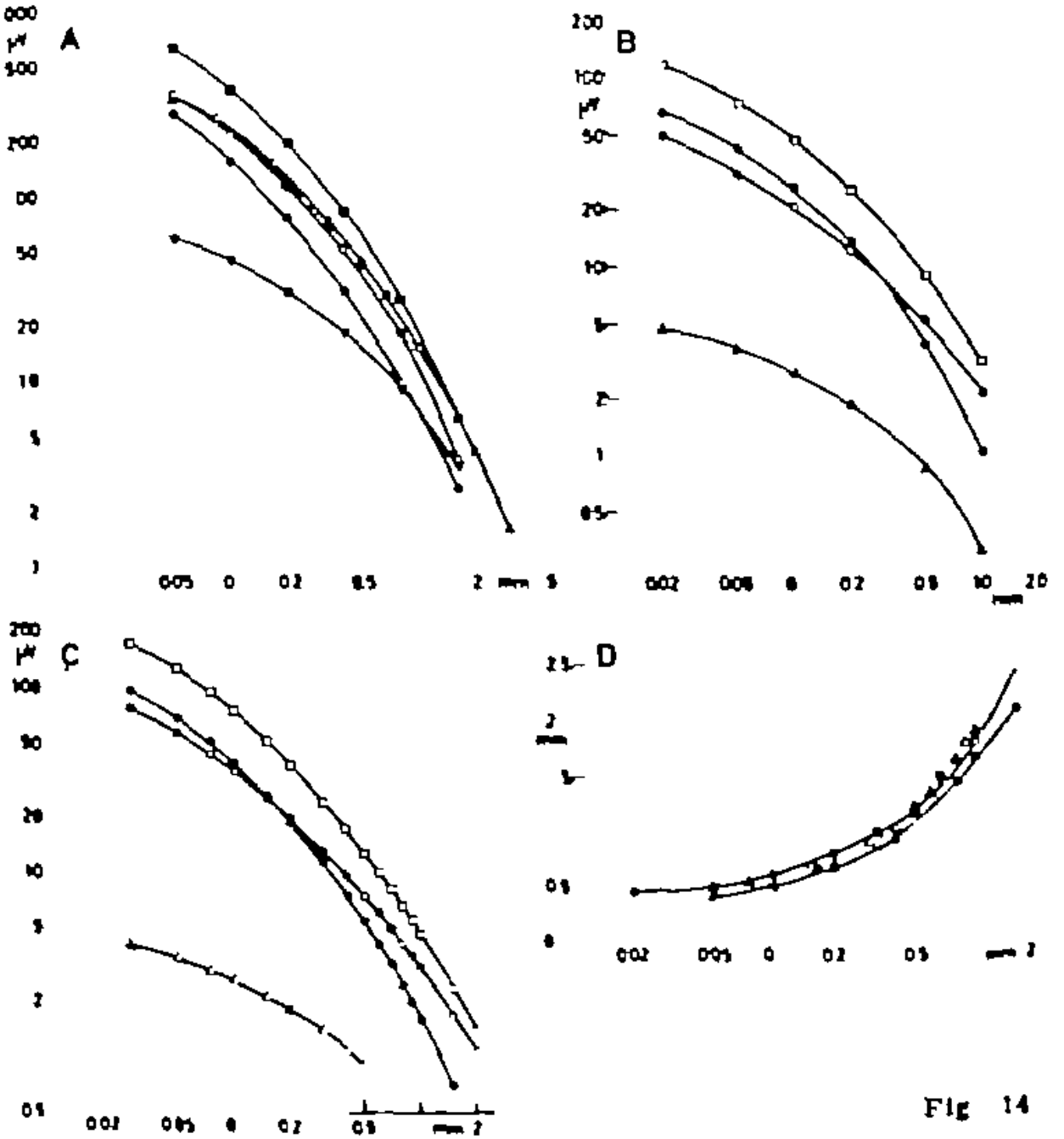


Fig 14

Fig 14 Radial decline (A B C) of the peak to-peak amplitude and of the amplitude of the three phases of the calculated muscle action potentials and the corresponding increase in the separation of the peaks (D)

A: frog Fig 13 A B C

△——△ peak to-peak amplitude (Fig 13 A B)

■——■ peak to peak amplitude

●——● initial positive peak

○——○ negative peak

▼——▼ terminal positive deflection.

B rat (Fig 13 D)

□——□ peak to-peak amplitude

●——● initial positive peak

○——○ negative peak

△——△ terminal positive deflection.

C man (Fig 13 E)

□——□ peak to-peak amplitude

●——● initial positive peak

○——○ negative peak

△——△ terminal positive deflection.

D·

■——■ frog Fig 13 A B

●——● frog, Fig 13 C

○——○ rat Fig. 13 D

Ordinates A B C amplitude in μV

D separation in mm.

Abscissa distance from the axis of the fibre (in mm)

experiments with single frog muscle fibres in Ringer s solution (Håkansson 1957a) In human muscle the duration of the positive-negative deflection of motor unit spike potentials increased by 75 pe cent over 0 38 mm (Buchthal Guld and Rosenfalck 1957a)

The absolute values of the calculated potentials depend on the radii and conductivity ratio assumed $\varphi_e(r s)$ being proportional to the squar of the radius and to the conductivity ratio (cf 174) Comparing the maximum peak to peak values of Fig 13 (333 μV and 610 μV for the frog fibres 114 μV for the rat fibre and 171 μV for the human muscle fibre) with the findings of Håkans son (1956 1957a) (800-2900 μV average 1800 μV) and Krnjević and Miledi (1958) the calculated values are rather low in case of the rat fibre *If we assume a somewhat larger fibre radius* e g 0 065 mm for the frog 0 030 mm for the rat and 0 05 mm for man the maximum amplitudes are 490 μV and 860 μV (frog) 215 μV (rat) and 395 μV (man) Higher amplitudes are obtained if a correction is introduced to compensate for the 15 25 per cent reduced amplitude of the intracellular action potentials of the frog As to the rat muscle fibre Krnjević and Miledi (1958) did not use isolated fibres but recorded from the surface of a diaphragm.

The density of the membrane current i_m was calculated from (162) Inserting the second derivates of the intracellular action potentials (Fig 12) the conductivity σ_i and the radius a we find that in the rat muscle fibre i_m has maximum values of +0 2 and 0 1 mamp/cm^2 in man +0 25 and 0 2 mamp/cm^2 and in the frog +0 35 and 0 3 mamp/cm^2 in one fibre and +0 8 and 0 6 mamp/cm^2 in anothe The values are compatible with the maximum value of the radial current density 100μ from an isolated frog muscle fibre as estimated by Håkansson (1957a 0 3 mamp/cm^2)

I. The relation between the extracellular action potentials and the thickness of the fibre

To evaluate the influence of the radiu of the fibre on the calculated extracellular action potential knowledge is required of the dependence

of the spatial second derivative of the intracellular action potential $\left(\frac{d^2V_i}{dx^2}\right)$ on the radius

Muscholl (1957) found the time course of the intracellular action potential to be independent of fibre diameter in strips of the rat diaphragm. Also the conduction velocity U was independent of fibre thickness. Therefore (18) shows that $\frac{d^2V_i}{dx^2}$ is independent of the thickness too. Hence the conductivity σ_i being the same in thick as in thin fibres (174) shows that the action potential from a fibre has an amplitude which is proportional to the square of the radius of the fibre. Therefore the amplitude distance relationship of Fig. 14 B can be used to calculate the relationship between fibre radius and the amplitude of the action potential recorded just outside the fibre (Fig. 15).

The amplitude A increases according to:

$$A \quad k\, a^{1.7}$$

where a is the radius of the fibre. No data is available on which this relation can be tested.

In frog muscle the conduction velocity of a single fibre immersed in a Ringer bath increased proportionally to the fibre diameter (Hâkansson 1956). Hâkansson found that the duration of the extracellular action potential increased slightly with the fibre diameter but no mention was made as to a corresponding change of the intracellular action potential (Hâkansson 1957a). Assuming the time course of the intracellular action potential to be independent of the fibre thickness the influence of the radius on the extracellular potential is obtained when (174) is expressed in temporal instead of in spatial terms. Introducing t and u defined as:

$$x \quad U \cdot t$$

$$U \, u$$

where U denotes the conduction velocity.

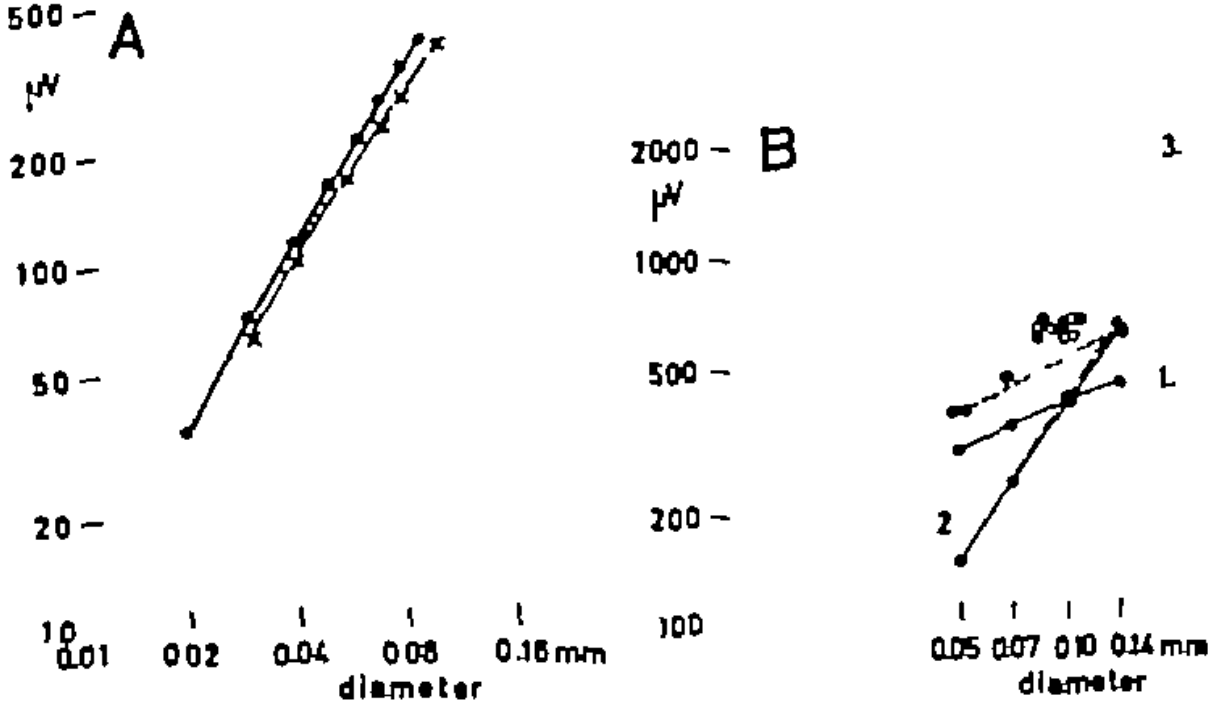


Fig 15 Relation between the peak to-peak amplitude of the calculated action potential and the diameter of the muscle fibre

A. calculated amplitude just outside a rat (●—●) and a human (x—x) muscle fibre assuming the time course and conduction velocity of the intracellular action potential to be independent of fibre thickness

B amplitude at a distance of 38 μ from the surface of a frog muscle fibre

1 calculated assuming (i) the conduction velocity to be proportional to the thickness of the fibre and (ii) the time course of the intracellular action potential (Fig 11 D E) to be independent of the thickness of the fibre

2 calculated assuming (i) the conduction velocity to be proportional to the thickness of the fibre and (ii) the density of the membrane current (Fig 13 D) to be independent of the thickne s of the fibre

Full lines radius of the fibre 50 μ

Broken lines radius: 65 μ

3 measured by Hâkansson (1956 Fig. 9) from isolated frog muscle fibres in Ringer s solution.

$$(184)\quad \varphi_e(r\ t) \quad \frac{a^2\sigma_i}{U^2\sigma_e} \int_{-\infty}^{\infty} \frac{\frac{d^2V_i}{du^2}}{\sqrt{(t-u)^2+(r/U)^2}}\, du$$

Inserting

$$(185)\qquad U \quad k\ a \quad \text{(Håkansson 1956)}$$

in (184) the action potential just outside the muscle fibre is

$$(186)\quad \varphi_e(a\ t) \quad \frac{\sigma_i}{k^2\sigma_e} \quad \int_{-\infty}^{\infty} \frac{\frac{d^2V_i}{du^2}}{\sqrt{(t-u)^2+\frac{1}{k^2}}}\, du$$

Thus the surface action potential should be independent of the thickness of the fibre Håkansson (1956) measured the action potential amplitude 38μ from the surface to avoid damaging the fibre Introducing (185) in (184) it is seen that

$$(187)\qquad \varphi\ (r\ t) \quad f(\frac{r}{a}\quad t)$$

i the potential is a function of the ratio between the radial distance r and the radius of the fibre a Therefore the relation between fibre thickness and action potential amplitude 38μ from the surface can be derived from the amplitude distance relationship (Fig 14 A) The calculated amplitude 38μ outside the fibre increase only slightly with fibre diameter (Fig 15 B) This is in disagreement with the findings of Håkansson (1956)

There are however indications that the electrical properties of the surface membrane depend on the thickness of the fibre (Katz 1948 Håkansson 1956 1957b Fatt 1964 Falk and Fatt 1964) Therefore the rising phase of the intracellular action potential might be faster in thick than in thin fibre This would be the case if we assume the density of the membrane current to be independent of or to increase with the diameter of the fibre; Inserting (185) in the expression (163) for the density of the membrane current

$$i_m(t) \approx \frac{\sigma_i}{2k^2a} \quad \frac{d^2V_i}{dt^2}$$

Hence assuming i_m to be independent of the fibre radius a $\frac{d^2V_i}{dt^2}$ is proportional to a:

(188) $$\frac{d^2V_i}{dt^2} \quad a\ g(t)$$

Introducing (188) in (186) shows that the amplitude of the action potential just outside the fibre is proportional to the thickness of the fibre From (184) (185) and (188) we get

(189) $$\varphi_e(r\ t) \quad a\ h(\frac{r}{a} \quad t)$$

From the amplitude distance relationship (Fig 14) and (189) the amplitude of the action potential lead off 38μ from the surface can be calculated for fibres of different size Fig 15 B shows how the amplitud increases with fibre diameter when i_m is assumed to be independent of the fibre diameter The increase would be larger if the membrane current density increased with the diameter of the fibre

Håkansson s (1956) data follows a relation somewhere between the two possibilities calculated thin fibre obeyed (187) and thick fibres (189) or gave an even steeper relation.

II. Comparison between experimental and calculated radial decline of the extracellular action potentials

a. The frog muscle fibre

The action potential were studied by Håkan son (1956 1957a) around thick and thin fibres immersed in Ringer s solution. Ac cording to my calculations the action pot ntial depends on distance r and fibre radius a according to

$$\varphi_e(r\ t) \quad a^{\alpha}\ h(\frac{r}{a} \quad t)$$

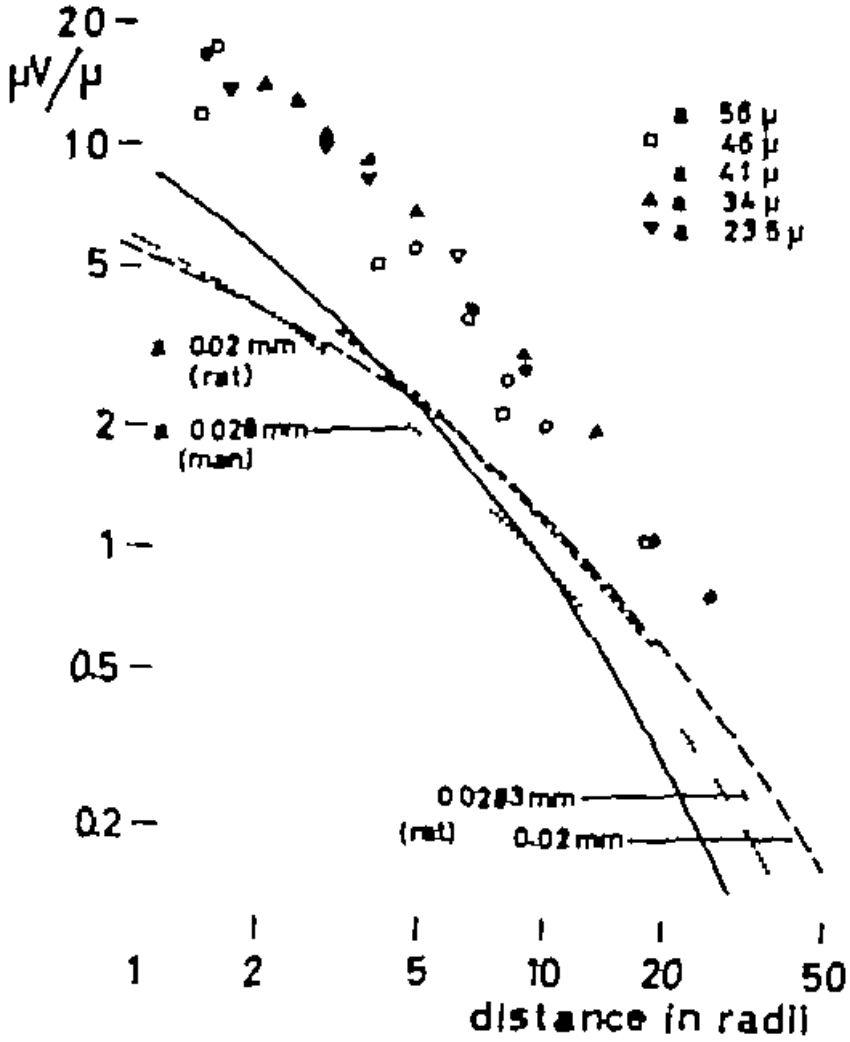


Fig 16 Calculated (full and broken lines) and experimentally determined radial decline (5 different symbol Häkansson (1956 195 a)) in peak to-peak amplitude of th ction potential of a muscle fibre
Full line frog muscle fibre (r dius 0 05 mm)
Broken lines two rat muscle fibres of different radius
Dotted lin human muscle fibre (radius 0 028 mm)
Ordinate peak to peak amplitude divided by th radius of the fibre (μV/μ) (cf (189))
Abscissa distance from the axis of the fibre in units of th radius.

α being bout 1 (cf (189)) To test this conclusion Håkansson s data and the calculated results (Fig 14 A) were plotted as a function of the distance from the axis of the fibre in units of the fibre radius and the amplitude of the action potential was divided by the radius (Fig 16) The data from 4 of the 5 fibres differed very little one fibre had a slightly reduced amplitude The course of the calculated relationship parallels that of the experimental points but th cal culated amplitudes are about half the experimental Up to a distance of 10 radii from the axis the calculated amplitude declined inversely with the distance at larger distances (10 50 radii) the decline was with the inverse square of the distance

Plotting the data calculated on the action potentials of human and rat muscle in the same way (Fig 16) we observe a slightly slower radial decline the amplitude declining to one tenth of its maximum value within 15-20 radii

b Human muscle fibres

In human muscle the radial spread of the spike component of motor unit potentials of fibrillation potentials and of chemically elicited action potentials has been studied (Buchthal Guld and Rosenfalck 1957a Rosenfalck and Buchthal 1962 Buchthal and Rosenfalck 1966 Ekstedt 1964) Multile d electrodes were used for recording to ensure defined distances between the different recording sites The distance between the row of leads and the active fibre(s) was un known. In our study of the spike of the motor unit potential (Buchthal Guld and Rosenfalck 1957a) this distance was deduced by assuming linear amplitude distance relationship on logarithmic cales s in the case of isolated frog muscle fibres (Håkansson 1957a) This a sumption w s objected to by Krnjević and Miledi (1958) who found th t it did not gre with the theoretical relation ship obtained by Lorente de Nó (1947a)

In fact the calculated amplitude distance relationships of Fig 17 A indicate that the assumption of a linear relationship between the l garithm of the spike amplitude and th logarithm of the recording distance might lead to an ov restimate of the recording

distance and hence of the size of spike generator In the following the number of fibres contributing to a spike potential in human muscle is evaluated from the calculated action potentials obtained from intracellular action potentials of human and rat muscle

No data is available regarding the radial decline of the action potential of an isolated fibre of rat muscle immersed in a conducting fluid However the calculated relationship given in Fig 14 B agree with the findings of Krnjević and Miledi (1958) on the surface of the rat diaphragm the amplitude of the action potential of the single muscle fibre decreased to 10 μV or less within about 0 5 mm (Fig 17B) The positive sharp-wave of denervated human muscle (Jasper and Ballom 1948) exhibit a similar amplitude variation (Fig 17 B) suggesting that they represent the activity of a single fibre (Buchthal and Rosenfalck 1966) Let us consider a single muscle fibre with a radius of 0 028 mm which represents an average value in man (Buchthal Guld and Rosenfalck 1955) Taking into account the 10 times higher specific external resistance and the anisotropy which exist in situ (p 50) we obtain from Fig 14 B and C the amplitude distance relationship of the action potential of the fibre and the variation in amplitude along line perpendicular to the fibre direction (Fig 17)

The calculated variation in amplitude along a line perpendicular to the fibre axis decreases and becomes slower when the distance to the line is increased (Fig 17 C) the amplitude decreases to one tenth within 0 35 mm on a line touching the surface of the fibre within 0 6 mm on a line 0 1 mm from the fibre axis and within 0 9 mm on a line 0 2 mm from the axis Thus the motor unit spike potential of small amplitude (235 2400 μV Buchthal Guld and Rosenfalck 1957) and small fibrillation potentials (125-870 μV Buchthal and Rosenfalck 1966) could theoretically represent the activity of a single muscle fibre at a distance of 0 1 0 4 mm from the electrode (Fig 17 C)

The maximum peak to peak amplitudes calculated for the human muscle fibre with a radius of 0 028 mm were 2200 μV and 3200 μV in situ for σ_r/σ_z 10/3 and σ_r/σ_z 0 4 and 0 2 respectively

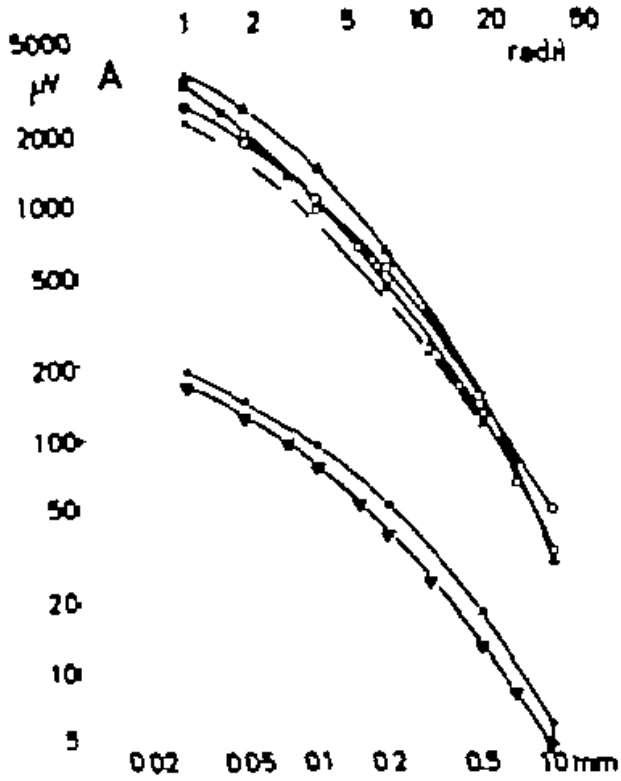
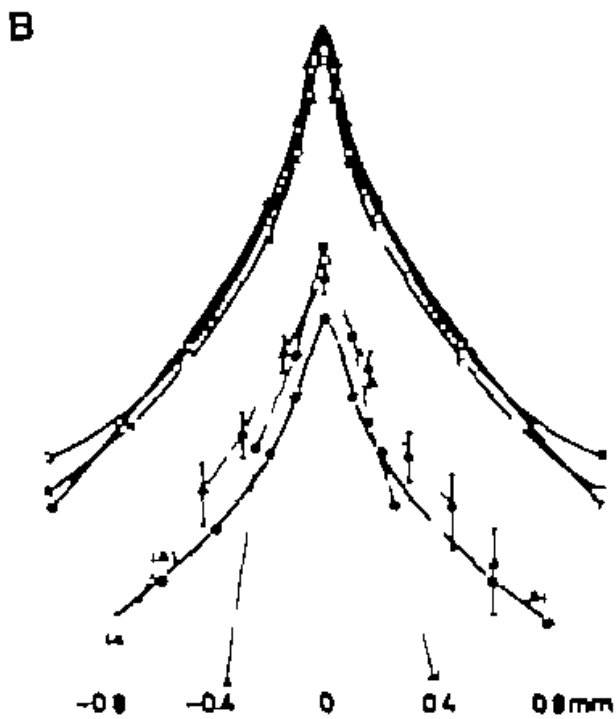

1 2 5 10 20 50
rad/s
5000
μV
A
2000
1000
500
200
100
50
20
10
5
0.02 0.05 0.1 0.2 0.5 1.0 mm

Fig 17


B
-0.8 -0.4 0 0.4 0.8 mm


C
0.8 -0.4 0 0.4 mm 0.8

Fig 17 A. Radial decline in the peak to-peak amplitude of the action potential calculated for a rat muscle fibre(●——●) and a human muscle fibre(▼——▼) in an isotropic conductor and in an anisotropic cond c tor of 10 times reduced conductivity:

o——o : rat σ_r/σ_z 0 4

△——△ rat σ_r/σ_z 0 1

×——× man σ_r/σ_z 0 4

□——□ : man σ_r/σ_z 0 1

radius of fibre 0 028 mm

Ordinate peak to-peak amplitude in μV;

Abscissae distance from the axis of the fibre in mm (below) and in units of the radius (above)

B Variation in peak to peak amplitude along a line perpendicular to the fibre axis and touching the fibre o——o △——△ and □——□ calculated for the same fibres s in A taking into account anisotropy and reduced conductivity

●——● the rat fibre under isotropic condition normal conductivity

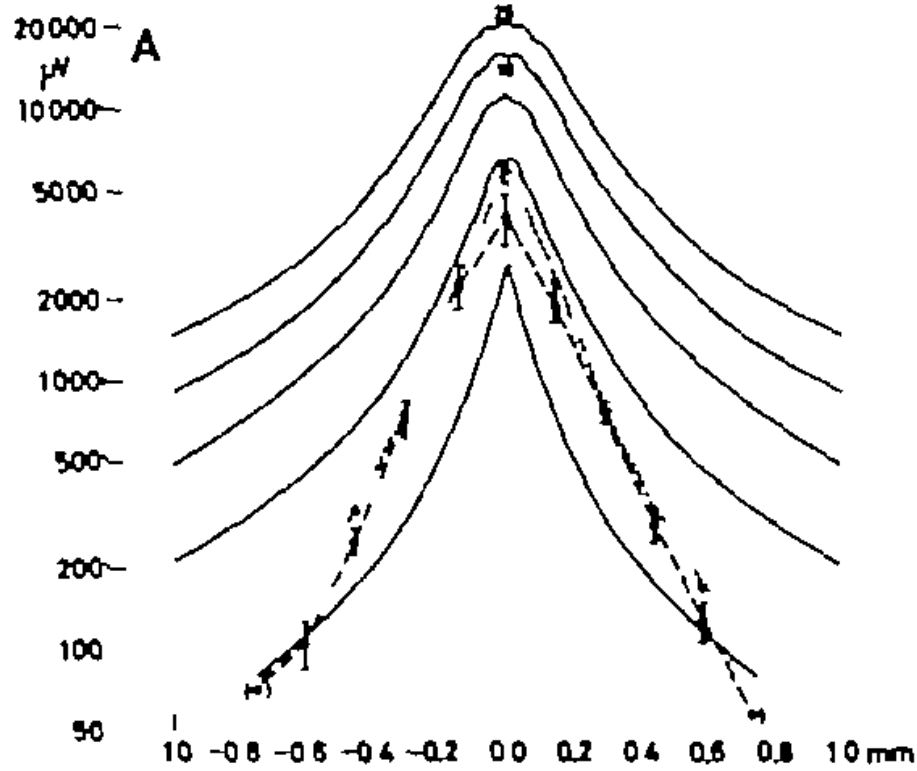
■——■ data from a rat muscle fibre Krnjević and Miledi (1958 Fig 5)

▲——▲ data from 7 positive sharp-waves of denervated human mu cle Buchthal and Rosenfalck (1966 Fig 8)

C Variati n in peak to-peak amplitud of the cal culated action potential (see B) along lines per pendicular to the fibre axis at different distance from th fibre (indicated in mm at th curves)

□——□ d ta from 33 motor unit spike potentials with amplitud s of 238 2400 μV (Buchthal Guld and Ro nfalck 1957a)

●——● data from 16 di or triphasi fibrillation potential with amplitudes of 135-570 μV (Buchthal and Rosenfalck 1966)


A
20000 –
μV
10000–
5000 –
2000 –
1000–
500–
200–
100
50
10 –0.8 –0.6 –0.4 –0.2 0.0 0.2 0.4 0.6 0.8 1.0 mm

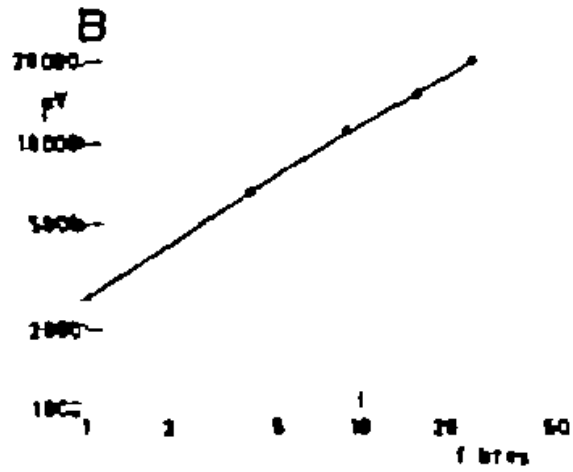

B

Fig 18

(Fig 17) If we consider a fibre with radius 0 050 mm about the upper limit in normal mu cle (Buchthal and Rosenfalck 1963) the maximum amplitudes are 4900 μV and 6400 μV and a radius of 0 070 mm as can occur under pathological conditions would give 7200 μV and 9200 μV Ekstedt (1964) pointed out that the insulating wall of epoxy resin surrounding the leads of the multielectrode will increase any voltage by a factor of two according to the method of electrical images (Morse and Feshbach 1953) Under these condi

Fig 18 Action potential amplitudes (peak to-peak) for groups of synchronously firing muscle fibres in situ cal culated from an intracellular action potential of rat muscle (Fig 11 C) The radius of the single fibre was 0 028 mm anisotropy and reduced conductivity of the muscle tissue were taken into account (Fig 17 B)

A. Full lines Calculated variation in amplitude along a line perpendicular to the fibre direction and touching the bundle of fibres The number of fibres in the bundl is indicated by the figures on the curves Broken lines Variation in amplitude of action poten tials of high voltage recorded along a multilead elec trode in human muscle

●- - - -● 25 motor unit spikes (Buchthal Guld and Rosenfalck 1957a) Maximum amplitude: 2 5-10 4 mV

○- - -○ 9 fibrillation potentials of denervated muscle (Buchthal and Rosenfalck 1966) Maximum amplitude 1 4-8 5 mV

Abscissa distance along the lin passing the fibre bundle (in mm) referred to the point with maximum amplitude

B: Peak to-peak amplitude of the calculated action potential at the surface of the bundle as a function of the number of fibres in the bundle

tions the calculated amplitude of the action potential of a single normal muscle fibre might attain value of 10-13 mV

To further elucidate this problem calculations were performed to give the variation in action potential amplitude past a small bundle of muscle fibres firing in complete synchrony in situ Single fibre contributions (Fig 17) were superimposed taking into account the different distances to the various fibres of the bundle The maximum amplitude as obtained on the surface of the bundle increases less than proportionally to the number of fibres 25 fibres gave 7 7 times higher amplitude than one fibre (Fig 18) Hence the relative decline in peak to peak amplitude along a line touching the fibre bundle is the smaller the more fibres are active synchronously the amplitude decreasing to one tenth within 0 35 mm for a single fibre within 0 6 mm for 9 fibres and within 0 8 mm for 25 fibres Taking into account the doubling of the calculated maximum amplitude of the action potential due to the wall effect of the multilead electrode (Ekstedt 1964) (Fig 19) we find from the theoretical analysis (Fig 18) that high voltage fibrillation potentials (1 4-6 5 mV) and motor unit spikes (2 5-10 4 mV) which contain a smooth positive-negative deflection of short duration (100-200 μsec) are likely to be derived from the activity of a single muscle fibre lying in contact with the needle electrode The alternative that these spikes represent the synchronous firing of a small group of muscle fibres (a subunit) was arrived at previously from histological evidence and from recording with multilead electrodes (Buchthal Guld and Rosenfalck 1957a Buchthal and Rosenfalck 1966)

The question then arises whether the present estimate of the possible contribution of a single muscle fibre to the motor unit spike potential is compatible with the histological evidence for the existence of subunits within the motor unit containing 10 fibres on the average (Buchthal Guld and Rosenfalck 1957a) This question can only be answered if knowledge is available on the temporal dispersion and the amplitude of the action potential from the fibres contributing significantly to the motor unit potential. With 10 muscle fibres per subunit with 780 fibres per motor unit (in biceps brachii Christensen 1959) and with a diameter of the motor unit territory

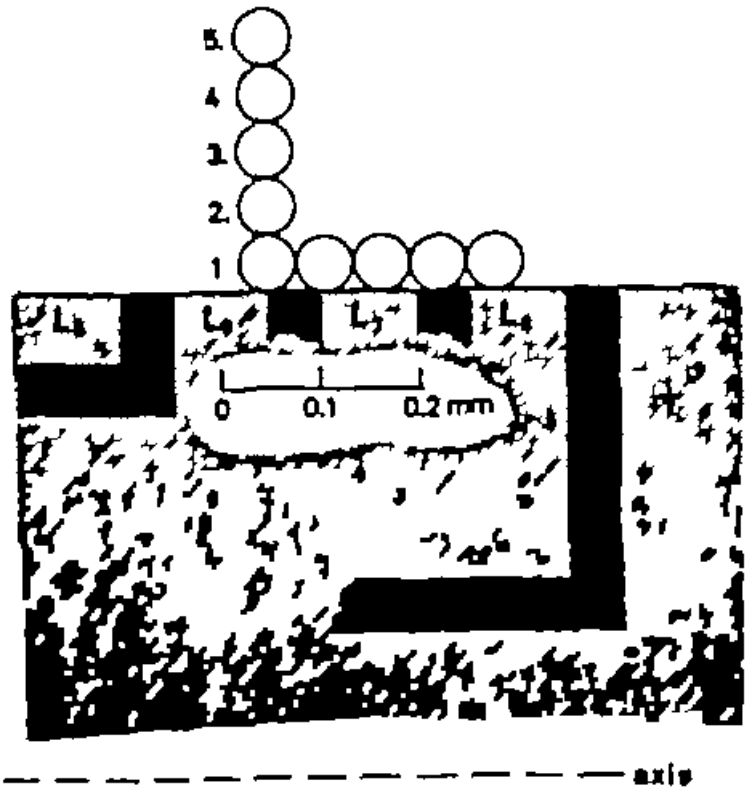


Fig 19 Size of human muscle fibre relative to the multilead electrode (Buchthal Guld and Rosenfalck 1957a) Fibres with a radius of 0 028 mm are shown in cross section positioned near le ds L_5 L_8 The broken line indicate the axis of the cannula of the multil ad el ctrode

of 5 mm (Buchthal Guld and Rosenfalck 1957b) the average distance between the diff rent subunits is $5/\sqrt{75}$ 0 6 mm. A similar stim te (0 7 mm) w btained from the multiel ctrode analysi of the spik components f the mot unit potential (Buchthal Guld and Rosenfalck 1957) The temporal dispersion of clearly di stinguishable spike was 0 5 2 3 msec (see also Ekstedt (1964))

Let us conside a small electrode (30-50 μ diameter) placed in contact with subunit (10 fibres) firing voluntarily On account of the rapid radial declin of the peak to-peak amplitud of the action potential of singl fibre (Fig 17A) other subunits do not contri

bute significantly to the main components of the motor unit potential The distance to the axis of the fibres of the subunit varies between 0 028 mm and 0 140 mm a range over which the calculated peak to-peak amplitude declines by a factor of three The temporal dispersion of the action potentials of the different fibre of the subunit is determined by the dispersion of the motor endplates and the variation in conduction velocity unless the action potentials interact and are propagated synchronously Histological findings in infants (Christensen 1959) suggest that the dispersion of the motor end plates of a subunit is maximally 5 mm in the adult This would cause a temporal dispersion of 0 4-2 4 msec (25 40 mm from the endplates) since the conduction velocity as measured over electrical ly activated muscle fibres had an average value of 4 0 m/sec and varied by 0 2 0 5 m/sec within the same muscle (Buchthal Guld and Rosenfalck 1955a) Hence double spikes triple spikes and other composite motor unit potentials of high voltage (1 mV or more) recorded on an electrode 30 µ in diameter (Ekstedt 1964) might represent the summation of temporally dispersed action po tentials of fibres from a single subunit In fact Ekstedt obtained distance between th spike sources which could be as small a 60 µ judging from the variations in action potential amplitude along the multielectrode

PART 3

DISCUSSION

Section 1

Introduction

The first detailed mathematical analysis of the spread of ction potentials was that of Lorente de Nó (1947a) followed by studies of Krakau (1957 1959) Mauro (1960) Plonsey (1964 1965) and Clark and Plonsey (1966)

The aim of my study was to relate the extracellular action potential originating from singl muscle fibre in a volume conduc tor to the intracellularly recorded action potential Simple consi derations and Lorente de Nó (1947a) de cription of the spre d of nerve action potentials in volume conductors showed that the second derivative of the intracellular action potential appeared in this re lationship (Ro enfalck 1957)

Extracellular ction potentials of single mu cle fibres were calculated from intracellular ction potentials of frog and rat muscle though the degree of pproxim tion btained was not known. Clark and Plonsey (1966) used th rel tions between modifi d Bessel func tion and their symptoti behaviour to evaluate the core-conductor model. Similarly I have derived the Lorent de Nó model th core conductor model and the di o tripol model as diff rent degrees of approximation to the rigorou math matical model.

In the mathem tical tre tment of potential fi lds of active cy lindrical fibre it is assumed th t these fields satisfy Lapl ce s equation (e g Helmholtz 1853 Craib 1928 Benoit 1937) This is the case because phase hifts are negligible in the external medium and within the fibre and hence the fields are qu si stati (Clark and Plonsey 1966) I derived the applicability of Laplace s equation from the Maxwell field quations fo conducting media assuming the

conductors to be isotropic and homogeneous and using the electrical parameters of nerve and muscle fibres

In whole nerve and muscle the action currents of a fibre flow mainly through the extracellular space the passive fibres having membrane impedance which makes them act almost like insulators (Buchthal Guld and Rosenfalck 1955b ee also p 125) This accounts for the low conductivity of nerve and muscle tissue as compared with that of Ringer s solution (Geddes and Baker 1967) Furthermore it explains the different conductivity of the tissue in the fibre direction and transversely Hence also in situ Laplace s equation is applicable if the boundary conditions are introduced that all fibre act as insulators towards the action currents of the others Thus taking into account that the high dielectric constant of muscle at audiofrequencies is due t the membrane capacitance of the fibres (Schwan and Cole 1960) the conditions for the applicability of a quasi stationary field appear more favourable than in the recent analysis of Plonsey and Heppner (1967)

To evaluate the significance of the anisotropy for the spread of action currents in muscle I consid red a fibre surrounded by a con ductor which was anisotropic but homogeneous The field equation corresponding to an anisotropic but homogeneous external medium can be transformed to a Laplace equation by a simple change of variables

Therefore the present m thematical analysis was essentially a study of solutions of Lapl ce s equation satisfying a boundary condition at the surface of the excitable fibre The radial gradient of the intracellul potentials g ve the density of the current passing the surface of the fibre and h nce the radial surface gradient of th extracellular potential According to potential theory (Kellogg 1929) this gradient determined the external potentials uniquely

Section 2

The potential field within an active fibre the membrane current and the core conductor model

Some of the quantitative tests of the theory were based on simulated intracellular action potentials chosen so as to approximate experimentally recorded potentials in a mathematically simple way The calculated radial variation in potential within a muscle fibre was less than 0 1 mV and in a giant nerve fibre it did not exceed 0 4 mV With this small radial variation the density of the longitudinal current within the fibre also varies little over the cross section These results justify depicting the interior of the fibre by an axial ohmic resistance as in the core conductor model

The applicability of this model was supported by the calculations of the density of the membrane current the membrane current of the rigorous mathematical model deviates at most a few per cent from that given by the core conductor model i e the membrane current can be considered proportional to the second derivative of the intracellular action potential. A similar relation stating that the membrane current is proportional to the second derivative of the transmembrane potential has been used in calculations of action potentials based on the Hodgkin-Huxley equations (see e g Noble 1966)

The limitations of the core conductor model appear when the extracellular action potential are considered. The model is linear and does not give information about the radial decline of the action potential field Furthermore the extracellular potential predicted by this model is monophasic like the intracellular potential and is proportional to it.

Since nerve and muscle action potentials lead off from a volume conductor are triphasic or diphasic (Craib 1928 Lorente de Nó 1947a Håkansson 1957 Murakami Watanabe and Tomita 1961) the applicability of the core conductor model to extracellular condition is restricted to the case of a fibre surrounded by a thin layer of the conductor

These conclusions were also arrived at by Clark and Plonsey (1966) in their mathematical evaluation of the core conductor model In an unlimited volume conductor an external longitudinal resistance per unit length of fibre cannot be defined

Section 3

The extracellular action potentials in an unlimited conductor

A The shape of the action potential

Action potentials of nerve and muscle fibres lead off in a volume conductor differ from the intracellular action potentials (i) their amplitude is at most one tenth of the intracellular potential and (ii) their shape is triphasic or diphasic the two first phases coinciding with the phase of depolarization of the intracellular action potential (Håkansson 1957 Murakami Watanabe and Tomita 1961) These differences can be accounted for in terms of the Lorente de Nó type of line integral model using intracellular action potentials recorded from human rat and frog muscle fibres (Fig 20)

> The shape of the extracellular action potential in an unlimited conducting medium can be predicted by simple arguments based on the spread of the action current (see e g Offner 1954) ahe d of the depolarized region of th fibre current flows out of the fibre i.e the potential de reases radially and is therefore positive since t is zero far from the fibre In the depol rized region conditions are oppo ite current flows into the fibre i.e the potential decreases towards the fibre and is negative in this region This explains the two main phases f th extracellular ction potential Finally currents which l ave the fibre in th region under repolari ati n give ri e to a terminal posi tiv deflection.

Considering the compound cti n potentials of bull frog n rve Lorente de Nó (1947a) derived th triphasic sh pe of the pot tial lead off in a volum conduct r from the monophasic surface action potentials recorded with the nerve in ir or oil Hi calculations showed that in the xt rnal medium the compo d nerv acts as a giant fibre which conducts continuously To a count for the di or triphasic shape of extracellular action potential Woodbury (1961) described the potential in t rm of a solid angle formulation (Plonsey 1965) assuming step variations in th membrane potential. According to this model th potential i proportional t the solid angle subtended by that cross ection of the fibre delimiting the ctive region o it s proportional to the diff rence between two

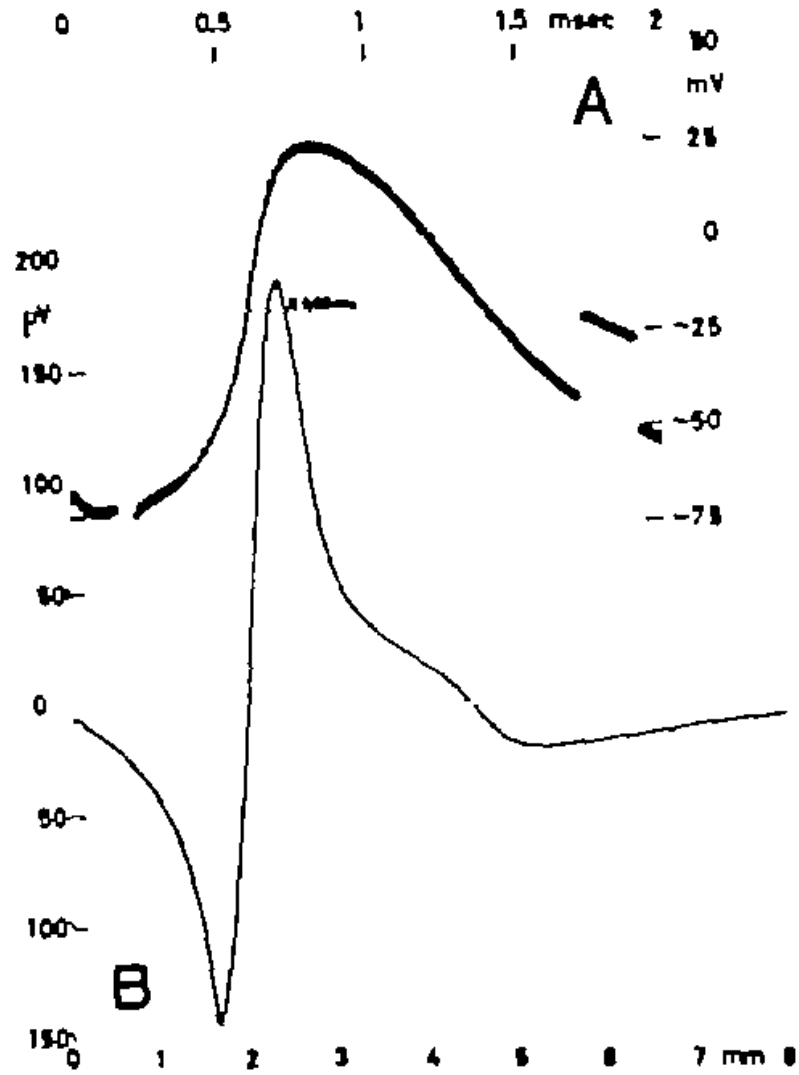


Fig 20 The temporal (and spatial) relationship between the intracellular action potential of a frog muscle fibre (A) and the calculated extracellular action potential obtained with an approximation like Lorente de Nó s (B)
A intracellular action potential (Fig 11 B)
B calcul ted action potential just outside the surface i e 0 05 mm from the axis of the fibre
Ordinate right intracellul r potential in mV
left extracellular potential in µV
Abscias above time in msec below distance along the fibre in mm conduction velocity 3 3 m/sec

solid angles of this type Mauro (1960) also used the solid angle model but the generator consisted of two plane sheaths depolarized in a narrow band By qualitative graphical solution he found that the external potential became monophasic rather than triphasic as the separation between the two membranes of the generator in creased and became large as compared with the width of th de polarized region Model experiments supported this conclusion Mauro s description of the triph sic external potential is illustrated by model experiments in which the longitudinal potential change is larger in the external than in the int rnal medium This is un fortunate since in fact the opposite is the case Moreover in the model experiments the amplitudes of the positive deflection are small as compared with the negative one even when the width of the depolari ed zone is 15 times the thickness of the generator In fact the initial positive d flection of the extr cellular action potentials of nerve and muscle fibres has about the same amplitude s the negative deflection (Håkansson 1956 1957a Mi rakami Watanabe and Tomita 1961) The simplest models that have been used to account for the shape of extracellul r nerve and muscl action potentials are the dipole and the tripole model (Craib 1928 Wilson MacLeod and Barker 1933) Neglecting th formal character of these models the question has been argued as to wheth r the axis of the dipole w s oriented parall l with the fibres or perpendicular to them (Gilson and Bishop 1937 Bishop 1937) The question is not pertinent when the dipole are considered to represent fictive generators of the ction potential

The extracellul action potential f single frog muscl fibre in Ringer' solution w re diphasic (Håkansson 1957a) My calculations gave triphasic potential (Fig 13). However th terminal positive defl ction wa small and protracted. Th discrepancy could be due to the fact that the intracellular action potentials used in the calculation were derived from singl muscl fibres in situ and not from isolated fibres. Th extr cellular action potentials of fibres on the surface of the toad sartoriu were triphasic as long as the microelectrode had not impaled the fibre (Murakami et al 1961) Murakami et al explain diphasic extracellular action potentials found

after impalement as due to local inactivation of the exitable mem trane This explanation does not apply to the findings of Håkansson (1957) An alternative cause of the lack of a terminal positive deflection is the short length of the fibres The triphasic action potential calculated from the intracellular potential extend over 7 mm of fibre length the two main phases covering 3 5 4 5 mm (Fig 12) Devi tions from the calculated shape can therefore be expected when recording closer than 7 mm from the end of the fibre In fact the triphasic action potential changed to a diphasic one near the tendon end of frog muscle fibres and near the closed end of the motor nerve terminals (Katz and Miledi 1965)

As to the shape of extracellular action potentials of mammalian muscle fibres Buchthal and Rosenfalck (1966) found 36 of 128 fibrillation potentials of denervated human muscle to be triphasic the voltage of the third phase being 10 per cent of the peak-to-peak voltage This agrees with my calculations (Fig 13)

In the theoretical analysis the action potentials were considered to be distant from the site of initiation. Since the depolarization propagates to both sides the terminal positive deflection recorded extracellularly must be more pronounced near the site of initiation than distant from it the late currents leaving the fibre being restricted to a small length and hence having a higher density The fibrillation potentials recorded by Buchthal and Rosenfalck (1966) in or near the endplate zone often had an enhanced positive terminal phase

B The relation between peak to-peak voltage and fibre thickness

The marked difference between th peak deflection of intracellular muscle action potentials and the peak to peak voltage of th extracellular action potentials of a fibre imm rsed in an unlimited volume conductor w s accounted for by calculations on the Lorente de Nó type of expression for the action potentials

The difference between intracellularly and extracellularly recorded amplitudes can also be derived by simple expressions From Ohm s law we expect

(190) $$\frac{A_e}{A_i} \quad \frac{r_e}{r_i}$$

where A_e and A_i are the amplitudes of the extracellular and intracellular action potential and r_e and r_i the extracellular and intracellular impedance Let n denote the number of radii within which most of the extracellular action current flows (190) can then be written

$$\frac{A_e}{A_i} \quad \frac{\sigma_i}{\sigma_e} \quad \frac{1}{n^2 \quad 1}$$

According to Katz (1948) $\sigma_i/\sigma_e \approx 1/3$ From experiments like those of Håkansson (1957a) we learn that action currents mainly flow within about 10 radii from the fibre axis i e n ≈ 10 This leads to

$$\frac{A}{A_i} \approx \frac{1}{300}$$

With A_i 120 mV we get

$$A_e \approx 400 \ \mu V$$

which is of the order found experimentally Even if half the current were to flow within 3 radii from the axis th amplitude of the extracellular action potential would maximally be only 2 5 mV as is seen by inserting $r_e/r_i \quad \frac{\sigma_i}{\sigma_e} \quad \frac{1}{2(3^2 \quad 1)}$ in (190)

The calculated relation between the thickness (2a) of the fibre and the amplitude (A) of the action potential recorded just outside the fibre is given by

$$A \quad k \, a^{1 \ 7}$$

provided the time course and conduction velocity of the intracellular action potential as well as th conductivity σ_i are independent of the fibre size (cf Muscholl 1957) N data is as yet available on mammalian muscle fibre on which this relation can be tested.

For frog muscle fibres the theoretical analysis lead to

$$\underline{A} \text{ independent of } \underline{a}$$

provided the intracellular action potential is independent of the thickness of the fibre Since this result is in disagreement with the findings of Håkansson (1956) an alternativ assumption was made i.e that th density of the membrane current is independent of fibre siz This lead to:

$$A \quad h\, a$$

Håkansson s findings gave a smaller increase in amplitude with fibre radius in th thin fibres and a l rger variation in the thick ones (Fig 15 B) His findings are in favour of an increased density of the membrane current with increasing fibre diameter An experimental study of the relationship between the fibre diameter and the density of the membrane current would be of interest in view of the decrease in specific resistance of the resting membrane with increasing thickness of the fibre (Katz 1948 Falk and Fatt 1964)

The maximum amplitude of the calculated action potentials was 610 μV fo the frog muscle fibre (Fig 11 D E 13 C) when a radius of 80 μ was assumed With a radiu of 70 μ the maximum value in the fibres studied by Håkansson (1956) the amplitude would be 1000 μV In fact Håkansson (1957a) report d maximum amplitudes of 800 2900 μV A deviation from a circula shape of the fibre cross section might contribute to these high amplitudes (Håkansson 1956) It would be of interest to extend the theoretical analysis to fibres of ellipti al cross section to evaluate this effect.

C The radial decline of the action potential

I Frog muscl fibres

Lorent de Nó s (1947a) theoretical analysis of the spread of action potential in a volume conductor was used by Buchthal Guld and Ro enfalck (1957a) to estimate how the di meter of the generator

of the action potential and the length along the generator over which the action currents extend affect the radial spread of the action potential. Thereby it was possible to explain the larger spread of the ction potential of the bullfrog nerve than that of a frog muscle fibre by the large diameter of the nerve and the broad region along the nerve over which the action currents extend Krakau (1957) suggested the use of the Fourier transform of the extracellular action potential to test the model developed by Lorente de Nó He showed that the transform should decline radially as $K_0(\frac{r\omega}{U})$ i e the same vari tion as obtained with the m re general model (p 47) Krakau (1959) demonstrated that the data from one of Håkansson s experiments followed the expectations closely for frequencies between 425 and 2975 Hz In the present study extracellular action potentials w re calcul ted using the theory derived by Lorente de Nó (1947a) except that the intracellular action potential wa used and not the surface action potential recorded with the fibre in air or oil. The calculated radial decline in th amplitude of the action potential agreed with Håkansson s finding (Fig 16)

Even dipole model can fairly well account f r the shape and radial decline of the action potential of a frog muscle fibre This is to be expected from the pronounced positive and negative peak of the distribution f the fictive sources and sinks obtained with the Lorente de Nó type of model (Fig 12)

II. Human muscl

Not until th completion of the first edition of my manuscript I got re cordings of intracellular ction potential of human muscle fibres with a resolution in time suffici nt to allow the second derivative to be obtained as t must be if the extracellular action potentials are to be calculated Therefore I performed th calculations on an intracellul action potential f a rat diaphragm recorded by Muscholl (1957) The results from rat and human muscle differed little from calculation on frog mu cle The radial spread of the action currents around isolated mammalian mu cle fibres has not been studied experime tally The spread of the action currents

around a fibre in an isolated rat diaphragm (Krnjević and Miledi 1958) is satisfactorily described by the calculations (Fig 17 B)

a. The generator of high voltage spike potentials

The spread of action currents around the fibres in human muscle has been studied by means of multilead electrodes (Buchthal Guld and Rosenfalck 1957a Rosenfalck and Buchthal 1962 Buchthal and Rosenfalck 1966 Ekstedt 1964 Stålberg 1966) My calculations of the variation in action potential amplitude along lines perpendicular to a single active fibre or a group of synchronously firing muscle fibres showed that the high voltag spikes of motor unit potentials and of fibrillation potentials could arise from the activity of a single muscle fibre In these calculations the high specific resistance and the anisotropy of the muscle tissue were tak n into account. A different estimate of the size of the generator of high voltage spikes was obtained by Buchthal Guld and Rosenfalck (1957a) who studied motor unit potentials electrophysiologically and correlated their finding with histological specimens Buchthal and Rosenfalck (1966) found the same radial decline in voltage for large fibrillation po tentials as for motor unit spike potentials therefore the high-voltage spikes of denervated muscle were assumed to originate similarly from the synchronous activity of a group of up to 10 30 muscle fibre a subunit

Ekstedt (1964) interpreted high voltage motor unit spikes and chemically induced spike potentials as arising from single mu cle fibres He rejected the subunit concept a being derived from observations on human muscle only under special pathological con ditions He did not find indications of a pi rcing of a subunit with the Janus electrode which allowed recording from opposite ides of the needle Finally he postulated th t the voltage decrement curves recorded by Buchthal Guld and Rosenfalck (1957a) were distorted by cros talk in th multilead electrode resulting in an apparent in crease of the spread of the action potentials Krnjević and Miledi (1958) did not find it compatible with the alculations of Lorente de Nó (1947a) to assum the linearity u ed by Buchthal et al in the analysis of the amplitude distance curves (cf p 104) This assump-

tion leads to an overestimate of the number of fibres involved and Krnjević and Miledi find it more likely that no more than one or two fibres contribute to the spike These problems were discussed by Buchthal and Rosenfalck (1966) Cross talk was larger in the multilead electrode used by Ekstedt (1964) than in that of Buchthal and co-workers and cannot account for the difference between the voltage decrement curves The difference between the decrement curves of Ekstedt and those of Buchthal and co-workers must be related to the selection of the spikes to be studied in that Ekstedt required that they had to satisfy some single fibre criteria Furthermore Ekstedt studied the voltage decrement over 0 3 mm in steps of 0 06 mm whereas Buchthal et al. covered 0 6 0 75 mm in steps of 0 15 and 0 3 mm

Further evidence supporting the single fibre interpretation was found in studies of the effect of d tubocurarine on motor unit spike potentials satisfying the single fibre criteria of Ekstedt. A fractionation of such potentials was never seen (Ekstedt and Stålberg 1967)

In the calculations on groups of synchronously conducting fibres I assumed that the action currents of the individual fibre do not pass through adjacent fibres to any significant degree Thi is true as long as the adjacent fibre are in the resting state (Buchthal Guld and Rosenfalck 1955b)

> During the early phase of repolarization the specific resistance of the muscle membrane is about 1 per cent of the resistance of the resting membrane (Fatt and Katz 1951) The decrease in resistance begins at the time when th initial positive phase of the extracellular action potential has reached its peak the membrane current being mainly capacitive up to this point (Murakami et al 1961)
>
> How much th membrane resistance decrease during the positive-negative deflection of the extracellular action potential is not known but probably it does not fall much below that measured in the early phase of repolarization (Cole and Curtis 1939 Hodgkin and Huxley 1952) Even when the membrane resistance i only 1 per ent of the resting value the transverse resistance of the fibre is larger than that of the adjacent inter space Let us consider 1 cm length of a quadratic "fibre 50 μ thick and let u assume the xtracellular space locally to amount to only 6 pe cent of the muscle volume i.e half the overall valu (cf. Buchthal Guld and Rosenfalck 1955b)

The interspace then has a width which is 3 per cent of the fibre thickness that is 1 5 μ The transverse ohmic resistance of the fibre is $\frac{2\ 50\ \Omega\ cm^2}{0\ 0050\ 1\ cm^2}$ 12000 Ω neglecting the resi stan e of the sarcoplasm. The resistance of the interspace is

$$\frac{87\ \Omega\ cm\ \ 0\ 0050\ cm}{0\ 00015\ 1\ cm^2} \qquad 2900\ \Omega$$

The membrane capacitance changes insignificantly during acti vity (Cole and Curti 1939) Hence the reactance of the c tive fibre at 5000 Hz is

$$\frac{1}{2}\ 2\ 6\ 10^{6}\ 0\ 0050\ 1\ 2\pi\ 5000 \qquad \Omega \qquad 5000\ \Omega \quad 1\ e$$

even at this high frequency larger that the resistance of th int rspace The time constant of fibre plus interspace is

$$2900\ \frac{1}{2}\ 2\ 6\ 10^{6}\ 0\ 0050\ 1\ s\ c \qquad 19\ \mu\ sec$$

This is short as compared with the duration of the po itive negative deflection of the extracellular action potential

Therefore also when the adjacent muscle fibres are active the action currents of a fibre pass mainly through the extracellular space Under these conditions it does not seem likely that the ac tion potentials of adjacent muscle fibres in situ interact to a degree that affects their propagation and results in a complete synchroniza tion a has been assumed previously (Buchthal Guld and Ro enfalck 1955a 1957a) An interaction between the action potentials of two large (30 μ) non medullated nerve fibres (Carcinus maenas) in paraffin oil was demonstrated by Kats and Schmitt (1940) This condition favours the interaction a does the reduction of the con ductivity of the saline in which the prepar tion was initially soaked. Complete synchrony wa obtained if th fibre had the same con duction velocity when stimul ted separately If the velocitie differed but no more than 10 per cent an out-of phase synchrony was ob- served In view of these findings and of the uncertaintie involved in the calculations above it would be of interest to study experiment ally whether or not there is an int r ction in muscle The experi ments of Tamai Abe and Goto (1961) on the interaction between

neighbouring fibres in fibrillating skeletal muscle of the frog were made with a time base which was too slow to allow a measurement of the temporal dispersion of the potentials

The temporal dispersion of the action potentials of the fibres of a subunit that could be expected in the absence of interaction was estimated to be at most 2 4 msec (p 112) This is also the maximum interval between the high amplitude double spikes and "triple spikes recorded by Ekstedt (1964) Let us theoretically consider subunits in which the interaction does not remove the temporal dispersion. As long as the t mpo al dispersion is small g 0 4 msec the compound potential of the subunit can only be distinguished from that of a single fibre by the occurrence of abrupt changes in slope on the positive-negative d flection (Fig 21 A) With increasing temporal dispersion the subunit potential recorded just outside the subunit g ts irregular (Fig 21 B and C) though not necessarily polyphasic. The shape and amplitude of the subunit potential depend on the recording electrode as well With concentric needle electrodes (area of inner core 0 1 0 4 mm^2) isolated spike components in th motor unit potential were considerably more difficult to obtain than with the 30 μ leads of a multielectrode (Ekstedt 1964) The spike amplitudes were about 5 times smaller and the duration of the positive n gative deflection w s twice that found with the 30 μ leads These ffects are to be expected from the radial chang of the action potential of th single fibre (Fig 1 A and p 105) though the spike amplitudes obtained with concen tric el ctrode appear rath r small and might be due to the different distortion of the action potentials by the large recording surface of the concentric l ctrode as compared with that caused by the multi lead l ctrode An experimental comparison f these distortions would be of interest. Ekstedt (1964) refers to unpublished obse vations by Ekstedt and Stålberg on the wall eff ct produced by the insulato in which the 30 μ leads are embedded He illustrates th influence of the electrode area by connecting seven leads of th multi lectrode distributed over 0 37 mm but gives only a single example A 20 per cent de rease in spike amplitude was observed.

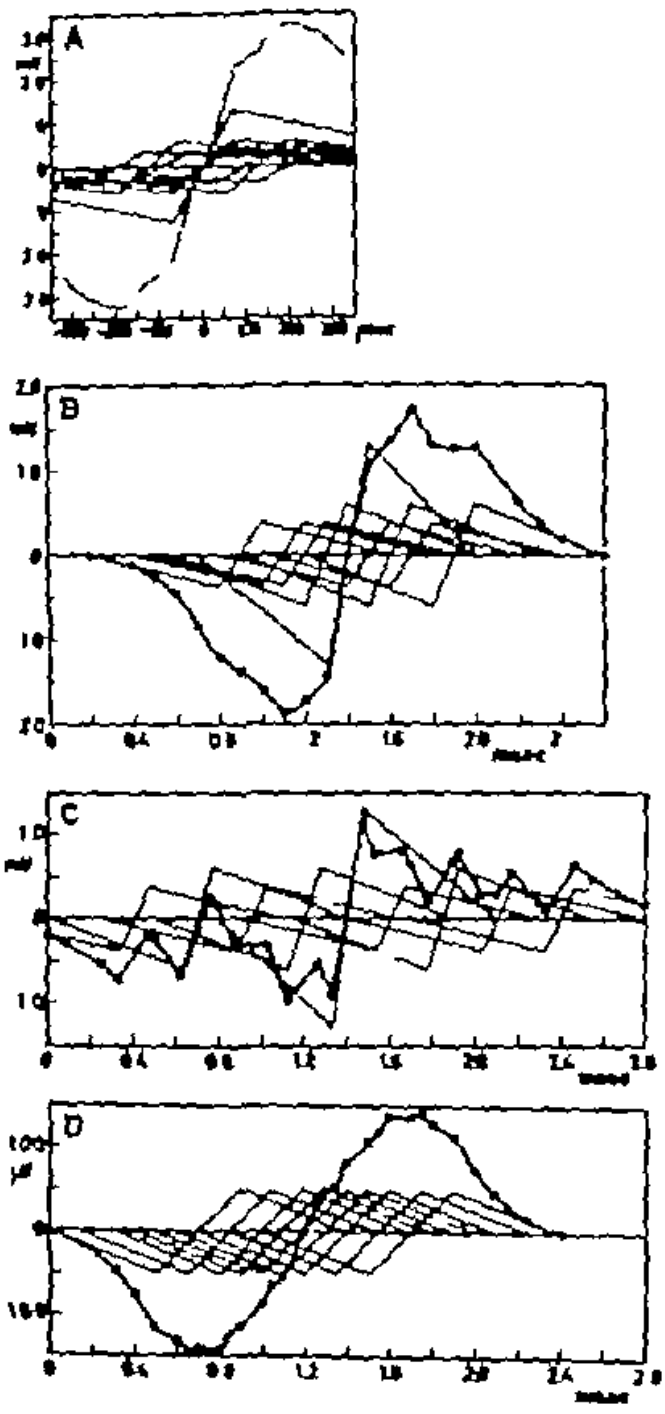


Fig 21

Subunit potentials constructed by summation of 9 temporally dispersed single fibre potentials
A. temporal dispersion 0 4 msec Rise time of single fibre potential 140 μ sec to simulate recording with a small electrode near the fibres
B temporal dispersion 1 msec Rise time of single fibre potential 200 μ sec to simulate recording with a concentric electrode near the fibres
C temporal dispersion 2 msec Rise time of single fibre potential 140 μ sec to simulate recording with a small electrode near the fibres
D temporal dispersion 1 msec. Rise time of single fibre potential 400 μ sec to simulate recording with a concentric electrode 0 8 mm from the fibres

At a distance of e g 0 6 mm from the subunit the increased duration of the positive negative deflections of the action potentials of the single fibres (Fig 14 D) effects a subunit pot ntial in which irregularitie are hardly detectable even when the temporal disper sion within the subunit is 1 msec (Fig 21 D) Under these circum stances the peak to-peak amplitude is about three times and the duration of the positive negative deflection about twice that of the action potential of a single fibre

In summary: the present result that a single muscle fibre can give rise to the high voltage motor unit spike potential containing fast and smooth positive negative deflection is not incompatible with the subunit concept. Even if the fibres do not interact the compound potential of the subunit is not necessarily polyphasic but humps and other irregularities occur

b Application

The knowledge regarding the radial change in shape and amplitude of the action potential of a single muscle fibre as obtained experi mentally and by th calculations can be used to derive information about the location of the fibres of a motor unit from the shape and amplitude of the motor unit potential. Thus Buchthal Guld and Rosenfalck (1957b) used an el ctrode containing 12 l ad (0 1 mm 1 5 mm 0 5 mm apart) to delineate the territory of the muscle cross section within which the fibre of a motor unit were located It appears from Fig 17 A and 14 D that the presence in th motor unit potential of a positive negative deflection with an amplitude above 50 μV and lasting less than 300-400 μsec indicates that an active fibre (or a subunit) is within a distance of less than 1 mm from the recording lead. Such criteria were used in the study of the limb mu cle f man where the diameter of the motor unit territory averaged 5-10 mm (Buchthal Erminio and Rosenfalck 1959). The accuracy of the delineation might be increased by using smaller leads and requiring a higher amplitude and a shorter duration of the positive negative deflection but the number of re cording sites would then have to be increased correspondingly to cover the same stretch in th muscle

Section 4

The muscle fibre in air

The action potential of a fibre surrounded by a thin layer of conducting fluid was derived in terms of the intracellular action potential. The theoretical expression was within few per cent identical with that obtained from the core conductor model both for muscle fibres and for nerve fibres

$$\varphi_e(b) \approx \frac{r_e}{r_i} V_i(z) \tag{191}$$

This model leads to surface action potentials which are higher in amplitude than the intracellular action potential if the external longitudinal resistance per unit length (r_e) exceeds the internal (r_i). This does not imply that the surface potential may increase indefinitely. It cannot exceed the electromotive force of the "battery" of the membrane of the fibre. In isolated frog muscle fibres in moist air the amplitude of the surface action potentials was the same as that measured intracellularly with the fibre in Ringer's solution (Håkansson 1957a). Theoretically this is to be expected if the external resistance with the fibre in air is large as compared with the internal resistance, that is if $(r_e/r_i) \gg 1$. According to (82)-(83) this condition is fulfilled if the thickness (b-a) of the conducting layer satisfies the relation:

$$b-a \ll \frac{\sigma_i a}{2\sigma_e}$$

i.e. if $b-a \ll 8.3\,\mu$ assuming $\sigma_i/\sigma_e = 1/3$ and $a = 50\,\mu$. From the conduction velocities in air and in Ringer's solution Håkansson (1957a) actually calculated a thickness of $2.3\,\mu$. The surface action potential in air had about half the rise time (from 20 to 100 per cent of peak) of the intracellular potential recorded from fibres in Ringer's solution (Håkansson 1957a). This was unexpected in view of the slower propagation along the fibre in moist air

Theory (p 38) predicts about the same rise time of the surface action potential as of the intracellular potential No experimental data are available about the rise time of the intracellular action potential with the fibre in moist air

Section 5

Conclusion future work

The mathematical analysis of potential fields presented in this study deals with continuously conducting circular cylindrical fibres of infinite length. Without doubt this is a very simplified type of po tential field. Still it proved to be useful. Though the evaluation related to excised muscles of man rat and frog gave similar re sults calculations based on an intracellular action potential of human muscle in situ ought to be made to support my interpretation of the extracellular spike potentials In connection with the numerical evaluations related to isolated muscle fibres reference was made to the fact that the fibres are elliptical rather than circular in cross section. Hence it seems of interest to extend the mathe matical analysis to elliptical fibres Furthermore one could take into account the finite length of the fibre and the fact that action potentials are propagating towards both ends

Next it would be of interest to perform a mathematical ana lysis of the action potentials of a single myelinated nerve fibre in a volume conductor

The potential field of the active soma of a nerve cell has been treated in detail by Rall (1962) assuming the soma to be spherical and to have one or a number of cylindrical dendrites attached to it (p 13) Rall (1962) assumed the soma to be a sink of current and the membrane of the dendrites to remain passive throughout Still more complicated is the field of a pool of neurones Rall (1962) pointed out that in superposing the potentials of several neighbouring neurones one had to consider the cell spacing and the extent of dendritic branching A qualitative analysis of such fields was made by Lorente de Nó (1947b) Closed fields arise in case of spherical symmetry s when the somas are located in the central zone of the pool while thin dendritic branches and tips are preponderant in the marginal zones If the pool does not show spherical symmetry ac tion currents spread outside it to produce an open field In view of the physiol gical importance attached to the effect of the action

currents of a cell on the excitability of neighbouring cells a mathe matical analysis of the potential field of a pool of active neurones seems desirable

The problems referred to in this Section cannot be solved ana lytically They are too complicated mathematically Numerical methods have to be used like those indicated by Rall (1962) and by the present study The impedance of the passive membrane and the properties of active membranes as described e g by the Hodgkin Huxley equations have to be taken into account to calculate the di stribution of the virtual sources and sinks of the extracellular ac tion potentials

APPENDIX 1

Comparison between the potential generated by a point source and a linearly extended source

Let us consider an electrostatic potential generated by a linear source along the z axis the charge density being

$$\rho(z) \quad \begin{cases} \frac{q_0}{2c}(1 + \alpha z) & \text{for } c \leq z \leq c \\ 0 & \text{for } |z| > c \end{cases}$$

Thus q_0 represents the total charge of the source The potential $\varphi(r\ z)$ produced by this distribution is

$$\varphi(r\ z) \quad \int_c^c \frac{q_0(1+\alpha s)}{2c\sqrt{(z\ s)^2 + r^2}}\, ds$$

Solving this integral we get

$$\varphi(r\ z) \quad \frac{q_0}{2c}\Big[(1+\alpha\ )\log \frac{z + c + \sqrt{(z+c)^2 + r^2}}{z\ \ c + \sqrt{(z\ c)^2 + r^2}}$$

$$\alpha\left(\sqrt{(\ +c)^2 + r^2} \quad \sqrt{(\ \ s)^2 + r^2}\right)\Big]$$

The potential corresponding to a source with constant density ρ is obtained by putting $\alpha \quad 0$

$$\overline{\varphi}(r\ z) \quad \frac{q_0}{2c}\log \frac{+c + \sqrt{(z+c)^2 + r^2}}{c + \sqrt{(\ \ c)^2 + r^2}}$$

We therefore have

Table A 1

Percentage difference between the potential of a point source (φ_p) and of a linear source of constant density ($\bar{\varphi}$)
(Length of the source 2c)

r	x 0	x ±c	x ±2c	x ±4c
0 4c	52	20	8	2
0 6c	30	11	7	2
0 8c	19	5	6	2
1 0c	13	2	5	2
1 2c	10	0	3	2
1 5c	7	1	3	2
2 0c	4	2	1	1

Table A 2

Percentage difference between the potential of a point source (φ_p) and of a linear source with zero density in one end (φ)
(Length of the source 2c)

r	x 4c	x 2c	x 0	x 2c	x 4c
0 4c	7	11	52	21	9
0 6c	7	11	30	19	9
0 8c	7	10	19	18	8
1 0c	7	10	13	16	9
1 5c	7	9	7	11	9
2 0c	6	8	4	8	7

$$\varphi(r\ z) \quad (1+\alpha z)\ \bar{\varphi}(r\ z) \quad \frac{\alpha q_0}{2c}\left(\sqrt{(z+c)^2+r^2} \quad \sqrt{(z\ c)^2+r^2}\right)$$

The potential generated by the total charge q_0 when concentrated at $z \quad 0$ is:

$$\varphi_p(r\ z) \quad \frac{q_0}{\sqrt{r^2+z^2}}$$

Tables A1 and A2 show that the error is le s than 10 per cent when the line source is replaced by a point source This applies to radial distances greater than half to three quarters of the length of the source and at axial distances larger than one to two times the length of the source

In these evaluations the charge density ρ of th line source was assumed either to be constant (Table A1) or to increase linearly from zero along the source (Table A2) If the charge density changes polarity along the source the total charge may become zero In such case the point source approximation is inappli cable Therefore if a linear charge distribution of variable density has to be approximated by a series of equidistant point charges the intervals must be chosen so that polarity is constant within each of them.

APPENDIX 2

The potential produced by a circular ring of charges of constant density

Let us consider a total charge Q distributed uniformly along a circle of radius a i.e the density of charge is $Q/2\pi a$ per unit length. The electrostatic potential generated by these charges is with the notation indicated in Fig 22 given by

$$\varphi(r\ z) \quad \frac{Q}{\pi} \int_0^{\pi} \frac{d\theta}{\sqrt{z^2+a^2+r^2 \quad 2ar\cos\theta}}$$

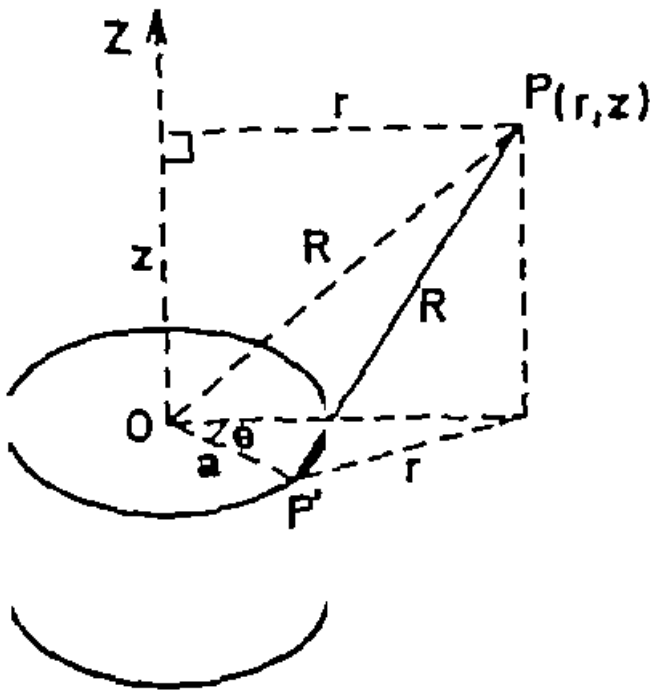


Fig. 22. Parameters used in the calculation of the potential produced by a circular ring of charges of constant density

ference is still smaller Thus in a plane one radius from that of the charges the difference is less than 2 per cent at axial distances $r > 1\ 3a$

Thus when Lorente de Nó (1947a) replaced a cylindrical distribution of charges by a linear one on the axis the error was negligible even close to the cylinder

SUMMARY

This monograph presents a mathematical analysis of intracellular and extracellular potential fields of active muscle fibres and of giant unmyelinated nerve fibres. The aim of the study was

(i) to derive the relation between the extracellular action potentials and the intracellular potential

(ii) to account for the shape duration amplitude and radial decline of the extracellular action potentials and

(iii) to study the relation between the present mathematical theory and other models the core-conductor model (Hermann 1879) introduced to account for the electrotonic spread of current along nerve and muscle fibres and the theory developed by Lorente de Nó (1947a) to explain the distribution of the action currents of nerve in volume conductors The theory of Lorente de Nó (1947a) was used previously by Buchthal Guld and Rosenfalck (1957a) and by Krakau (1957 1959) to interpret the radial decline of the extracellular action potentials of frog muscle fibres

On th basis of the Maxwell field equations it was shown that the potential fields of fibres along which conduction of action potentials takes place at a constant speed satisfy Laplace s equation if the intracellular and extracellular volume conductors are homogeneous and isotropic. In solving Laplace s equation the fibre was assumed to be infinitely long and to have a circular cross section so that the fields were symmetrical around the axis of the fibre Fourier transforms were used to obtain the formal solution The field within the fibre was derived the action potential measured just inside the surface giving the boundary condition. Th density of the membrane current was deduced from the radial gradient of the intracellular potential field at the surface of the fibre and could be written as a sum of two terms one of them being identical with the density of the membrane current as obtained from the core

conductor model With the current density thus derived as boundary condition the extracellular action potentials were deduced. The com plicated expression for the potential field contained an integral in volving modified Bessel functions of the second kind Considering the asymptotic behaviour of these functions at small arguments approximations to the potentials were obtained suitable for a numerical evaluation and easier to interpret than the true solution. One approximation was a line integral similar to that arrived at by Lorente de Nó (1947a) in his analysis of the spread of nerve action currents The other approximation was a surface integral

To compare the present findings with those of Lorente de Nó (1947a) the special case was considered when the fibre was sur rounded by a thin layer of the conductor The surface action potential could be considered a sum of two terms one of which was iden tical with the extracellular potential expected from the linear core-conductor model Furthermore the density of the membrane current was identical with that derived from this model In the analysis of the theory of Lorente de Nó (1947a) an expression was derived which compared the true membrane current with that arrived at by his theory As to the radial decline of the extracellular action potentials of the rigorous three dimensional model the high frequency components (4000 6000 Hz) decayed 8-10 times faster than the low frequency components (250-300 Hz)

Muscle is an anisotropic volume conductor of low conductivity inactive fibres acting almost as insulators The reduced conductivity by itself gives rise to an about 10 times higher amplitude of the extracellular action potential in situ than in Ringer's solution. The anisotropy caused a radial constriction of the potential field by 25 45 per cent and a further increase in the amplitude of the action potential recorded just outside the fibre by 35 60 per cent depend ing on the degree of anisotropy

The quantitative tests and the application of the mathematical theory were based on measured intracellular action potentials as well as on potentials simulating them.

The radial variation of the intracellular action potentials was negligible in muscle fibres as well as in unmyelinated giant nerve

fibres i.e the longitudinal current density varied insignificantly over the cross section of the fibre The membrane current was to good approximation proportional to the second derivative of the intracellular action potential i.e identical with that derived from the linear core conductor model. Furthermore the surface action potential of a fibre surrounded by a thin layer of conductor deviated insignificantly from that predicted by the core conductor model.

In the case of an extended external conductor the validity of the Lorente de Nó type of approximations to the extracellular field was confirmed by the finding that the membrane current calculated from them differed only insignificantly from the true value Calculations based on measured intracellular muscle action potentials lead to extracellular action potentials triphasic in shape the two main phases being of similar amplitude and the terminal positive deflection being 5-10 per cent of the peak to-peak value The peak to-peak amplitude declined to one tenth within about 0 5 mm from the fibre axis It was deduced that the amplitude of the action potential recorded just outside a rat muscle fibre increases almost as the square of the radius of the fibre and for a frog muscle fibre a proportionality between amplitude and radius was derived The difference between rat and frog muscle was due to the different relationship assumed between fibre diameter and conduction velocity The amplitudes calculated for th frog muscle fibres were too small by a factor of about two possibly due to the low resting and action potential of the fibres on which the calculations were based. The calculations relating to rat and human muscle accounted for the amplitude variation past a single fibre of the diaphragm observed by Krnjević and Miledi (1958) as well as for the volume conduction of spontaneous positive sharp-waves of human muscle studied by Buchthal and Rosenfalck (1966)

Finally the calculations showed that high voltage fibrillation potentials (1 4 6 5 mV Rosenfalck and Buchthal 1962) and motor unit spikes (2 5 10 8 mV Buchthal Guld and Rosenfalck 1957a) of human muscle can be derived from the activity of a single muscle fibre as suggested by Krnjević and Miledi (1958) and by Ekstedt (1964) This result is not incompatible with the existence of sub-

units within a motor unit It differs from the view previously held (Buchthal Guld and Rosenfalck 1957a Buchthal and Rosenfalck 1966) in that the present study indicates that interaction between the action potentials of adjacent muscle fibres is not likely to affect their conduction to a degree that complete synchrony results This problem needs experimental testing

The models used previously to describe the spread of nerve and muscle action potentials (the Lorente de Nó model the core conductor model and the di or tripole model) could be accounted for as different degrees of approximation to the rigorous mathematical model. This was shown by using the asymptotic behaviour and the relations between modified Bessel functions referred to by Clark and Plonsey (1966) They studied the applicability of the core-conductor model and their conclusions are supported by my findings. The core-conductor model could be applied extracellularly only when the fibre was surrounded by a thin conducting layer

The extracellular action potentials calculated from the approximation similar to that developed by Lorente de Nó (1947a) were triphasic That the action potentials recorded from isolated frog muscle fibres were diphasic (Håkansson 1956 1957) does not invalidate the calculations because the action potential changed from a triphasic to a diphasic configuration as the recording electrode approached the tendon end of the fibre (Katz and Miledi 1965)

In evaluating the relation between the amplitude of the action potential and the fibre thickness I assumed that the density of the membrane current was independent of the thickness Better agreement with the experimental findings would obtain if the density of the membrane current increases with the thickness of the fibre

RESUMÉ

Denne monografi indeholder en matematisk analyse af intra og extracellulære aktionspotentialfelter for umyeliniserede nervefibre og for muskelfibre Formålet med arbejdet var:

(1) at udlede sammenhængen mellem de extracellulære aktionspotentialer og det intracellulære potential

(2) at gøre rede for de extracellulære aktionspotentialers form vo righed amplitude og radiale dekrement samt

(3) at sammenligne den foreliggende matematiske model med (a) kerneleder modellen (Hermann 1879) der benyttes til forklaringen af elektrotonus hos nerve og muskelfibre og med (b) den mate matiske model Lorente de Nó (1947a) udviklede for at redegøre for spredningen af nerveaktionsstrømmene i en volumenleder Buchthal Guld og Rosenfalck (1957a) og Krakau (1957 1959) har tidligere benyttet Lorente de Nó s model til at gøre rede for den radiale aftagen af aktionspotentialerne fra isolerede frømuskelfibre

Først vises det på basis af Maxwell s ligninger at det intra såvel som det extracellulære aktionspotentialfelt skal tilfredsstille Laplace's ligning, idet det antages at aktionspotentialerne for planter sig med konstant hastighed hen langs fiberen og at volumenlederne er homogene og isotrope

Ved løsningen af denne ligning antage fiberen at være uendelig lang og at have et cirkulært tværsnit hvorfor felterne er rotationssymmetriske om fiberaksen. Ved den formelle løsning benyttes en Fourier transformation. Feltet inde i fiberen udledes idet man be nytter potentialet målt lige indenfor overfladen som grænsebetingelse Membranstrømtætheden bestemmes derefter udfra det intracellulære l ns gradient ved fiberens overflade og det vises at den kan skrives som en sum af to led hvoraf det ene er identisk med den membranstrømtæthed der fås udfra kerneleder modellen. Med den således bestemte membranstrømtæthed som grænsebetingelse udledes

derefter det extracellulære aktionspotentialfelt Den komplicerede løsning indeholder et integral hvori der indgår modificerede Besselfunktioner af 2 art. Ved brug af disse funktioners asymptotiske opførsel ved små argumenter viser det sig muligt at udlede approximationer til feltet der er egnet til numerisk behandling og lettere at fortolke end den sande løsning Den ene approximation var et linieintegral af samme type som det Lorente de Nó (1947a) ledtes til i sin analyse af spredningen af nerveaktionsstrømme og den anden approximation var et overfladeintegral

For at kunne sammenligne de foreliggende resultater med Lorente de Nó s (1947a) betragtes det specielle tilfælde hvor den ydre leder omkring den excitable fiber kun er et ganske tyndt lag. Det vises at overfladeaktionspotentialet kan betragtes som en sum af to led hvoraf det ene er identisk med det den lineære kerneledermodel vil give Desuden er membranstrømtætheden den samme som følger af denne model I en analyse af Lorente de Nó s allerede omtalte matematiske model foretages en sammenligning mellem den sande membranstrømtæthed og den der følger af hans model

Med hensyn til den radiale aftagen af det extracellulære aktionspotentialfelt således som den beregnes udfra den tredimensionale model vises det at de højfrekvente komponenter (4000 5000 Hz) aftager 8-10 gange hurtigere end de lavfrekvente (250 300 Hz)

Muskelvæv er en anisotrop volumenleder med lav ledningsevne da inaktive fibre virker næsten som isolatorer Den reducerede ledningsevne giver i sig selv anledning til at det extracellulære aktionspotential har en ca 10 gange større amplitude in situ end i Ringeropløsning Anisotropien bevirker en radial kompression af aktionspotentialfeltet på 25-45 procent og en yderligere forøgelse af amplituden på aktionspotentialet målt lige udenfor fiberen på 35 80 procent alt efter graden af anisotropi

Ved den numeriske afprøvning af den matematiske model benyttes dels virkeligt målte intracellulære aktionspotentialer dels simulerede potentialer Det vises at den radiale variation af det intracellulære felt er forsvindende i både muskel og nervefibre og at derfor den longitudinale strømtæthed varierer ubetydeligt over

fiberens tværsnit. Det vises endvidere at membranstrømtætheden med god tilnærmelse er proportional med den anden afledede af det intracellulære aktionspotential dvs identisk med den der følger af den lineære kernoledermodel Hvad angår det tilfælde da fiberen kun er omgivet af et ganske tyndt ledende væskelag viser beregningerne at overfladeaktionspotentialet kun adskiller sig uvæsentligt fra hvad der følger af kernoledermodellen.

Ved beregningerne vedrørende en ubegrænset ydre volumenleder bekræftes det tilladelige i at anvende den tilnærmelse til det extracellulære aktionspotentialfelt der er af samme type som Lorente de Nó s idet det f ex demonstreres at den membranstrømtæthed der følger af denne approximation, kun afviger ubetydeligt fra den sande værdi. De beregninger der derefter foretages udfra de faktisk målte intracellulære aktionspotentialer giver extracellulære potentialer som er trifasiske idet de to første faser har amplituder af samme størrelsesorden mens amplituden for den afslutte nde positive fase kun beløber sig til 5 10 procent af totalamplituden, Totalamplituden aftager til mindre end en tiendedel indenfor ca, 0 5 mm fra fiberaksen.

Beregningerne viser d suden at amplituden af det aktionspotential der måles lige udenfor en muskelfiber fra en rotte skulle vokse omtrent som kvadratet på fiberens radius mens man for en frømuskelfiber lede til simpel proportionalitet mellem amplitude og radius Denne forskel m llem rotte og frø skylde de forskel lige sammenhænge mell m propageringshastighed og fibertykkelse som er anvendt ved beregningerne De beregnede amplituder er for frømuskelfiberen ca halvdelen af det eksperimentelt fundne hvad der sandsynligvis hænger sammen med det lave hvile og aktionspotential hos de fibre hvorpå beregningerne er baseret. Hvad rotte muskelfiberen angår kan beregning rne gøre rede for den amplitudevariation Krnjevic og Miledi (1958) iagttog hen over overfladen af diafragma når de passerede den enkelt aktive fiber Beregningerne på det intracellulære aktionspotential fra human muskel kan redegøre for volumenledningen af de spontane positive potentialer sådan som den r iagttaget af Buchthal g Rosenfalck (1966) i denervered muskler ho mennesket. Det følger endvidere t

fibrillationspotentialer med stor amplitude (1 4 8 5 mV Rosenfalck og Buchthal 1962) og høje spidspotentialer i de vilkårlige potentialer hos mennesket (2 5 10 8 mV Buchthal Guld og Rosenfalck 1957a) kan repræsentere en enkelt fibers aktivitet som forestået af Krnjević og Miledi (1958) og af Ekstedt (1964) Dette resultat er ikke uforeneligt med existensen af underenheder (sub-units) i den motoriske enhed selvom det afviger fra den tidligere opfattelse (Buchthal Guld og Rosenfalck 1957a Buchthal og Rosenfalck 1966) Den foreliggende analyse viser at det er lidet sandsynligt, at vekselvirkningen mellem nabofibres aktionspotentialer påvirker deres propagering i en sådan grad at de synkroniseres fuldstændigt.

Hovedresultatet af det foreliggende arbejde er at det er muligt at udlede de forskellige modeller der hidtil er anvendt ved beskrivelsen af nerve og muskelaktionspotentialfelter Lorente de Nó's model kerneledermodellen og di og tripolmodellen som forskellige grader af tilnærmelse til den strenge 3 dimensionale matematiske model Dette vises ved at gøre brug af de modificerede Bessel funktioners asymptotiske opførsel og relationerne der gælder mellem dem og som Clark og Plonsey (1966) har benyttet i deres analyse af kerneledermodellens anvendelighed. Deres konklusioner støttes af mine resultater Kernelederen kan kun anvendes extracellulært når fiberen er omgivet af et ganske tyndt ledende væskelag.

De extracellulære aktionspotentialer der beregnes udfra den approximation der er af samme type som den Lorente de Nó (1947a) udviklede er trifasiske Den tilsyneladende modstrid med de difasiske aktionspotentialer som Håkansson (1956 1957) registrerede fra isolerede frømuskelfibre gør ikke beregningerne unanvendelige Katz og Miledi (1965) har nemlig vist at aktionspotentialets form ændres fra at være trifasisk til at blive difasisk når registreringselektroden nærmes fiberens sarcemme

Ved udledningen af sammenhængen mellem aktionspotentialamplitude og fibertykkelse blev det antaget at membranstrømtætheden er uafhængig af tykkelsen Bedre overensstemmelse med de eksperimentelle fund vil man få hvis membranstrømtætheden voksede med fibertykkelsen.

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The subject of this monograph arose from studies on the motor unit potentials of normal human muscle initiated and conducted by Professor dr med. F Buchthal in the Institute of Neurophysiology of the University of Copenhag n These studies aimed at accounting for the duration shape and amplitude of the motor unit potenti ls in terms of the summation of the spatially and temporally dispersed action potentials of the individual fibres of the unit. Therefore knowledge of the volume conduction of the action potential of a single muscle fibre was required. My mathematical analysis of thi problem intended to derive an approximation to the action potential in terms of an expression containing the second derivative of the intracellular action potential but otherwise similar to that deduced by Lorente de Nó to account for the spread of the compound action potential of a nerve Several attempts at obtaining an approximation of this type had to be discarded since they lead to integrals which I was unable to interpret.

My work has been much stimulated by the members of the Institute Profe sor Buchthal has with never failing energy introduced me to the biological problems and to scientific methods has encouraged inspired and teased me whatever was most appropria Our joint collaboration with the m ticulously wo king and ever ing Chr Guld E E provided detailed experimental information the el ctrophysiology of the m tor units Prof sor dr med. O Sten-Knudsen and I have often di cu sed common mathemati problems He pointed out th weaknesses in the first edition of the manuscript. Prof sso Buchthal sought the expert advice Profe sor dr techn. H Lottrup Knudsen when we encount problem of evaluating the influence of the anisotropy of the tissu on the spread of the a tion currents He provid the solution of th problem which I hav presented in a version. Prof sor Lottrup Knudsen and the late Professor

R Petersen made valuable suggestions after reading the first edition of the manuscript Dr med Lise Engbæk supplied me with the intracellular action potentials of frog muscle Dr E Muscholl Main permitted me to use one of his recordings from rat muscle and Dr H P Ludin Bern provided the action potential from human muscle At a time when my mathematical problems looked too discouraging I decided to try to evaluate by numerical methods alone the degree of approximation that was obtained by means of my modified Lorente de Nó model This involved the application of a digital computer and I wa given valuable dvice on numerical methods as well as on computer problems by Mr B Svejgaard Nielsen M Sc. Head of the Section on Information Processing at the H C. Ørsted Institute However in the end a simpler solution was found as described in this monograph For critical reading of my manuscript and for correcting my English I am in debt to M. A Lennox Buchthal M D Mr F Riis skilfully made the illu strations and Mrs E M Fischer typed with rapidity and excellence the several editions of the manuscript. I thank most heartily for all the help hereby given to me in completing this thesis

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I dedicate this book to my wife Anneliese Rosenfalck E E and to Professor Dr H Pau Düsseldorf Without their support it had never appeared

Bagsværd December 1968

Poul Rosenfalck

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LIST OF SYMBOLS

(figures in brackets refer to the page on which each symbol is introduced)

a	radius of nerve or muscle fibre (19)
A	amplitude of action potential (99)
A_e	amplitude of intracellular action potential (121)
A_i	amplitude of extracellular action potential (121)
$\vec{A}$	vector potential (17)
$A(p)$	coefficient of integration (20 28 35)
b	radius of nerve or muscle fibre plus a layer of conducting fluid (34 35)
$\vec{B}$	magnetic induction (15)
$B(p)$	coefficient of integration (20 28 35)
c	velocity of light (15)
$C(p)$	coefficient of integration see (99) (47)
curl $\vec{A}$	$(\frac{\delta A_z}{\delta y} \quad \frac{\delta A_y}{\delta z} \quad \frac{\delta A_x}{\delta z} \quad \frac{\delta A_z}{\delta x} \quad \frac{\delta A_y}{\delta x} \quad \frac{\delta A_x}{\delta y})$ (15)
$\vec{D}$	electric displacement (15)
div $\vec{A}$	$\frac{\delta A_x}{\delta x} + \frac{\delta A_y}{\delta y} + \frac{\delta A_z}{\delta z}$ (15)
$\vec{E}$	strength of electric field (15)
grad φ	$(\frac{\delta \varphi}{\delta x} \quad \frac{\delta \varphi}{\delta y} \quad \frac{\delta \varphi}{\delta z})$ (17)
$G(x)$	simulated intracellular action potential (58 59 62)
$G'(x)$	$\frac{dG}{dx}$ (60)
$\vec{H}$	strength of magnetic field (15)
$\vec{i}$	current density (15)
i_e	longitudinal current in external conductor (25)
i_i	longitudinal current in the fibre (core) (25)
i_m, $i_{m\,1}$	density of membrane current (22 45)

i_r density of radial current (22)

i_z density of current in the fibre direction (22)

I_1 I_2, I_3 strength of current source (65 67)

$I_0(rp)$, $I_1(rp)$, $I_2(rp)$ modified Bessel functions of the first kind order 0 1 and 2 (20 22 23)

$J_0(kr)$, $J_1(kr)$, $J_m(kr)$ Bessel functions of the first kind order 0 1 and m (43)

K complete elliptic integral of the first kind (33)

$K_0(rp)$, $K_1(rp)$ modified Bessel functions of the second kind order 0 and 1 (20 28)

m surface membrane of nerve or muscle fibre (23)

p independent variable of Fourier transforms (18)

Q total charge of circular ring (139)

q_0 total charge of source (135)

r distance from axis of fibre (19)

R distance (32)

r_e ohmic resistance per cm length of external conductor (25)

r_i ohmic resistance per cm length of intracellular fluid (25)

x independent variable (length) (30)

t time (17)

u independent variable by Fourier transformation (30)

U conduction velocity of action potential (17)

V_e surface action potential in oil or air (25 42)

V_i intracellular action potential (19)

rat muscle 89 123
resting potentials 89

shape 11 13 95 117 118
simulated action potentials 14 58 70 74 76 81 83 84 85 88
solid angle model 12 117
spike potentials 14 104 110 124 129
spread of action potentials
    experiments on 10
    calculated 11
subunit 110 124 127
surface action potential 12 36, 42 83 130

temporal dispersion 111 125
tendon end action potentials 120
three dimensional model 14
time constant 16 53
triple spikes 112 127
tripole model 65 67 91 119

voltage maximum 56 98 105 122
voltage versus thickness of fibre 14 98 120
volume conduction
    experiments on 10 124
    theories on 11
volume conductor 9
    anisotropic 14 50
    limited 35 130
    unlimited 28 85 117

wall effect 109 127

ACTA PHYSIOLOGICA SCANDINAVICA
SUPPLEMENTUM 322

Studies on the Mode of Action of α-Methyldopa

BY
MATTS HENNING

GÖTEBORG 1969

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FROM THE DEPARTMENT OF PHARMACOLOGY
UNIVERSITY OF GÖTEBORG, SWEDEN

Studies on the Mode of Action of α-Methyldopa

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MATTS HENNING

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This summary is based on studies reported in the following papers

1 M Henning Interaction of DOPA decarboxylase inhibitors with the effect of α methyldopa on blood pressure and tissue monoamines in rats. Acta pharmac. et tox 1969 in press.

2. M Henning Blood pressure and noradrenaline levels after treatment with α methyldopa, α-methyldopamine and α methyl-*m*-tyrosine J Pharm Pharmac. 1967 *19* 775–779

3 M Henning and L. Svensson Adrenergic nerve function in the anaesthetized rat after treatment with α methyldopa. Acta pharmac et tox. 1968 *26* 425–436.

4 M Henning and P A van Zwieten Central hypotensive effect of α-methyl dopa. J Pharm Pharmac. 1968 *20* 407–419

The papers are referred to by their arabic numerals in the text i e Paper 1 Paper 2 etc.

Abbreviations used. DA = dopamine DOPA = 3 4-dihydroxyphenylalanine 5-HT = 5-hydroxytryptamine α MD = α-methyldopa α-MDA = α methyldopamine, α MmT = α-methyl-*m*-tyrosine α MNA = α methylnoradrenaline

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ELANDERS BOKTRYCKERI AKTIEBOLAG

INTRODUCTION

The arterial blood pressure in mammals varies as the product of cardiac output and peripheral resistance. Among the components influencing these factors the neural control through the autonomic nervous system is perhaps the most important. The main determinants of cardiac output are heart rate and stroke volume. The former is governed by sympathetic and vagal impulses which act on the basal rhythmicity of the heart muscle cells, stroke volume is mainly adjusted by alterations in sympathetic activity (review by Sarnoff and Mitchell 1962). Total vascular resistance is determined by a balance between the effects of nervous vasoconstrictor discharge and the basal tone of the vessels, with contributions from locally produced vasoactive substances (review by Folkow, Heymans and Neil 1965). The morphological and physiological basis for this autonomic control of circulation has been extensively studied in recent years. The development of specific and sensitive biochemical assay systems for the determination of monoamines in tissues and body fluids, as well as the introduction of the fluorescence method for visualizing these amines in histological preparations, has greatly added to the clarification of functional and structural aspects of the sympathetic nervous system throughout the body. This subject has been covered in several reviews e.g. Euler (1959, 1966), Hillarp (1959, 1960), Carlsson (1966), Norberg (1967). The use of refined physiological techniques has expanded our knowledge of central nervous cardiovascular control considerably; the information has been summarized by e.g. Uvnäs (1960), Folkow *et al* (1965).

As an introduction to this summary, a brief review will be presented of some relevant anatomical and functional points in the regulation of blood pressure. Special attention will be drawn to the importance of the sympathetic nervous system.

The peripheral division of the sympathetic system will be considered first. It has long been known that the transmission of nerve impulses to the effector cells from postganglionic sympathetic nerves is mediated by a humoral mechanism, and the work of Euler (1946, 1948) established that the transmitter is noradrenaline (NA). However, certain sympathetic fibres are cholinergic, notably those subserving a vasodilator function (see Uvnäs 1960). By means of the fluorescense method it has been found that the NA in sympathetic nerves is located in intensely fluorescent enlargements on the postganglionic nerve terminals, the varicosities. The varicose terminals are enclosed in an autonomic ground plexus (Hillarp 1946, 1960) in close contact with the effector cells. In the heart, the rhythm-regulating structures, the conduction system and the muscle cells are abundantly supplied by NA-containing

vasoconstrictor tone and hence arterial blood pressure by excitation and inhibition, respectively of the spinal neurons. Alterations in cardiac activity are conveyed through sympathetic accelerator and parasympathetic inhibitory pathways. The contractile force of heart muscle may be specifically influenced (Rushmer 1962).

Higher levels of central vasomotor control are found in e.g. the mesencephalon, the hypothalamus and the cerebral cortex. The type of regulation exerted by these centres often is of a more complex nature than at lower levels. Reflex patterns comprising differentiated vasoconstrictor influences, sometimes with concomitant vasodilatation in other vascular beds, have been described. The hypothalamus is often ranked with the vasomotor centre in the medulla oblongata as the most important level of superior autonomic circulatory control. Excitatory and inhibitory responses of vessels and heart have been obtained on stimulation of various hypothalamic areas and its integrative function is evident from the fact that most of the corticofugal pathways involved in blood pressure control pass through or are relayed in this region. In addition the majority of the efferent fibres from the hypothalamus seem to have synapses in the medullary vasomotor centre. However some neurons project directly on the spinal sympathetic centre. Among cortical regions of importance for cardiovascular regulation are the motor and premotor areas, the anterior part of the temporal lobes and certain sections of the limbic system. Thus, Löfving (1961) observed that stimulation in the cingulate gyrus may elicit pronounced alterations in sympathetic tone. Of special interest is the possibility of a differential vasoconstrictor control by a selective excitation of pathways to either muscle, renal or intestinal vessels.

The precise morphological basis of cardiovascular regulatory activity at suprabulbar levels is only partly understood. Studies with the histochemical fluorescence method have demonstrated extensive NA-containing terminal networks in the relevant regions of the brain stem, the mesencephalon and the hypothalamus. 5-HT terminals have been found in the brain stem and biochemical determinations have shown high concentrations of this amine in e.g. the hypothalamus. In some instances it has been possible to trace complete neuron systems which may correspond to pathways with similar localisation previously demonstrated with e.g. neurophysiological techniques. Short NA and 5-HT-containing neurons with both the cell bodies and the nerve terminals located in the lower brain stem, sometimes innervating autonomic nuclei, may be involved in cardiovascular reflex activity at these levels. Ascending catecholamine and 5-HT neurons have been shown to run from these regions to e.g. the hypothalamus, the limbic system and the neocortex. Further studies will no doubt provide more informations regarding the precise functional significance of such monoamine pathways.

Aim of the present investigation

Drugs which influence the formation, storage and release of monoamines are often found to exert profound effects on autonomic neuronal mechanisms. This may

reflect an interference with monoaminergic neuron systems of the kind previously mentioned. Many currently used antihypertensive agents seem to have such effects and, as evident from the survey presented above, their action may be located at several different levels of central cardiovascular control. An important aim in studying the mechanisms of action of such drugs should therefore be to localize the effect of the drug within the regulating systems. In addition, it may be of considerable relevance to establish the precise locus of the effect on adrenergic neurons thus may have important therapeutical implications, e.g. in cases of possible interactions with other drugs.

The DOPA analogues L-α methyl-DOPA (α-methyldopa, α MD) and L-α methyl *m*-tyrosine (α MmT) interfere with the metabolism of the biogenic monoamines in several respects. They may thus be assumed to influence the function in neurons utilizing these amines as neurotransmitters. The abundant literature on α MD and α-MmT has been summarized in several recent review articles (Muscholl 1966, Holtz and Palm 1966, Stone and Porter 1966, 1967, Sourkes and Rodriguez 1967, Kopin 1968). However, in spite of several years of concentrated research, the mechanisms underlying the hypotensive action of α-MD are not clearly understood.

It is commonly agreed that the effect of α MD is mediated through an interference with the sympathetic system. However, the exact site of action has not been established. Opinions differ as to the function of the peripheral sympathetic nerves after treatment with α MD (see reviews mentioned above and Paper 3). On the other hand, several obvious effects of the drug on the central nervous system have been reported.

The interrelation of the alterations in monoamine metabolism and the functional effects of α MD is also subject to debate. It has been suggested that the amines formed on metabolism of the drug may take over the function of DA and NA in the brain (Carlsson and Lindqvist 1962), as well as that of NA in peripheral sympathetic nerves (Day and Rand 1964). The activity on the adrenergic receptors of the amines formed from α MD appears to be less than that of NA and it has been proposed that the α-methylated derivatives act as substitutes or "false" transmitters in the sympathetic nerves, thereby explaining the hypotensive effect of α-MD (Day and Rand 1963, 1964). This hypothesis seems to have become widely accepted (see reviews by Crout 1966, Muscholl 1966, Stone and Porter 1966, Sourkes and Rodriguez 1967, Kopin 1968).

The studies summarized here were undertaken to examine the relationship of the hypotensive action of α-MD and the effects of the drug on monoamine metabolism. Basic to the hypothesis of false transmitters is that the lowering of blood pressure depends on the formation of amines from α MD. It was found that decarboxylation of the drug in the central nervous system is a prerequisite for the antihypertensive effect (Paper 1), thus pointing to the involvment of a central mechanism in the action of α-MD on blood pressure. When the NA levels in peripheral tissues were examined in parallel with blood pressure after a single dose of α-MD (Paper 2), there was no

clear-cut correlation in time between these parameters. The assumed false transmitters do not lower blood pressure by themselves (Brunner *et al* 1966, 1967 Paper 2) and the function in peripheral sympathetic nerves is only slightly impaired at the time of maximal fall in blood pressure (Paper 3) These results seemed to point to the importance of an effect of α MD on central mechanisms. In Paper 4 direct evidence was obtained showing that α-MD is capable of lowering blood pressure by acting via the central nervous system

METHODOLOGICAL CONSIDERATIONS

The methods used have been described in detail in the individual papers and only a summary will be presented here.

Experiments on anaesthetized animals

In Paper 3 preganglionic stimulation of the cervical sympathetic trunk was performed in rats anaesthetized with urethane (1.2 g/kg body weight s.c.). A Grass stimulator model S4 was employed and stimulation parameters were 5 V 1 msec rectangular pulses with frequencies from 0.4–25.6 imp./sec in geometrical succession using bipolar platinum electrodes. The response of the inferior eyelid was recorded with a Grass force displacement transducer model FT03. A graduated coil spring was inserted in the system connecting the eyelid to the transducer allowing for a convenient adjustment of the initial tension to 0.50 g. Since this device permitted a certain amount of movement of the eyelid, the contractions were regarded as semi-isometrical. Blood pressure was taken from the left carotid artery to a Statham model P23Dc pressure transducer. The contractions of the eyelid and the blood pressure were recorded on a Grass Model 5 Polygraph via suitable preamplifiers.

In Paper 4 changes in blood pressure were recorded from a femoral artery with similar techniques after infusion of drugs into the left vertebral artery of cats under chloralose anaesthesia (60–70 mg/kg i.p.). Since cannulation of the vertebral artery involved thoracotomy artificial respiration was applied throughout these experiments by means of a positive pressure respiration pump. An attempt was made to evaluate if the artificial respiration was adequate by stopping the pump temporarily at intervals throughout the experiments and observing the spontaneous respiratory movements of the animals.

Blood pressure recording in conscious rats

Previous studies on α methyldopa indicated that the hypotensive effect of this drug was difficult to demonstrate in anaesthetized animals. Most of the techniques available in the literature for recording of blood pressure in conscious animals were adapted for use in small animals e.g. rats. In addition, several previous investigations on the effects of α MD on monoamine metabolism had been performed in rats, allowing a direct comparison with the present findings.

The methods for measuring blood pressure in conscious rats fall in two main categories. True arterial blood pressure may be recorded through a catheter implanted

in a suitable artery Indirect estimations are possible by recording the pressure required to occlude the circulation to a peripheral tissue in which the blood flow can be qualitatively monitored, e.g. by plethysmography or auscultation in a paw or the tail. The more direct method of measurement was chosen to avoid erroneous results after administration of vasoactive drugs. Popovic and Popovic (1960) have described a convenient preparation which involves cannulation of the thoracic aorta via the left carotid artery This method, with some modifications, was used in the present study and a brief description will be given of some technical details.

In Sprague-Dawley rats weighing 180–250 g anaesthesia was induced by pentobarbital (Nembutal® Abbott) 30–40 mg/kg i p and the left carotid artery was carefully isolated and ligated A polyethylene catheter (as a rule Portex Tubing PP 25 outer diameter 0 80 mm) shaped as described by Popovic and Popovic (1960) was introduced into the artery and pushed distally until the tip reached the aorta. The catheter was then withdrawn a few mm and secured by double ligatures. The free end was passed subcutaneously between the eye and the ear on the left side of the animal and exteriorized midway between the ears on the back of the neck. A piece of an injection needle of suitable gauge ending with a short piece of heat-sealed polyethylene tubing was attached. The animals were kept in individual cages and their catheters were flushed at least 1–2 times daily with about 0.5 ml of a solution of heparin (25 IU/ml in 0 9 / saline) Body weight, which as a rule showed a transient decrease during the first 1–2 days after the operation, was checked frequently and animals not displaying a normal gain in weight were discarded. As a rule no recordings were performed for the first two postoperative days, although no systematic variation could be detected in repeated measurements beginning immediately after recovery from anaesthesia. For recording of blood pressure, the catheter protruding from the rat was clamped by means of a hemostat with both tips protected by polyethylene tubing (to avoid damage to the catheter), the heat-sealed piece removed and the needle adapter joined to a length of tubing which permitted free movements of the rat in its cage. The connection to the transducer (Statham P23Dc recording on a Grass Model 5 or Model 7 Polygraph) was made through a 3-way stopcock to enable periodical flushing with saline-heparin solution as required The entire system was filled with this solution. Owing to the small diameter of the catheter and the length of the system only average pressures could be recorded Basal values of blood pressure were obtained by continuous recording for 10–40 minutes 3–4 times daily for at least 2 days, usually at a paper speed of 0.25 mm/sec. During the first few minutes the blood pressure of the rats tended to be somewhat higher than at later intervals. The initial five minutes of each recording were therefore always disregarded and the average of the following 5–10 minutes was taken as the level of blood pressure Blood pressure varied little at various times of the day or during subsequent days Daily recordings of blood pressure were made in nine rats analysis of variance with two criteria of classification did not show any significant variation between days (Table 1) The basal values were therefore

Table 1 Mean arterial blood pressure of 9 normal rats during four subsequent days. Analysis of variance using two criteria of classification shows that there is a significant variation between rats ($p < 0.01$) but not between days ($p > 0.10$).

Rat No.	Day 1	Day 2	Day 3	Day 4
1	105	105	120	120
2	105	110	110	115
3	100	95	95	115
4	125	110	115	115
5	135	120	120	135
6	115	120	120	110
7	115	115	120	115
8	100	110	110	105
9	125	115	105	110

estimated as an average of all basal recordings in individual rats and drug effects were expressed as changes from this mean in mm Hg. Care was always taken to follow the blood pressure to complete recovery after drug treatment and, usually recordings from 6 or more days after the last drug administration were included in the basal values. The mean value of all basal recordings in 57 normotensive rats was 112 mm Hg with a standard deviation of 13 8. This value is comparable to the values reported by Stanton and White (1965) and Stanton and Cooper (1966, 1967) (average basal pressure was 108 mm Hg, s.d. 10 7 in 100 rats recordings were made from a catheter in the abdominal aorta). Other investigators, using the same technique have found somewhat higher basal pressures (Weekes and Jones 1960, McIlreath De Graw and Kadar 1965) However in these studies, both experimental conditions and animal strains were different.

The influence of an i p injection of 2.0 ml 0 9% saline is shown in Fig. 1 The changes in blood pressure are small and statistically insignificant (t-test)

In spite of daily flushing with heparin solution as indicated above the arterial catheters were eventually occluded by thrombus formation around the tip. The average time during which reliable blood pressure recordings could be taken was about 15 days with a range of 10–40 days.

Induction of arterial hypertension in rats

Several possibilities for induction of experimental hypertension in rats have been described (for reviews, see Green 1963 Plummer 1967). Studies of drug effects on blood pressure have mostly been performed with renal hypertensive animals the technique described by Sokabe and Grollman (1963) which involved a partial infarction of one kidney and a contralateral nephrectomy was used. The operations were performed on young Sprague Dawley rats of either sex (body weight 80–100 g) under pentobarbital anaesthesia (Nembutal® Abbott, 30–40 mg/kg). Through a

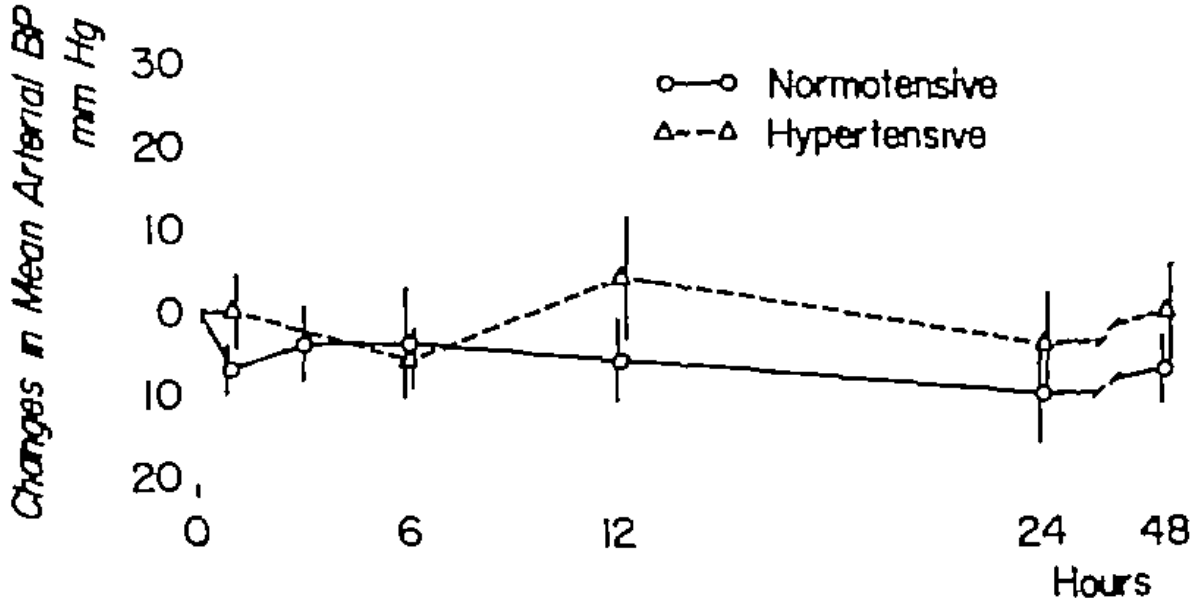


Fig. 1 Effect of an i.p. injection of 2.0 ml 0.9% saline to normal (circles) and hypertensive (triangles) rats. Basal pressures were 111 mm Hg (s.e.m.=5.0, n=4) and 170 mm Hg (s.e.m.=5.4 n=9), respectively

longitudinal incision the right kidney was exstirpated and an anterior branch of the left renal artery was ligated in order to produce an infarction of the ventral half of the kidney The operative mortality was about 20 per cent. About 80 per cent of the surviving animals slowly developed an arterial hypertension reaching stable high blood pressure levels 4–6 weeks after the operation The hypertensive rats increased their body weight slower than normal animals and also showed gross pathological signs of hypertensive disease, e g. left ventricular hypertrophy

Recording of blood pressure in these animals was performed as described above for normotensive rats and hypertension was defined as the average basal blood pressure exceeding 140 mm Hg. This corresponds to a mean basal blood pressure of the normotensive rats plus two times the standard deviation of this value Using this criterion, 65 out of 78 rats cannulated after operation for induction of hyper tension were hypertensive with an average basal blood pressure of 167 mm Hg (standard deviation 19 2) A few of these animals had a basal blood pressure in excess of 200 mm Hg. The results of an i.p injection of saline is shown in Fig. 1 the changes were not significant.

Biochemical determination of monoamines in tissues

Tissue levels of the following amines were determined after homogenization and extraction in ice-cold perchloric acid. The organs from two animals were always pooled The individual methods have been described in the references listed below

noradrenaline (Bertler Carlsson and Rosengren 1958)

dopamine (Carlsson and Waldeck 1958 modified according to Carlsson and Lindqvist 1962)

5-hydroxytryptamine (Bertler 1961 modified according to Andén and Magnusson 1967)

α-methyldopamine (Carlsson and Lindqvist 1962).

Drugs used

atropine sulphate
carbachol chloride
chloralose
eserine salicylate
DL-α-hydrazino-α-methyl β-(3 4-dihydroxyphenyl) propionic acid (MK 485)
4-(m-chlorophenylcarbamoyloxy)-2 butynyltrimetylammonium chloride (McN A 343)
L-α-methyl-3 4-dihydroxyphenylalanine (α methyldopa)
D-α-methyl-3 4-dihydroxyphenylalanine (D-α methyldopa)
DL-α methyl-*m* tyrosine
(±)-α-methyldopamine hydrobromide
(−)-noradrenaline-d-bitartrate
pentobarbital sodium (Nembutal®)
DL-seryl 2,3 4-trihydroxybenzyl-hydrazine hydrochloride (Ro 4-4602)
tyramine hydrochloride
urethane.

The doses given refer to the salts

Statistical methods

Tests of significance were conducted using Student s t test and analysis of variance with one or two independent criteria of classification (Davies 1949).

RESULTS AND DISCUSSION

Introductory remarks

α-MD was synthesised with the aim of obtaining a DOPA decarboxylase inhibitor and it was believed that such a compound might be therapeutically useful in the treatment of hypertension (Sourkes 1954). DOPA decarboxylase inhibition by α MD *in vivo* was demonstrated by Dengler and Reichel (1957) and Westermann Balzer and Knell (1958) The closely related, possibly identical enzyme (Rosengren 1960) catalyzing the decarboxylation of 5-HT was also inhibited by α-MD (Westermann *et al* 1958 Smith 1959 1960) These properties of α MD are also shared by α MmT which is however considerably less active than α MD (Porter Totaro and Leiby 1961 Hansson and Clark 1962, Carlsson 1964) The pharmacology of decarboxylase inhibitors has been extensively reviewed by Clark (1959) Sourkes (1966) and Sourkes and Rodriguez (1967)

α-MD may also inhibit monoamine synthesis at the stage where tryptophan or tyrosine are hydroxylated (Sharman and Smith 1962, Roos and Werdinius 1963 Ross and Haljasmaa 1963 Pletscher Burkhard and Gey 1964 Pletscher and Gey 1964 Nagatsu Levitt and Udenfriend 1964 Udenfriend 1966) It should be pointed out that α MD is a relatively weak inhibitor of tyrosine hydroxylase and α-MmT is only slightly active in this respect.

Treatment of animals with α MD or α MmT results in a decrease in the levels of DA and 5-HT in various tissues, as first reported by Murphy and Sourkes (1959) and Smith (1959 1960) Soon after this it was found that tissue levels of NA were also lowered by these drugs (Oates *et al* 1960 Sourkes *et al* 1961 Hess *et al* 1961 Kuntzman *et al* 1961) These findings are substantiated in numerous later studies (for references, see reviews mentioned above) Only the laevo isomers are capable of inducing amine depletion and the lowering of NA is more long lasting than that of DA and 5-HT α MmT seems to be more active than α-MD The mechanism underlying the depletion of catecholamines by these drugs has been subject to considerable argument (see review by Muscholl 1966) The lack of specificity of the enzymatic mechanisms in the synthesis of DA and NA allows for a metabolism of the α-methylated amino acids to form their corresponding amines. It is now generally agreed that α MD and α MmT lower catecholamine levels chiefly by a mole for mole displacement of these amines from their intraneuronal storage sites by the amine metabolites of the α methylated acids (Carlsson and Lindqvist 1962, Maitre and Staehelin 1963 Muscholl and Maitre 1963 Andén 1964 Shore, Busfield

and Alpers 1964 review by Muscholl 1966) Inhibition of synthesis may contribute to the effect, particularly in the case of 5-HT lowering after α-MD (Sharman and Smith 1962, Roos and Werdinius 1963 Carlsson 1964 Pletscher *et al* 1964).

After treatment with α MD the depleted amines are replaced by metabolites of α MD i.e. α methyldopamine (α-MDA) or α-methylnoradrenaline (α-MNA) Both compounds have been identified in the brains and peripheral tissues of animals given α MD (Carlsson and Lindqvist 1962, Muscholl and Maitre 1963 Porter Totaro and Burcin 1965 Torchiana *et al* 1965) In addition, the presence of small amounts of α-methyl-adrenaline in the adrenal medulla was reported by Muscholl (1965) In the case of α MmT the depleting agents are α methyl-*m*-tyramine and metaraminol both of which have been found in brain and peripheral organs of experimental animals treated with α MmT (Carlsson and Lindqvist 1962, Andén 1964).

Evidence is accumulating that α methylated amines are stored in the same manner as normal transmitters. Their displacement of NA appears to be stoichiometric (for references, see above) and it is evident from histochemical studies that α-MNA (*Hillarp and Malmfors 1964 Malmfors 1965*) as well as *metaraminol* (*Jonsson* and Ritzén 1966) are taken up and accumulate in adrenergic nerves after administration of the corresponding amino acids. α-MNA appears to have an intracellular distribution closely resembling that of NA following pretreatment with reserpine neither of these amines are taken up by the granules but appear to be located extra granularly Furthermore injected radioactive α-MNA and metaraminol are taken up by the adrenergic nerve fibres and retained by a particulate fraction of sympathetically innervated tissues (Lundborg and Stitzel 1967*a* 1967*b*), indicating a granular storage of these amines. In addition, reserpine blocks the granular uptake of ^{3}H-α MNA. The time course of this blockade, measured by means of ^{3}H NA uptake, recovers simultaneously with the adrenergic function (Lundborg and Stitzel 1967*b* 1968) The uptake and storage of α-MNA and metaraminol by the adrenergic nerves may thus be regarded as a partial fulfillment of the criteria for their identification as false transmitters Another requirement necessary for this is that a presumed transmitter substance should be released by nerve stimulation. Muscholl and Maitre (1963) were able to demonstrate that α-MNA formed from α-MD was released during stimulation of the sympathetic nerves in isolated rabbit hearts. Crout *et al* (1964) have made similar observations with regard to metaraminol which had replaced NA in the hearts of cats

These findings suggest that the amine metabolites of α-MD and α MmT function as pseudo-transmitters, as was originally suggested by Carlsson and Lindqvist (1962) with respect to the central nervous system. Later Day and Rand (1964) hypothesized a similar mode of action of α MD in the peripheral nervous system Day and Rand found α MNA to have less vasoconstrictor activity than NA and suggested that the substitution of α MNA for NA would diminish the effectiveness of sympathetic impulses to the blood vessels, thus leading to a reduction in blood

pressure. Basic to this concept is that the new transmitter has less receptor activity than the natural substance This problem has been the subject of several investigations The two drugs were found largely equipotent in raising the blood pressure of the pithed rat (Ahlquist 1948, Muscholl and Maitre 1963 Maitre and Staehelin 1963) or the anaesthetized dog (Sydow 1959) and in increasing the heart rate in the anaesthetized cat (Gaddum Peart and Vogt 1949). Ahlquist (1948) also examined the direct receptor activity of these amines in the heart and the blood vessels in animals of various species and found α MNA less effective than NA A similar relation has been found for the blood pressure increases after intravenous infusion of the two amines to conscious humans, NA being 3–4 times more potent than α-MNA (Mueller and Horwitz 1962) Day and Rand (1964) found α MNA 2–3 times less potent than NA in increasing the arterial blood pressure in several laboratory animal species under anaesthesia. In the conscious rat, α MNA had a pressor activity of about a third of that for NA (Brunner *et al* 1967) Holtz and Palm (1967) studied these relations extensively and reported that the vasoconstrictor effect of α-MNA relative to that of NA in the anaesthetized cat was dose-dependent In small doses, the two amines were equipotent while in higher dose ranges α MNA was a weaker vasoconstrictor The chronotropic action of the two amines in anaesthetized dogs was identical over a wide dose range (Sugarman *et al* 1968) The widely different conditions of these experiments, as well as possible differences in species and target organ sensitivity make it difficult to estimate the potency of α MNA as a substitute for NA at the vasoconstrictor nerve endings. However it seems quite obvious that α MNA is only little less effective than NA and, judging from the above-mentioned studies, a factor of 2–4 would seem a reasonable estimate

Homeostatic mechanisms often tend to oppose drug induced changes in the function of various systems in the intact organism. This is especially obvious in the control of blood pressure a drop in pressure at baroreceptor level may elicit reflex vasoconstriction and cardioacceleration, counteracting the change. In view of this a purely peripheral site of action of α-MD is not likely Physiological impulse flow in sympathetic nerves is normally only a fraction of that observed at maximal activity (Celander 1954) and it appears reasonable that the nerves are capable of increasing their frequency 2–4 times to compensate for the lower receptor activity of the new transmitter released after treatment with α MD Obviously this compensatory mechanism does not operate The present studies were initiated by considerations of this kind and it was felt necessary to examine critically the arguments in favour of a possible mechanism of "false" transmission in peripheral sympathetic nerves.

The theory that the α MNA formed from α-MD acts as a pseudotransmitter which produces a functional impairment in sympathetic nerves and hence a decrease in blood pressure seems to be widely accepted at the present time. Several recent review articles have emphasized the importance of this concept for explaining the antihypertensive effect of α MD (Crout 1966 Muscholl 1966 Stone and Porter

1966, 1967 Sourkes and Rodriguez 1967 Kopin 1968) On the other hand Holtz (1963 1965) has pointed out certain observations which are difficult to reconcile with this hypothesis (cf also Holtz and Palm 1966)

Interaction of decarboxylase inhibitors with the effects of α-methyldopa on blood pressure and monoamines

A main prerequisite for the false transmitter theory is that prevention of the formation from α-MD of a false transmitter should abolish the hypotensive action Since only a few studies of this kind have been performed and, in addition, have yielded contradictory results, this assumption was re investigated in Paper I The experiments were set up in two series, studying the effect of two different decarboxylase inhibitors on the action of α MD on blood pressure and tissue monoamines in rats. One group of animals was given hydrazino-methyldopa (MK 485) which has previously been shown to inhibit preferentially peripheral decarboxylase (Porter *et al* 1962) Another group received seryl-2,3 4-trihydroxybenzyl-hydrazine (Ro 4-4602). This inhibitor when given in large doses, inhibits both peripheral and central decarboxylase (Pletscher and Gey 1963 Burkhard *et al* 1962, 1964). The inhibitors were given alone or in combination with α MD and in repeated doses to ensure a sufficient degree of enzyme inhibition In addition α MD was studied separately A significant reduction of mean arterial blood pressure was observed 1–12 hours after α MD (100–200 mg/kg i p.) maximal effect occurred after 3–6 hours. In rats pretreated with the decarboxylase inhibitor Ro 4-4602, the fall in blood pressure which occurred 1–6 hours after α-MD administration was completely prevented. This is in contrast to the results obtained from rats which received MK 485 in combination with α MD 100 mg/kg i.p. in spite of the lower dose of α-MD used, no protective action of the decarboxylase inhibitor was observed in these experiments. In both groups there was a delayed hypotensive effect when the two drugs were given in combination This was also evident after the decarboxylase inhibitors alone Tissue monoamine levels were decreased by α-MD after both doses. It is of interest to note that femoral muscle NA content, i.e. the transmitter content of the vasomotor nerves of this tissue, was not altered and that the lowering of heart NA amounted to only 30–40 per cent of the controls at the time of maximal effect on blood pressure. The interaction of the two decarboxylase inhibitors with the effects of α MD on tissue monoamines was difficult to assess due to changes in these amines brought about by the inhibitors alone. These relations are discussed in detail in Paper I However it was established that both inhibitors completely prevented the formation of α MDA in the heart after α MD while that in the brain was significantly reduced by Ro 4-4602 only

This observation should be viewed in conjunction with the results obtained in the blood pressure experiments. In animals pretreated with Ro 4-4602 the hypotensive action of α-MD was inhibited but this effect was unchanged after pretreatment with

MK 485 These findings show that the decarboxylation products of α-MD play an important role in mediating the blood pressure effect of the latter drug. Further it may be concluded that decarboxylation of α MD in the central nervous system is necessary for its hypotensive action. However the question whether α MDA or α-MNA or both are responsible for the effect must be left open, as must also the possibility of these amines acting by false transmitter mechanisms in central neurons. It may also be pointed out that the findings do not exclude the contribution of a peripheral component such an action must be of little importance by itself (see below). This was shown also by the fact that the NA levels in the heart and the femoral muscle were slightly or not at all reduced at the time of maximal fall in blood pressure after α MD

Relationship of blood pressure and monoamine levels after α-methyldopa

If the depletion of the NA stores in sympathetically innervated organs is due to a substitution of this amine for α MNA which then acts as a less active false transmitter one would expect a rather close time correlation between the effect of α MD on NA level and on blood pressure The validity of this assumption was tested in Paper 2. Blood pressure and tissue monoamine levels were examined in parallel following the administration of a single large dose of α-MD to rats. After an i p injection of 400 mg/kg the blood pressure of normal as well as renal hypertensive rats was significantly reduced for 1–12 hours with maximal effect after 3–6 hours. Normal values were reached 24 hours after the administration of α MD The rats were sedated and showed a tendency to develop hypothermia during a period of time corresponding to the fall in blood pressure.

The same dose of α MD caused a transient fall in brain DA and 5-HT levels. This decrease was about 75 per cent of the control value and the time course of this depletion was rather similar to that of the blood pressure effect. Brain NA was also lowered but with a slower onset and a longer duration than DA and 5-HT minimum values were observed after 6–12 hours and the levels were still depressed after 72 hours. Heart NA was not maximally depleted until after 24–48 hours and did not return to normal until after 72 hours These biochemical determinations were made using normotensive rats. Supplementary studies were also performed in rats with renal hypertension using the same methods (see under Methodological considerations). The results are presented in Table 2. Analysis of variance showed that the values from hypertensive rats were not significantly ($P>0.05$) different from those obtained at corresponding intervals in normal rats. However femoral muscle NA was decreased significantly more ($P<0.005$) in hypertensive rats than in normal animals 24 hours after α MD

A comparison of the effects of α-MD on blood pressure and tissue monoamine levels shows that the former action was apparent at a time when there was only a small depletion of brain NA and an insignificant lowering of heart NA (cf also the

Table 2. Levels of noradrenaline (NA) in brain, heart and femoral muscle dopamine (DA) in brain and 5-hydroxytryptamine (5-HT) in brain normally and after an i.p. injection of α-methyldopa (α MD) or α-methyl-*m*-tyrosine (α MmT) to normotensive and hypertensive rats. The values are means in μg/g and number of experiments is shown in brackets. Variance and standard deviation was calculated with analysis of variance.

	Brain			Heart	Femoral muscle
	NA	DA	5-HT	NA	NA
Untreated					
Normotensive	0.39	0.62	0.35	0.78	0 10
	(10)	(9)	(6)	(6)	(10)
Hypertensive	0.40	0.72	—	0.73	0.08
	(10)	(8)		(14)	(9)
α-MD 400 mg/kg					
Normotensive					
after 6 hours	0.07	0.20	0.13	0.54	0.08
	(2)	(3)	(3)	(3)	(5)
after 24 hours	0 15	0.54	0.27	0.29	0.09
	(3)	(3)	(3)	(3)	(5)
Hypertensive					
after 6 hours	0.10	0.24	0.10	0 46	0.06
	(3)	(3)	(3)	(3)	(3)
after 24 hours	0.07	0.57	0.31	0 19	0.05
	(3)	(3)	(3)	(3)	(3)
α-MmT 400 mg/kg					
Normotensive					
after 6 hours	0.08	0 23	0.25	0.31	0.04
	(5)	(5)	(5)	(5)	(5)
after 24 hours	0.17	0.57	0.30	0.15	0 02
	(3)	(3)	(3)	(3)	(3)
Hypertensive					
after 6 hours	0.07	0.21	0.28	0.25	0 03
	(4)	(4)	(4)	(4)	(4)
after 24 hours	0.08	0.51	0.37	0.09	0.02
	(3)	(3)	(3)	(3)	(3)
Variance within groups	0.0026	0.0113	0.0031	0.0119	0.0002
Standard deviation	0.051	0.106	0.055	0.109	0.014

findings in Paper 1) The blood pressure had returned to normal values long before the NA stores were refilled. The same was true of the sedation and the hypothermia after α MD As stated in the introduction to this section, previous studies have established that the NA depletion after a single dose of α MD lasts for several days (cf also Paper 2). A number of earlier investigators have also noted that the hypo-

tensive effect of a single dose of the drug is of considerably shorter duration than the depletion of NA Directly comparable with the findings in Paper 2 are those studies which have been performed with similar techniques in conscious rats. Thus, Torchiana *et al* (1965) observed that maximal hypotensive effect occurred 2–6 hours after a moderately large dose of α MD to renal hypertensive rats return to control values was observed after 16 hours. Stanton and White (1965) and Stanton and Cooper (1966 1967) found that these events occurred 3 hours and 24 hours, respectively after α MD administration to normal as well as to hypertensive rats After repeated doses of α MD (hypertensive rats), the indirectly recorded blood pressure returned to control levels within 16 hours (Kroneberg 1963*a*) Similar results were obtained in conscious dogs (Goldberg, Da Costa and Oznki 1960 Kroneberg 1963*b*) Thus, there seems to be an obvious discrepancy between certain functional effects of α MD such as the lowering of blood pressure and the depression of monoamine levels in peripheral tissues. It should be pointed out, however that nerve function seems to be correlated to only a small fraction of the amine store (see Introduction) and it may be that this part of the store is influenced by α MD with a time course similar to that of the hypotensive effect. This functionally important depletion is then too small to be detected as a change in tissue NA levels when determined by the methods used in Paper 2.

Comparison of the effects of α-methyldopa and α-methyl-*m*-tyrosine on blood pressure and monoamine levels

Another implication of the false transmitter concept is that there should exist a parallellism between the receptor activity of the assumed transmitter and the magnitude of the hypotensive response This assumption was also tested in Paper 2 by comparing the effects of α-MD and α MmT on blood pressure The pressor activity of metaraminol in the conscious rat has been estimated to be about 10 times less than that of α MNA and about 20 times less than that of NA (Brunner *et al* 1966, 1967) Consequently α MmT should be proportionally more active than α MD in decreasing blood pressure. However after an i p injection of α MmT 400 mg/kg, normal as well as hypertensive rats responded with a moderate increase in blood pressure after 1–6 hours and no hypotension was observed even up to 72 hours after the injection This should be compared with the effect of α MD 200 mg/kg, as the α MmT used was a racemic mixture of the dextro and laevo isomers and only the latter are substrates for the decarboxylase *in vivo* (Sjoerdsma 1961 Porter *et al* 1961 Gillespie *et al* 1962, Sjoerdsma, Vendsalu and Engelman 1963) In similar experiments this dose of α MD lowered blood pressure significantly 3 12 hours after the injection in normal rats and 1–12 hours in hypertensive rats Both drugs produced roughly the same depletion of NA, as shown in Table 2 α MmT even appeared to be more active than α-MD in this respect, as also found by e g. Porter *et al* (1961) Udenfriend and Zaltzman-Nirenberg (1962) Andén (1964) Previous

studies on the cardiovascular effects of α MmT have not given conclusive results. Stone *et al* (1961) reported that α-MmT lacked an effect on blood pressure in renal hypertensive rats. Similarly Horwitz and Sjoerdsma (1964) found α MmT less effective than α-MD on acute administration to hypertensive patients however Holtmeier *et al* (1966) failed to reproduce these results and recent studies seem to indicate that α MmT may lower blood pressure on chronic administration to renal hypertensive rats (Brunner *et al* 1966 1967). Differences in species, dosage schedules or experimental conditions may partly account for these variable results. It might be noted, however that no pharmacological evidence of an impairment of sympathetic transmission has been obtained in animal experiments with α MmT despite a severe reduction in peripheral NA stores (Stone *et al* 1961 Andén and Magnusson 1964)

Effects of α methyl-DA on blood pressure and monoamines

It has long been known that sympathetic nerves are able to take up and store other amines than NA (for references, see Andén 1964 Andén *et al* 1964*c*). Utilizing this property of the nerves as well as the fact that catecholamines generally show a poor penetration through the blood-brain barrier it was considered possible to mimic the action of α-MD in peripheral sympathetic nerves by administration of the amine metabolites of α MD In Paper 2, the effect of α MDA was studied in normotensive rats which had been pretreated with α MDA (5 × 5 mg/kg s.c. during two subsequent days). No significant alteration in blood pressure was observed upon examination 16–18 or 40–42 hours after the last injection of α MDA. The treatment was found to produce a depletion of heart NA of at least the same magnitude as that observed after α-MD 400 mg/kg. Brain NA was not changed. A supplementary investigation was performed using rats with renal hypertension. α MDA was given for 3 days as indicated above and the blood pressure was followed during the first 12 hours after the last injection, i.e. the time corresponding to the hypotensive action of α MD (Table 3). No significant lowering of blood pressure occurred after α MDA during this period. Twelve hours after the termination of a similar treatment, four other

Table 3. Changes in mean arterial blood pressure of four hypertensive rats treated with (±)-α-methyldopamine (α-MDA) 5 × 5 mg/kg s.c. for 3 days. The changes are expressed in mm Hg from the individual basal values average initial pressure was 185 mm Hg (s.e.m.—4.1).

Hours after administration of last dose of α-MDA	3	6	9	12
Change in blood pressure mm Hg	+5	−6	−1	−5
s.e.m.	7.9	7.5	5.5	5.4

Table 4. Levels of brain noradrenaline (NA) and dopamine (DA) heart NA and femoral muscle NA in hypertensive rats, untreated or following pretreatment with (±)-α-methyl-dopamine (α-MDA) 5×5 mg/kg s.c. for 3 days. The animals were sacrificed 12 hours after the last dose of α MDA. The values are means in μg/g with s.e.m. and number of experiments is shown in brackets.

	Brain		Heart	Femoral muscle
	NA	DA	NA	NA
Controls	0.40	0.72	1 12	0 11
	0 022	0.051	0.040	0.008
	(10)	(8)	(8)	(6)
After α MDA	0.43	0.75	0.19	0.09
	0.012	0.034	0.014	0.012
	(4)	(4)	(4)	(4)

hypertensive rats showed a reduction of the heart NA to 17 per cent and of the femoral muscle NA to 84 per cent of the control animals. No change was observed in brain NA or DA. The absolute values are given in Table 4

Thus, it appears that depletion of NA by α MDA in peripheral tissues to a level even lower than that after α MD at maximal hypotensive effect is not accompanied by a reduction in blood pressure. α MNA is also devoid of antihypertensive properties (Brunner *et al* 1966, 1967)

Adrenergic nerve function at maximal hypotensive effect of α-methyldopa

If α MNA takes over the function of NA in the sympathetic nerve endings, the nerve function would be expected to decrease in proportion to the lower activity of the substitute transmitter Numerous studies have been performed to determine the influence of α MD on peripheral adrenergic function. Unfortunately investigations of this kind have yielded variable and sometimes conflicting results. This is possibly due to widely different experimental conditions, using several different animal species and after varying types of pretreatment schedules. A review of the findings from the literature has been given in Paper 3 This study was initiated to investigate if a sympathetic blockade of relevance to the antihypertensive effect of α MD should be easily demonstrable at the time of maximal fall in blood pressure after the drug Since this was found to occur 3–6 hours after a single i.p. injection of 400 mg/kg (Paper 2), a number of pharmacological tests of adrenergic nerve function were applied in anaesthetized rats after the same dose of α MD The responses of the lower eyelid to graded preganglionic stimulation of the cervical sympathetic trunk showed a tendency to be lower in treated rats but they were not significantly different from control animals. The blood pressure increases to graded doses of the

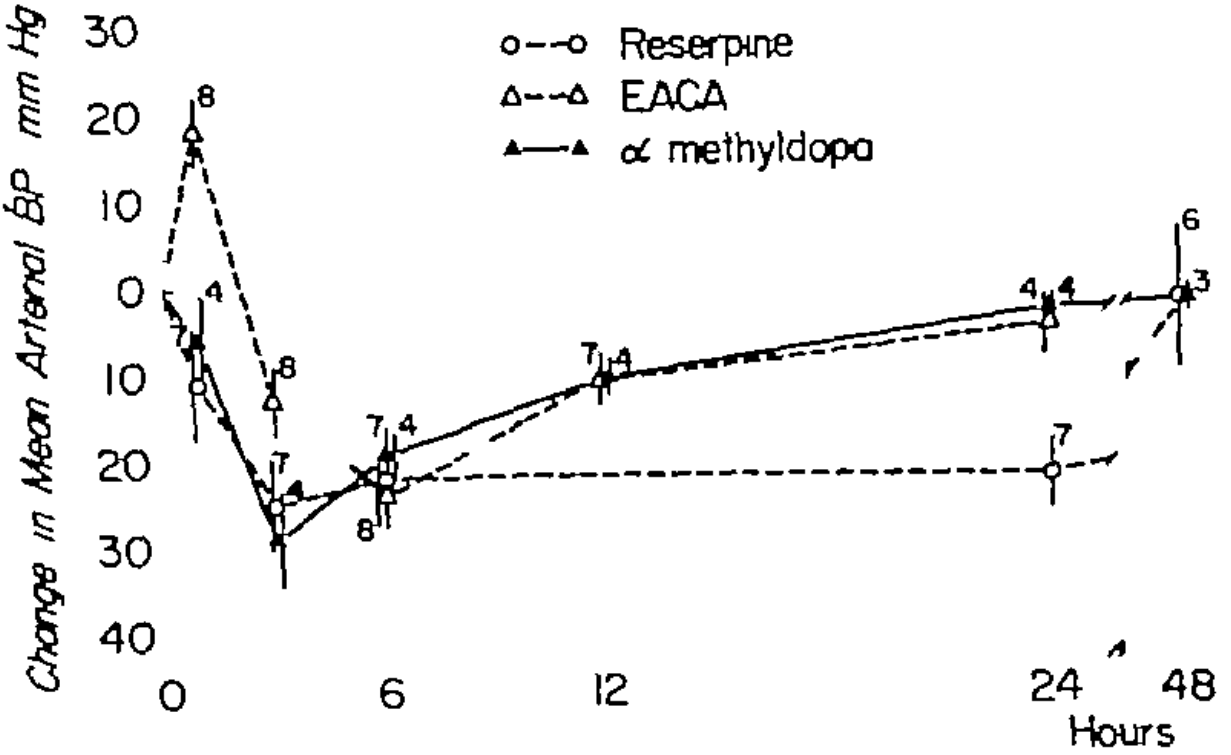


Fig. 1. Changes in mean arterial blood pressure of normotensive rats after i.p. injections of reserpine (Serpasil®) 10 mg/kg (circles), epsilon amino caproic acid (EACA) 2 g/kg (open triangles) and α-methyldopa 200 mg/kg (filled triangles). Blood pressure was recorded in conscious animals as described in Methodological considerations and basal pressures were 122 mm Hg (s.e.m.=4.2 n=7), 119 mm Hg (s.e.m.=3.5, n=6) and 121 mm Hg (s.e.m.=3.8, n=4), respectively. The data for EACA were taken from Andén, Henning and Obianwu (1968) and those for α-methyldopa from Paper 2.

sympathetic ganglion stimulant drug McN A 343 were slightly reduced by α MD but were likewise not significantly different from those obtained in untreated rats. The same was also true of the small contractions of the lower eyelid elicited by McN A 343. The response of the blood pressure to ganglionic stimulation by carbachol 0.15 mg/kg after atropine was significantly reduced. The blood pressure and eyelid responses to NA and tyramine were unaffected by α MD.

In summary these findings indicated that the responses of the peripheral adrenergic nerves may be slightly reduced at the time of maximal effect on blood pressure after α MD. This is also evident from the previous work summarized in Paper 3. Most investigators find an approximately normal sympathetic transmission when this was studied several hours after treatment with α MD. If on the other hand, the interval was relatively short, a moderate impairment of sympathetic function was sometimes observed. This effect then coincided with the period during which the blood pressure was lowered. It should be stressed, however, that such a blockade when it occurs is always considerably less prominent than that observed after adrenergic neuron blocking drugs or reserpine. Epsilon amino caproic acid (EACA) has recently been shown to produce an almost complete blockade of peripheral

sympathetic nerves equivalent to that observed after e.g. guanethidine leading to a reduction of the arterial blood pressure (Fig. 2 Andén Henning and Oblanwu 1968) In the present experiments, α MD lowered blood pressure to a similar extent yet α MD showed only a slight impairment of the peripheral sympathetic function. Similar relations are found for reserpine In spite of an almost complete adrenergic blockade in the rat (Andén *et al* 1964*b*) reserpine gives almost the same hypotensive response as α-MD on administration in the same doses to conscious rats (Fig. 2). These comparisons seem to indicate that the impairment of peripheral sympathetic function of α MD is not by itself of sufficient magnitude to account for the anti hypertensive effect of α-MD

Evidence for a centrally mediated hypotensive effect of α-methyldopa

The present results (Papers 1–3) do not support the false transmitter concept in its simplest outline. It was shown (Paper 1) that the hypotensive effect of α MD persisted when the decarboxylation of the drug was prevented peripherally but not centrally However if decarboxylation of α MD was blocked both peripherally and centrally the hypotensive effect was prevented There was no clearcut time relation between the NA depletion in peripheral tissues and the decrease in blood pressure after a single dose of α MD the latter effect being more rapid in onset and shorter lasting that the former (Paper 2) Peripheral adrenergic mechanisms were only slightly impaired at the time of maximal hypotensive effect (Paper 3) After treatment with α MDA or α MNA, peripheral stores of NA are depleted at least as much as after α MD but this depletion is not accompanied by a reduction in blood pressure (Paper 2, Brunner *et al* 1966, 1967) In view of these findings, central nervous mechanisms must be taken into account for explaining the antihypertensive effect of α MD An action on the peripheral sympathetic system might contribute to the final result although appearing insufficient alone. Previous studies have shown that administration of drugs into a vertebral artery in anaesthetized cats may be used to reveal centrally mediated effects of vasoactive drugs (v Zwieten, Bernheimer and Hornykiewicz 1966, Reis and v Zwieten 1967 Sattler and v Zwieten 1967) This method was employed in a series of experiments. The results are reported in Paper 4

In cats under chloralose anaesthesia, a small dose of α MD (20 mg/kg) was infused into the left vertebral artery This procedure resulted in a pronounced slowly developing decrease in systemic arterial pressure which was maximal after about 3 hours. In cats similarly prepared, the same dose of α-MD infused intravenously had no significant effect on arterial pressure This was also true of two other types of control experiments in which the dextro isomer of α MD (20 mg/kg) or 0.9 per cent saline was infused into the vertebral artery A large dose of α MD (200 mg/kg) infused into a systemic vein seemed to lower the blood pressure slightly after 3 hours but the change was not significant Tissue monoamine levels were also studied in these experiments and three hours after the infusion of α-MD 20 mg/kg into the vertebral

artery there was a significant although moderate reduction of brain NA and DA while brain 5-HT and heart NA remained normal. Intravenous infusion of the same dose of α MD showed a depletion of these amines which in every case was similar to that observed after infusion into the vertebral artery. Infusion of D-α MD had no effect on any of the amines examined. In an attempt to reveal possible regional differences in the lowering of brain monoamines by α MD (20 mg/kg) after the two types of administration, the lower brain stem and the forebrain were analyzed separately in some experiments. No difference between the effect of intra-arterial and intravenous infusion of α-MD was observed. In a few experiments, the level of α-MDA in the brain was also measured. Both types of infusion of the small dose of α MD gave rise to similar concentrations of α MDA.

Since infusion of saline or D-α MD into the vertebral artery had no effect on the blood pressure in identically designed experiments, this would indicate that the effect of L-α MD was specific. It should be pointed out, however, that it is not known whether in the cat D-α MD penetrates the blood brain barrier to the same extent as does the L isomer. A specific action of L-α MD is also suggested by the gradual onset of the effect and the fact that its time course and relative magnitude was similar to that seen after single larger doses of the drug to conscious animals (see Paper 4). The possibility that the observed effect on blood pressure of α-MD was due to a potentiation of anaesthesia seems to be excluded by the fact that a much larger dose of α MD intravenously infused did not produce a corresponding effect on blood pressure.

The effects of α-MD on tissue monoamines were poorly correlated to the hypotensive action. Whole brain NA and DA were equally lowered and the accumulation of α MDA was similar after both types of infusion. When the lower brain stem was analyzed separately there was still no significant difference in the amine content after the two routes of administration. It may be that a more extensive and detailed analysis of various parts of the brain stem could have disclosed a correlation between the lowering of blood pressure and the decrease in amine content. However, it is also possible that the hypotensive action observed in the present experiments is not primarily related to changes in gross monoamine levels. Further, as shown in Paper 4, the time correlation between the effect of α-MD on blood pressure and the depletion of monoamines is poor, the hypotensive effect occurring at a time when only a small depletion of brain NA had taken place. Apparently, there exists in the brain a structure receiving its blood supply from the vertebral artery which is influenced by infusion of α-MD in such a way as to bring about a reduction in blood pressure. The determination of the nature and exact localisation of this effect requires further investigation. As stated in the introduction to this section, previous investigators have paid little attention to this possibility for the mode of action of α MD. Jaju, Tangri and Bhargava (1966) studied the effect of injection of small doses of α MD into the lateral cerebral ventricle or of topical application to the floor of the fourth ventricle in anaesthetized dogs. Both types of administration were reported to give

a gradual though short lasting fall in blood pressure and also inhibited the responses to central vagal stimulation and to occlusion of the common carotid arteries. Of considerable interest are also the results of a recent study by Boakes Candy and Wolstencroft (1968) NA applied by iontophoresis to neurons in the brain stem of decerebrate cats, was found to excite some neurons and to inhibit others. Both these actions could be mimicked by α-MNA although the excitatory effects of this amine were weaker than those of NA. Further after α MNA had been applied, the excitatory response to NA was reduced or abolished for up to 20 minutes. This antagonism of the excitatory responses of brain stem neurons to NA is of particular interest in view of the findings in Paper 4 providing a possible explanation for the action of α MD in those experiments.

Animal experiments as well as clinical experience with α-MD have revealed that a number of its effects may be attributed to impairment of central nervous functions. Examples of this are quoted in Paper 4 cf also Sourkes (1965) and Hamon and Henning (1967) The last-mentioned study showed that α MD significantly decreased a conditioned avoidance response (CAR) in cats. Further α MD prevented the amphetamine-induced restitution of CAR in reserpinized cats which indicates an insufficient transmission mechanism in neurons engaged in the maintenance of CAR. The significance of such actions for the hypotensive properties of α-MD is difficult to establish but it may be of relevance that, in therapeutic doses, α MD interferes with certain reflex adjustments of blood pressure. This is true of the overshoot in blood pressure following the Valsalva maneuvre in hypertensive patients (Dollery and Harington 1962, Dollery 1963) the cardiovascular adjustments to exercise (Sannerstedt, Varnauskas and Werkö 1962) the reflex vasoconstriction after a cold test or exercise in normal subjects (Mason and Braunwald 1964) and of the pressor responses to noxious stimuli in hypertensive patients (Shapiro and Kritcher 1964), all of which were impaired or blocked during treatment with α MD These findings may be explained by interference at various levels of central vasomotor control as previously mentioned. They may also be interpreted in terms of impaired monoaminergic transmission in the structures involved in blood pressure control Thus effect of α-MD may represent a mechanism of false transmission as well as other types of influence on the neuron separately or in combination. Since the reflexes mentioned involve both central and peripheral divisions of the sympathetic system, a peripheral sympathetic blockade may contribute Little is known of the influence of α MD on this part of the system in man Although being more effective in reducing blood pressure in the standing position, α-MD seems to be less prone to give orthostatic side reactions than the purely peripherally acting adrenergic blocking drugs guanethidine and ganglioplegics (Dollery and Harington 1962, Smirk 1963 Holtmeier and Heilmeyer 1965, Prichard *et al* 1968). Nasal congestion inhibition of ejaculation and other signs of peripheral sympathetic blockade also seem to be less commonly encountered after α-MD Observations of this kind may be taken to indicate that peripheral adrenergic mechanisms are relatively intact during therapy

with α-MD in keeping with the findings in animal experiments. The results of the investigations reported in this summary may then be of relevance to the clinical effects of α MD as well the hypotensive action of the drug in man may be at least partly due to interference with central mechanisms of blood pressure regulation Further work is required to ascertain the significance of such an assumption such clinical studies may possibly be performed following similar lines of investigation

SUMMARY

Several currently used antihypertensive drugs are known to interfere with the function in the sympathetic nervous system and also to influence the metabolism of the biogenic monoamines. The interrelations of such actions as well as their possible relationship to the therapeutic effect are in many instances poorly understood. The studies summarized in this paper were aimed at considering these aspects with particular reference to α methyldopa (α-MD)

α-MD depletes tissue stores of catecholamines mainly through a displacement of these amines by the metabolites of α MD i e. α methyl DA or α methyl NA or both This replacement of the normal transmitter by α methylated amines has been related to the antihypertensive properties of the drug by assuming that the metabolites substitute NA at the sympathetic nerve endings in the heart and the blood vessels, thus serving as so-called false transmitters. Since the α-methylated amines are less active on the adrenergic receptors than the endogenous transmitter the effectiveness of sympathetic impulses would be diminished thus leading to a fall in blood pressure. A number of basic requirements for this hypothesis may be set up e.g.

(1) prevention of the synthesis from α MD of possible false transmitters should abolish the hypotensive action
(2) a reasonable time relation should be demonstrable for the depletion of NA in peripheral nerves and the decrease in blood pressure
(3) since the compounds proposed as false transmitters are taken up by the adrenergic nerves and displace the adrenergic transmitter they should also be active in lowering blood pressure
(4) a parallelism should exist between the receptor activity of the assumed transmitter and the magnitude of the hypotensive response
(5) the function in peripheral sympathetic nerves should decrease in proportion to the lower activity of the false transmitter and this functional impairment should be clearly demonstrable at the time of maximal fall in blood pressure

The validity of these assumptions was tested in different types of experiments using the same animal species (rat) for both functional and biochemical studies.

(1) The formation from α MD of possible false transmitters was prevented by pretreatment of the animals with two DOPA decarboxylase inhibitors, seryl-2,3 4-trihydroxybenzylhydrazine (Ro 4-4602) and α-hydrazino-α-methyl β-(3 4-dihydroxy

phenyl) propionic acid (MK 485) Administration of Ro 4-4602 completely inhibited the hypotensive effect and markedly reduced the accumulation of α methyl DA in the heart and in the brain after α MD On the other hand pretreatment with MK 485 had no effect on the hypotensive response or on the accumulation of α-methyl-DA in the brain but prevented the formation of this amine in the heart as much as Ro 4-4602. It was concluded that decarboxylation of α MD in the central nervous system is a prerequisite for its hypotensive action (Paper 1)

(2) The time relations of the hypotensive effect and the depletion of NA in tissues were studied after administration of a single dose of α MD (Paper 2). A significant fall in blood pressure was observed after 1–12 hours at this time there was only a minor decrease in the peripheral NA stores and maximal depletion of NA did not occur until about 48 hours after the administration, *i.e* at a time when the blood pressure had returned to normal levels.

(3) Previous work has shown that α methyl NA is devoid of antihypertensive activity (Brunner *et al* 1966 1967) and the studies reported in Paper 2 established that this applied to α-methyl DA as well, in spite of a reduction of the heart NA content exceeding that caused by α MD at the time of maximal fall in blood pressure. However brain NA was not altered by treatment with α-methyl DA

(4) Like α MD the closely related compound α methyl-*m*-tyrosine (α MmT) depletes NA from the stores through displacement by its decarboxylated amine products These substances possess considerably less receptor activity than the amines arising from α MD By the concept of false transmission as outlined above it would therefore seem that α MmT should be more potent than α MD in lowering blood pressure However on administration in equivalent doses to normal as well as hypertensive rats, α-MmT was found to lack anti-hypertensive properties (Paper 2).

(5) The function in peripheral sympathetic nerves was studied at the time of maximal fall in blood pressure after a single dose of α MD (Paper 3). At most a slight functional impairment was observed. This is in contrast to e g. reserpine and epsilon aminocaproic acid which in equi-hypotensive doses, have been found to produce an almost complete block of sympathetic transmission It seems that this discrepancy can only be explained by assuming that α MD in addition to a possible slight peripheral sympathetic blockade, also influences central blood pressure regulation. The results summarized above thus seemed to point to the existence of an important action of α MD on the central nervous system and it was thought of interest to investigate this component further A small dose of the drug was slowly infused into a vertebral artery in anaesthetized cats, resulting in a gradual and significant lowering of mean arterial blood pressure (Paper 4) Infusion of the same low dose of α-MD into a systemic vein did not affect blood pressure, neither did infusion of saline

or the dextro isomer of α MD into a vertebral artery These findings support the view that the hypotensive action of α MD is at least in part, of central nervous origin. The contribution of an action on the peripheral division of the sympathetic system cannot be excluded but is apparently of little importance at least in the rat. It would thus appear that in order to be relevant to the mechanism of action of α-MD the theory of false transmission must be extended to include central neurons. Further experiments are required to settle the question whether this is so or if the transmitter involved is α-methyl DA or α methyl-NA or both.

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GÖTEBORG
ELANDERS BOKTRYCKERI AKTIEBOLAG
1969

ACTA PHYSIOLOGICA SCANDINAVICA
SUPPLEMENTUM 323

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By
BODIL NIELSEN

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SUPPLEMENTUM 323

From the Laboratory for the Theory of Gymnastics, University of Copenhagen, Denmark

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Contents

Preface		7
Acknowledgements		8
List of symbols		9
Introduction		11
Chapter I	Methods	13
Chapter II.	Thermal gradients in the human skin	18
Chapter III	Average deep skin temperature and the regulation of sweating and skin blood flow	24
Chapter IV	Deep skin temperature, muscle temperature and the regulation of sweating during positive and negative work	39
Chapter V	General Discussion	50
Appendix:	Statistical analysis of the data	59
Summary		63
Dansk resumé		66
References		69

Preface

This study of thermoregulation during exercise is part of a series of investigations dealing with different aspects of the same topic, parts of which have been published earlier The main findings from these papers are discussed in the General Discussion in connection with the results from the present study

The series consists of the following papers:

1 Nielsen, B and M. Nielsen, Body temperature during work at different environmental temperatures. *Acta physiol. scand.* 1962 *56* 120–129

2. Nielsen, B and M. Nielsen, On the regulation of sweat secretion in exercise. *Acta physiol scand* 1965 a. *64* 314–322.

3 Nielsen, B and M. Nielsen, Influence of passive and active heating on the temperature regulation of man. *Acta physiol scand.* 1965 b *64* 323–331

4 Nielsen, B., Regulation of body temperature and heat dissipation at different levels of energy- and heat production in man. *Acta physiol. scand* 1966. *68* 215–227

5 Nielsen, B., Thermoregulatory responses to arm work, leg work and intermittent leg work. *Acta physiol. scand.* 1968 *72* 25–32.

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List of symbols

Temperatures in C

A bar over a symbol denotes mean value of the quantity

T	ambient air temperature.
T_{ds}	deep skin (intracutaneous) temperature.
$T_{(ds\ s)}$	average temperature differential between deep and surface temperature in the skin.
T_m	muscle temperature.
T	operative temperature, measured as the mean of walls and air temperature, since both were nearly the same
T_{es}	esophageal temperature.
T_{re}	rectal temperature.
T	skin surface temperature.
T_{ty}	tympanic membrane temperature.

Quantities for the heat balance equation expressed in kcal/hr

M	metabolic energy liberation ($V_{O_2} \times 4.9 \times 60$)
W	external work (kpm/mm $\times$ 1/427 $\times$ 60)
$H = M - W$	total heat production.
$(C + R)$	convective plus radiation heat exchange (positive for net loss)
E_l	evaporative heat loss from lungs.
E_{sw}	evaporative heat loss from skin sweat.
$E = E_l + E_{sw}$	evaporative heat loss (water loss g/hr $\times$ 0.582)
S	storage of body heat (positive for net heat gain by the body)

Miscellaneous

A_D	DuBois Area, body surface area m^2
HR	heart rate, beats/min.
h_c	combined heat transfer coefficient for radiation and convection kcal/hr $\times$ m^2 $\times$ ° C.
K	conductance of the peripheral tissues kcal/hr $\times$ m^2 $\times$ ° C (cf p 16)
$\dot{Q}$	cardiac output l/min.
$\dot{Q}$	skin blood flow l/min.
R_s	thermal resistance of skin ° C $\times$ hr/kcal.
Sw	sweat rate g/hr
$\dot{V}o$	oxygen uptake l/min.

Introduction

In a study of heat dissipation during uphill and downhill walking on a motor driven treadmill (B Nielsen 1966), it was found that in conditions where the skin temperature is the same the body regulates the sweat rate so that the heat dissipation equals the total heat production, in spite of differences in the level of internal body temperature and the mechanical events in the muscles.

It was suggested that the thermoregulatory centre obtains information on the rate of heat production through thermoreceptors "measuring" the temperature at some definite depth in the skin as proposed by Kerslake (1955) and Blockley (1966) The rationale for this concept is that the temperature in the skin below a thermal resistance must be increased proportionally to the heat flow through the tissues. Another possibility is that thermal receptors, placed at different depths in the skin, could be stimulated by thermal gradients across the skin, in proportion to the heat flow and, hence to the rate of heat production (Bazett 1949 1951) Thirdly it has been postulated (by Robinson et al. 1965) that thermal receptors in the muscles or in the veins draining the working muscles provide information to the heat dissipating centre.

The present study was designed to evaluate the above mentioned hypotheses. A method was worked out for the measurement of 1) local temperature gradients in the skin, 2) the average deep skin temperature, and 3) the average deep to surface skin temperature differential. In addition the muscle temperature in *vastus lateralis* was measured in one series of experiments.

The study is divided into three experimental parts, chapters II–IV which are discussed independently followed by a general discussion of thermoregulation during work.

Chapter two is a pilot study of the local thermal gradients measured in the skin on the ventral surface of the thigh, and on the chest. The changes in these gradients with variations in both heat production (different work loads) and environmental temperature were studied, in order to determine the depth at which an average deep skin temperature should be measured.

Chapter three consists of a study of "average deep skin temperature" in relation to sweat rate (sweat rates between 50 and 600 g/hour) at 3 metabolic levels at various environmental temperatures.

Chapter four describes the changes in "average deep skin temperature" average deep to surface skin temperature differences, and muscle temperature in relation to heat dissipation during positive and negative work. By walking or bicycling uphill (+) or downhill (−) the proportion of total heat production to metabolic energy liberation can be varied.

Chapter I

METHODS

The instrumentation in the present study is nearly identical to that used in our earlier studies and has been described in detail (B Nielsen and M. Nielsen 1962 1965 a B Nielsen 1966) Therefore, only a short survey of the methods and the estimated accuracy of the measurements is presented here.

The temperature measurements. The temperatures were measured with copper-constantan thermocouples. For each thermocouple a calibration curve was constructed. The calibration was performed in a stirred thermoregulated waterbath, with the single thermocouples tied to the bulb of a mercury thermometer The reference thermocouples were similarly fastened and kept in a thermos bottle. The thermocurrent was read on a Cambridge Spot Galvanometer or recorded on a 4-channel recording galvanometer (Kipp Micrograph BD 1) The calibrations were repeated for each environmental condition, and were frequently checked. The *rectal temperature* in 4 depths (T_{re}) and the deep *esophageal temperature* (T_{oe}) were measured with the thermocouples described by B. Nielsen and M Nielsen (1962) The measurements are considered to be accurate to ± 0 025° C. During the experiments the esophageal temperature was recorded continuously on the Micrograph while the rectal temperature, measured every 5 min, was calculated from the readings on the Cambridge Spot Galvanometer

The *skin surface temperature* (T_s) was measured either with a skin thermocouple or by a radiation receiver (B Nielsen and M. Nielsen 1965 a)

The *average skin surface temperature* (T_s) was obtained by weighting the 15 single measurements of T according to the size of the corresponding skin area (Hardy and DuBois 1938 a) The error on the single skin surface measurement is less than 0 10° C.

Deep skin temperatures The temperature in the skin (T_{sk}) was measured with copper-constantan thermocouples made by Ellab., Copenhagen. The last 10 cm of the measuring probe consisted of only one copper core (0 045 mm diam.) and one constantan core (0 15 mm diam.) covered by "thermolacquer" The total outer diameter was 0.25 mm the exposed thermosensitive junction being 0.5 mm long.

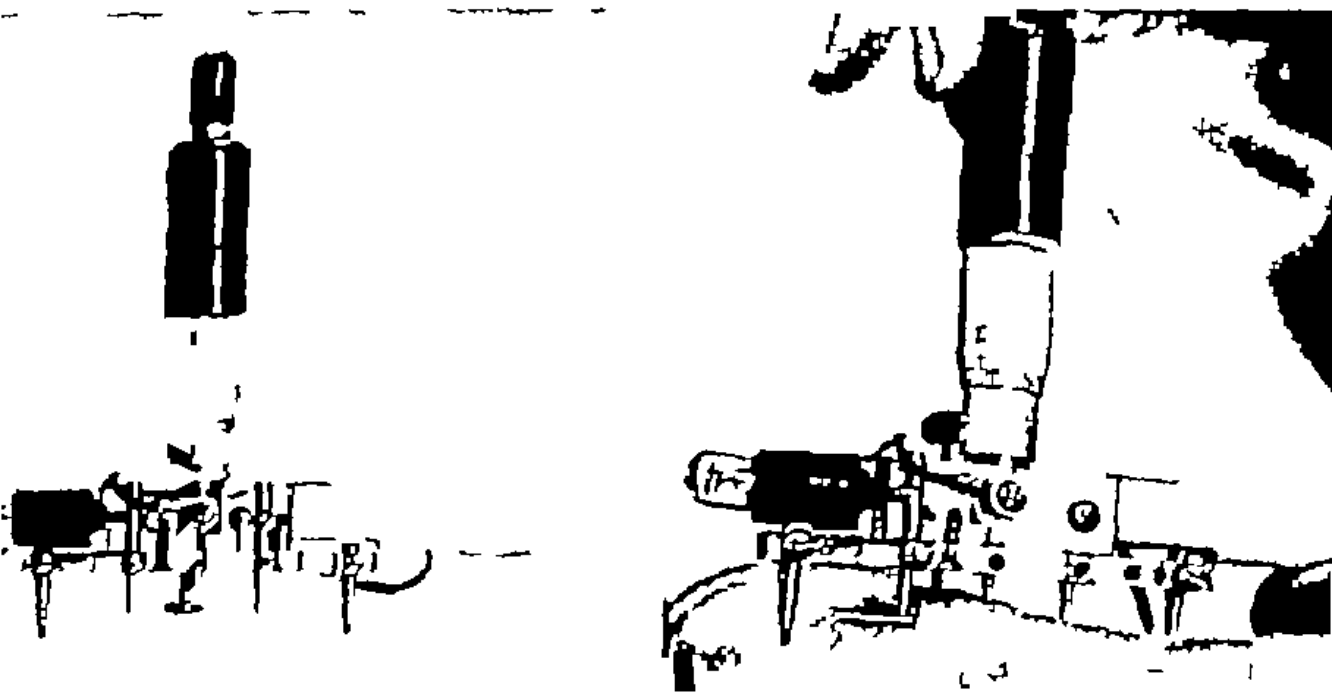


Fig 1

Left. Micrometer for measuring the distance between the intracutaneous thermocouple and the skin surface. Right. The micrometer placed on the needle—through which the thermocouple is inserted—in position for the measurement.

The thermocouple was inserted into the skin through a hollow needle (inner diam. 0.3 mm, outer diam. 0.5 mm) The procedure was as follows: A skinfold was picked up with the fingers and the hollow sharpened needle was pushed through the skinfold so that it protruded on both sides. The skin was then allowed to slide back into place. A micrometer provided with 2 grooved tips which could be placed on the cannula across the pierced skinfold, was placed on the needle (cf. Fig. 1) The micrometerscrew was then advanced until contact with the skinfold was obtained. This closed an electric circuit and lit a signal lamp. The reading on the micrometer at this point gave the vertical distance from the needle below the skinfold to the skin surface.

After this measurement the skin thermocouple was pushed through the needle; the needle was removed and the thermocouple was pulled back so that its tip lay midway between the two holes in the skin made by the needle. The thermocouple was then fixed in place with adhesive tape.

It is estimated that the depth of the thermocouple in the skin can be measured by this method with an accuracy of between 1/10 and 1/4 of a mm.

The skinfold thickness at the sites of measurement was measured with a

standard Harpenden skinfold caliper With some training it was possible to place 4 thermocouples in depths ranging from 0 8 mm to half the double skinfold thickness within an area of 2 × 2 cm (chap II)

The temperatures were continuously recorded on the above mentioned recording galvanometer (chap II and III) or on a Transocomp potentiometer recorder* (chap IV)

The single deep skin temperatures, calculated from the Micrograph record using the constructed calibration curves are considered accurate to ± 0 025° C, while within ± 0.1° when recorded with the Transocomp. (Accuracy ± 0.5 % for full scale deflexion, 50 C) The intracutaneous thermocouples were tested for errors due to heat conduction through the wires, but even a 10° C difference between the water surrounding the tip and the base of the 10 cm exposed wires did not influence the value recorded from the thermocouple.

The *average deep skin temperature* (T_{d_a}) was measured with thermocouples in 7 places (chest, abdomen, shoulder upper arm and forearm, thigh and calf) The skinfold thickness was measured at these 7 sites, and the needles through which the thermocouples were inserted were placed with the mid part at a *depth of half the skinfold thickness* The distance from the skin surface to the needle was measured with the apparatus described above. After insertion, the thermocouples were secured with tape as described The average deep skin temperature (T_{d_a}) was calculated as the simple average of the seven measured temperatures. There was no visible sign of skin irritation around the deep skin thermocouples, and the surface temperature above them was only slightly different from corresponding areas (subj TD average of 60 measurements. + 0 67° on the chest, and − 0.22° on the shoulder)

The *average temperature differential* across the skin ($T_{(d_a - s)}$) was calculated as the difference between T_{d_a} and the simple average of the seven of the fifteen skin surface temperatures which were measured exactly above the intracutaneous thermocouples.

Muscle temperature (T_m) was measured in 2 of the 3 subjects in the study reported in chapter IV Immediately after the work was stopped, a needle thermocouple (diam. 0.5 mm) was inserted 2.5 cm into the thigh in *vastus lateralis* The thermocouple was kept in 39° water till it was used. The temperature recording (Kipp Micrograph) was stable after $^1/_2$–1 min and did not change during the observation period of $^1/_2$–1 min The muscle temperature measurements are accurate within 0 05° C.

The external work. In the experiments reported in chapter II and

obtained through a grant from the Carlsberg Foundation.

III the subjects worked with 60 rev./min on the Krogh bicycle ergometer in which the saddle had been replaced by an armchair

In the treadmill experiments (chap. IV) the subjects walked or bicycled on an ordinary bicycle with direct transmission on the inclined treadmill uphill or downhill. (In the last mentioned situation the subject resisted the acceleration of the pedals) In the bicycle experiments the speed was kept constant at 8 1 km/hr The bicycle was geared so that the frequency of pedaling was 60 rev./min. The work load could be varied by changing the slope of the treadmill and by placing extra weights on the bicycle (1 2 or 3 × 11.5 kg) In the experiments where the subject walked or ran the speed was varied while the slope was kept constant at + or − 10°

The weight loss in the experiments was measured by the Krogh balance (Krogh and Trolle 1936) the error on the determinations being less than 5 g. The ergometer used in the experiments described in chapter II and III was suspended from a Krogh balance (M. Nielsen 1938) so that a continuous registration of the weight loss could be obtained. In the treadmill experiments the weight loss was determined from the weighings before and after the 45 min experimental period (cf p 39)

The sweat secretion (Sw) was calculated from the total weight loss by subtracting the respiratory losses due to evaporation and gas exchange.

The respiratory gas exchange was determined by the Douglas bag method, and air samples were analysed in duplicate on the Scholander apparatus (Scholander 1947)

The metabolic energy liberation (M) was calculated from the oxygen uptake using a caloric value of 4 9 kcal pr 1 oxygen, and the *total heat production* (H) was found by subtracting the caloric equivalent of the external work (W) (1 kpm = 1/427 kcal) from M

In the treadmill experiments (chap. IV) the total heat production during the experiment was calculated according to the formula.

H = M − W for positive (uphill) work and
H = M + W for negative (downhill) work,

where H is the total heat production in kcal/hour M is the metabolic energy liberation in kcal/hour and W is the external work converted to heat in kcal/hour The work was calculated as. $G \times v \times \sin \alpha$. G = weight of subject (or subject + bicycle + weights) in kg, v = speed m/min, α = inclination of the treadmill in geometrical degrees.

Skin blood flow Changes in skin blood flow as indicated by changes in the *conductance* of the peripheral tissue (K) (Burton 1934 Hardy 1937 Winslow et al. 1937) was calculated for the last 15 min of the experiment as

$$k = \frac{H - E_l - S}{\left(\frac{T_{rs} + T_{ee}}{2} - T\right) A_D} \quad \text{kcal/hr} \times \text{m}^2 \times {}^\circ\text{C}.$$

The modification $\frac{T_{rs} + T_{ee}}{2}$ instead of T_{rs} has been applied since it was found that during leg work T_{rs} was higher (0.2 – 0.4° C) than T_{ee}, probably due to heating of a large part of the pelvic cavity by blood returning from the working muscles (B Nielsen and M Nielsen 1962)

The ambient conditions *Air temperature* (T_a) was obtained from 2 mercury thermometers hanging behind and in front of the subject, and wall temperature was measured with a radiation receiver (B Nielsen and M. Niel- 1965 a) Since the walls and the air had nearly the same temperature, the *operative temperature* (T_o) was calculated from these temperatures as $\frac{T_{wall} + T_a}{2}$ T was measured 3 times during the last 30 min of the experimental period. The relative humidity was determined by an aspiration psycrometer

The experiments described in chapter two and three were carried out in a climatic chamber (B Nielsen and M Nielsen 1962) which could be maintained at any desired temperature level between 5° and 44° ± 1/4° C. The treadmill experiments (chap IV) were performed in a temperature controlled room kept at T 22½° ± 1° C

III, the subjects worked with 60 rev./min on the Krogh bicycle ergometer in which the saddle had been replaced by an armchair

In the treadmill experiments (chap IV) the subjects walked or bicycled on an ordinary bicycle with direct transmission on the inclined treadmill uphill or downhill. (In the last mentioned situation the subject resisted the acceleration of the pedals) In the bicycle experiments the speed was kept constant at 8 1 km/hr The bicycle was geared so that the frequency of pedaling was 60 rev./min. The work load could be varied by changing the slope of the treadmill and by placing extra weights on the bicycle (1 2 or 3 × 11.5 kg) In the experiments where the subject walked or ran, the speed was varied while the slope was kept constant at + or — 10°

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In the treadmill experiments (chap IV) the total heat production during the experiment was calculated according to the formula.

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where H is the total heat production in kcal/hour M is the metabolic energy liberation in kcal/hour and W is the external work converted to heat in kcal/hour The work was calculated as $G \times v \times \sin \alpha$. G = weight of subject (or subject + bicycle + weights) in kg, v = speed m/min, α = inclination of the treadmill in geometrical degrees.

Skin blood flow Changes in skin blood flow as indicated by changes in the *conductance* of the peripheral tissue (K) (Burton 1934 Hardy 1937 Winslow et al. 1937) was calculated for the last 15 min of the experiment as

Both the skin thermocouple and the radiation receiver gave a stable reading of skin surface temperature within 6–15 sec after being placed against or above the skin. However even these short applications affected the levels of deep skin temperatures (the temperatures generally decreased 0 1°–0.5°) in the region below the measured area for several minutes. The number of surface temperature measurements was therefore reduced to a minimum, and the values given in the graphs represent the average of only 3–5 measurements taken at intervals of about 5 minutes during the last half hour of the 1–1½ hours exposure to the given environment and activity level.

Twentyfour experiments on 4 subjects were carried out. No fundamental differences due to the variations in skinfold thickness were noticed

Subject	Average skinfold thickness mm		Number of exper
	Chest	Thigh	
J W	8.9	9.5	7
M C	13 6	13.6	7
K H N	4 7	6.8	6
G M	14.5	12.2	4

Results

Temperatures at different depths in the skin. In experiments where a thermal equilibrium could be established (as demonstrated by attainment of a constant level of internal temperatures) the highest deep skin temperatures ($T_{d.s}$) were, as expected, generally measured at the deepest sites (Fig. 2) In some experiments, however the temperature did not increase regularly with increasing depth, but showed reversals in the gradients. This phenomenon may have been due to a dislocation of the thermocouple during the work period or caused by contact of the thermocouple with minor bloodvessels in the skin. During rest in moderate warm and warm conditions the tissue layers studied received heat from the surface. (The temperature decreased with depth, Fig. 4)

The effect of different environmental temperatures on the temperature gradients in the skin is illustrated in Fig. 2 and in Fig. 3 In the last mentioned graph the differences from the surface temperature at various depths are shown. Each curve represents the gradient of a single experiment in conditions of heat equilibrium. At a constant work load

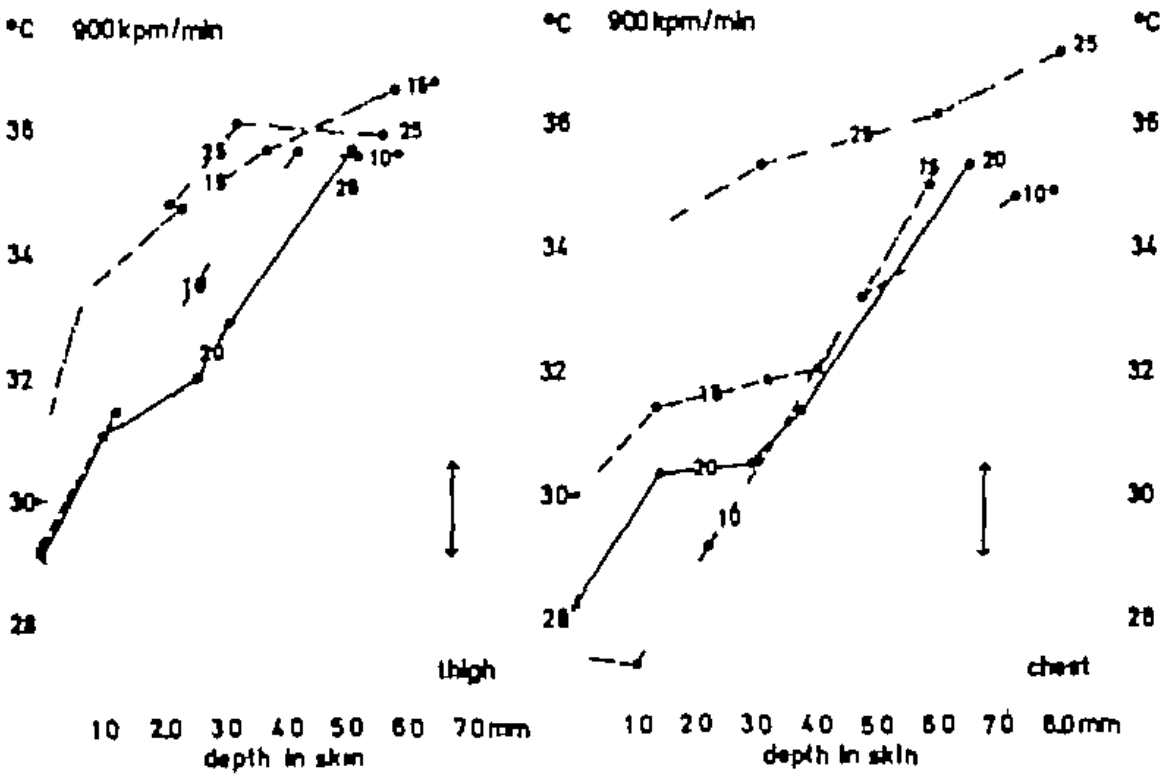


Fig 2

Temperatures at the skin surface and at various depths in the skin of the thigh (left) and the chest (right), during stable conditions of work at 10 15 20 and 25 operative temperature. (Single experiments). ↕ denotes ½ skinfold thickness. Subj. MC. Work intensity 900 kpm/min.

the increase in temperature with depth in the skin is generally smaller the higher the environmental temperature. (The 20° experiment deviates somewhat from the general pattern) This effect seems to be due to a temperature increase in all skin layers (Fig. 2) but the increase is greatest in the more superficial layers.

Increases in the rate of heat production produced by a higher work load also effects the temperature gradients in the skin. In Fig 4 the temperature gradient across the skin is seen to increase as the work intensity rises. The changes are brought about mainly by a temperature rise in the deepest skin layers. However the variations from one experiment to the next in surface temperature above the 4 thermocouples are too great to allow definite conclusions to be drawn as to which temperatures, deep or superficial, are changing the most.

Apparently no systematic difference exists between the temperatures of the two chosen skin areas the chest or the thigh

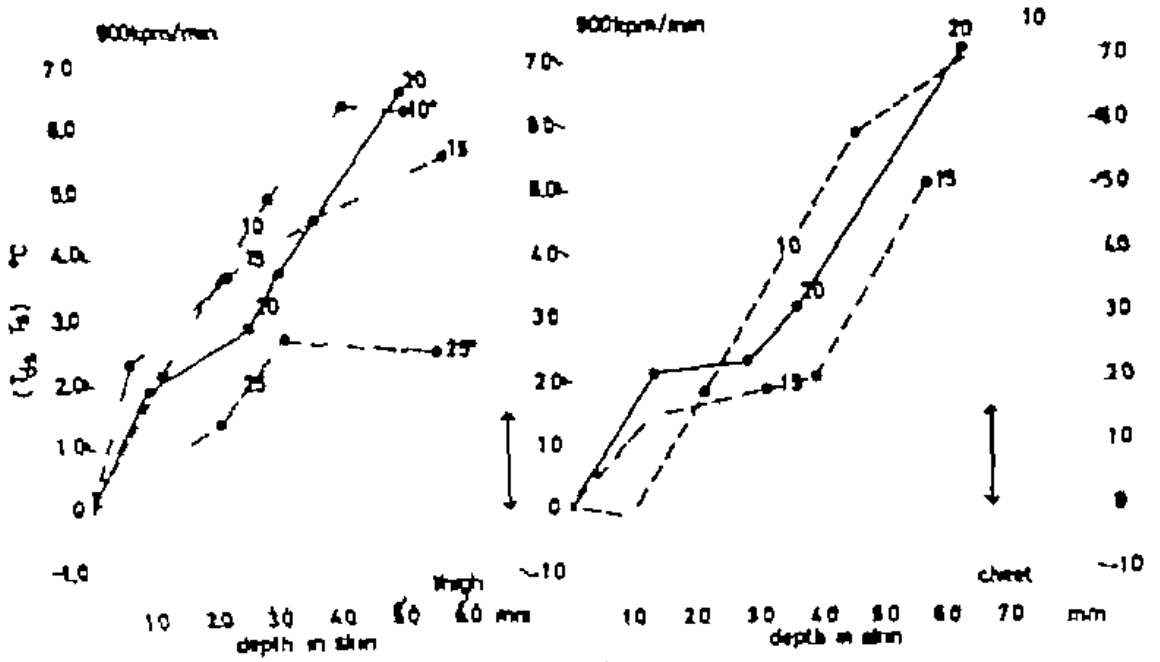


Fig 3

The gradient ($T_{cl} - T_o$) in stable conditions of work at 10 15 20 and 25 operative temperature. (Single experiments). ↕ denotes / skinfold thickness. Subj. MC. Work intensity 900 kpm/min.

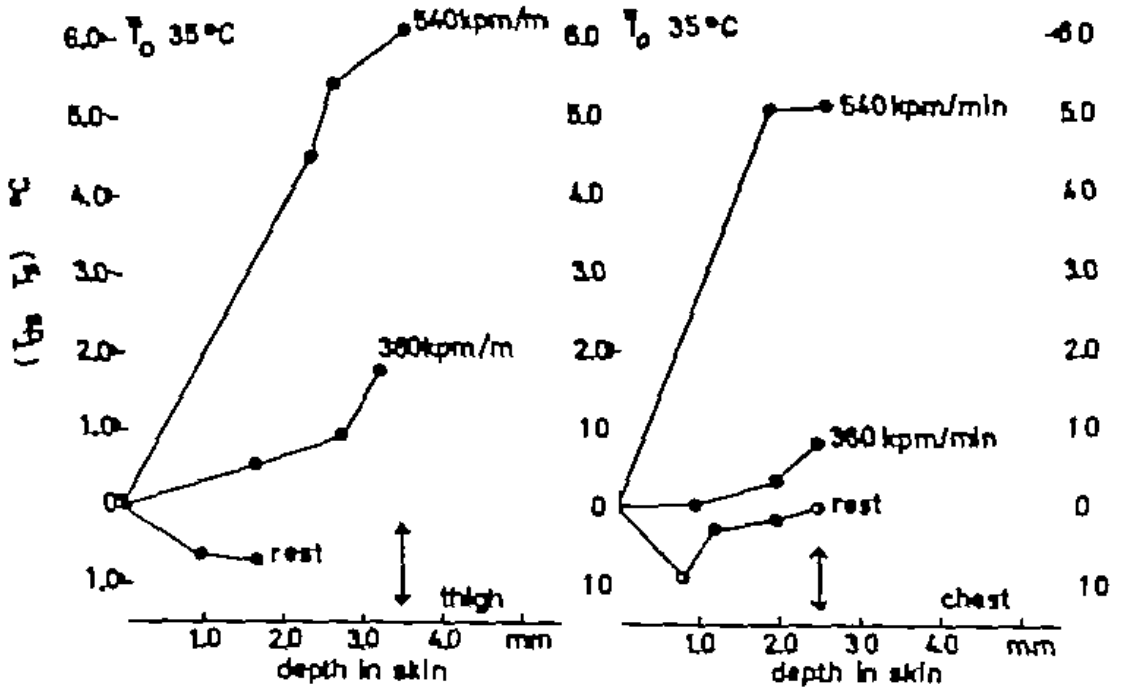


Fig 4

The gradient ($T_{cl} - T_o$) in stable conditions of rest and work of 360 kpm/min and 540 kpm/min. Operative temperature 35 C. (Single experiments). ↕ denotes /s skinfold thickness. Subj. KHN

Discussion

The intracutaneous temperature rises with increasing depth in the tissue. This finding is in good agreement with the results of Bazett and McGlone (1926, 1927) Mendelson (1936) Pennes (1948) and Reader and Whyte (1951) The very steep reversed gradients with 0.3–0.5° C temperature change for 0.5 mm change in depth in the most superficial (0–2 mm) skin layers, noticed by these authors, were not found in the present experiments. One source of error in the present study was that the skin areas in which the 4 intracutaneous couples were placed showed a slight flush and were 1–2 degrees warmer than the corresponding areas on the other side of the midline of the body However it is believed that in conditions of heat equilibrium the values for the different external temperatures and metabolic rates nevertheless are comparable and that the changes observed with different conditions also reflect those occuring in the undisturbed skin areas. When only one intracutaneous thermocouple was used in each skin area (cf p 15) no sign of irritation was noticed.

It appears, that the gradient across the skin is generally *smaller* the *warmer* the environmental conditions. This finding is in agreement with the more extensive studies of Bazett and McGlone (1927) and Reader and Whyte (1951)

Further also changes in the level of activity have a strong effect on the gradient, which becomes steeper with increasing work rate and heat production.

The skin on the thigh over the working muscles (bicycle ergometer) and the skin on the chest behaved in similar ways, and no special effect of blood returning from the working muscles through superficial veins (Cooper Randall and Hertzman 1951 cit. by Robinson 1963) has been noted.

The confirmation of the existence of even very steep gradients (up to 8°) in the skin and subcutaneous tissues in some situations draws more attention to the theory of Kerslake (1955) He calculated an average deep skin temperature and found a correlation between this calculated value and sweat rate in various experimental conditions of rest and work. In our earlier studies (B Nielsen and M. Nielsen 1965 a) we found that at a given sweat rate the average skin surface temperatures were very much lower (5–10°) during work when compared to rest values. This means that if sweat rate is controlled *solely* by the temperature of nerve endings situated deep in the skin (Kerslake 1955 Blockley 1966) these endings must be placed at a depth where the temperature in certain conditions exceeds that of the surface about 5–10 i.e. in the very deepest part of the tissue layer studied in the present paper

If the temperature in the deeper layer of the skin should play any part in the thermoregulation, it must contain thermosensitive receptors. In the studies of Bazett et al (1927 1930) Bazett (1951) Oppel and Hardy (1937) it was presumed that the receptors concerned with thermal sensation and thermoregulation were the "warm" and "cold spots" which could be correlated with Ruffini and Krause endorgans. These structures seemed to lie at depths of 0.3–0 6 mm and 0 15–0.20 mm respectively (Bazett et al. 1927 1930) These values are in good agreement with the receptor depth they calculated from thermal conductivity and temperature-sense reaction time and have later been confirmed by the electrophysiological studies of thermal sensations by Hensel and Zottermann and their coworkers, cf Hensel (1952) These receptors seem to be placed far too superficially to serve as the "deep skin temperature" receptors involved in the thermoregulation according to Kerslake's theory (1955) However the existense of morphologically distinguisable thermal receptors in the skin has not been verified (Zottermann 1959 Winkelmann 1960 Hensel 1966) —Evidence of Lele et al. (1954) Weddell et al. (1954) and Weddell and Miller (1962) seems to show that such specialized thermoreceptors do not exist in the common skin areas and that thermal sensations may be achieved from free nerve endings widely distributed but arranged in distinct layers through the tissue down to the subcutaneous border (Weddell and Miller 1962 Melzack and Wall 1962)

In conclusion it may be said that the present experiments and modern neurophysiological evidence seem to justify a study of the temperature measured as deeply as possible in the skin + subcutaneous tissues. The "average deep skin temperature" i.e. the average of measurements from different sites (chest, abdomen shoulder upper arm and forearm, thigh and calf) may then be evaluated with respect to its possible role in the regulation of sweat secretion in rest and work.

Chapter III

AVERAGE DEEP SKIN TEMPERATURE AND THE REGULATION OF SWEATING AND SKIN BLOOD FLOW

Sweat secretion during work occurs at a mean skin temperature ($\bar{T}$) which is much lower than the threshold of about 35° C for thermal sweating during rest. In certain conditions the differences in $\bar{T}$ between work and rest were found to be more than 10° C for the same sweat rate (B Nielsen and M Nielsen 1965 a) the skin temperature being lower when the work rate was higher

If the rate of sweat production during both rest and work is controlled solely by the temperature of nerve endings situated in the skin (Kerslake 1955) these receptors must lie as deep as possible so that the work and rest values of temperature coincide. This argument also follows from the results presented in chapter II which showed that the highest temperatures were found in the innermost skin layers in all subjects, even when their subcutaneous fat layers were of different thickness (table p 19) It was therefore decided to measure T_{d_i} as the average of the highest possible temperatures found in the tissues studied, namely the temperature measured at a depth corresponding to half the skinfold thickness rather than at a fixed depth. The "average deep skin temperature" (T_d) (cf. methods p 15) was measured during rest and work at different environmental temperatures in conditions for which the sweat rate was between 50g/hour and 600 g/hour in order to evaluate the possible role of T_{d_i} in the regulation of heat dissipation and skin blood flow

Procedure

The subject arrived in the morning, changed to shorts and entered the climatic room which the day before had been set to the desired temperature He inserted the rectal and esophageal thermocouples and then rested in the ergometer chair while the intracutaneous thermocouples were put in place An electric fan directed towards the chest of the subject produced an air movement of 0.5–1 m/sec at chest height it was started at the beginning of the actual experimental period in the cold and cool environments but was kept on also during the preparation period in warm conditions. In this way

he usually spent 1 hour at the experimental environmental temperature before the experiment started. The experiment lasted 60 min during which the subject rested or worked at a constant rate while the measurements were taken The weight loss, esophageal temperature and average deep skin temperature of trunk (3 localities) arm (2 localities) and leg (2 localities) were recorded continuously T_{re} was measured and pulse rate counted at 5 min intervals $\bar{T}$ and wall and air temperatures were measured 3 times during the last 30 min of the 60 min experimental period. After about 45 min of work samples for the determination of the respiratory gas exchange were collected and the relative humidity in the chamber was determined with an aspiration psycrometer

The experimental data presented in the graphs represent the values in stable conditions, i. e. the average of the values obtained from the 45th to the 60th min of the experimental period.

Subjects: Two of the three subjects in this study MC and KHN are the same as used in the temperature gradient studies, chapter II. Their data are presented below Max. V_{O_2} was estimated from the heart rate at submaximal work loads ($\dot{V}_{O_2}$) extrapolated to V_{O_2} at heart rate 196 (Asmussen and Hemmingsen 1958)

Subj.	Age yrs	Weight kg	Height cm	A_D m	Est max. $\dot{V}_{st}$ l/min	N of exp.
T D	19	70	189	1.94	3.0	20
K H N	21	65	181	1 83	4.2	13
M C	21	83	176	1.99	3.5	6

Results

Variations in sweat rate and skin temperature were produced by exposing the subject to different environmental temperatures at 3 metabolic levels (rest at 30 35 and 44° C work 540 kpm/min at 20 25 and 35° C and work 900 kpm/mm at 10 20 and 30° C)

In Fig. 5 and 6 the sweat rate is plotted against the mean skin surface temperature ($\bar{T}_s$) and the mean deep skin temperature ($\bar{T}_{ds}$) for subject TD and KHN It appears that the relationship between sweat rate and skin temperature is different for the 3 metabolic levels, the sweat rate being greater for a given $\bar{T}$ at higher work loads as found earlier The same separation is seen for the sweat rate/deep skin temperature relationship

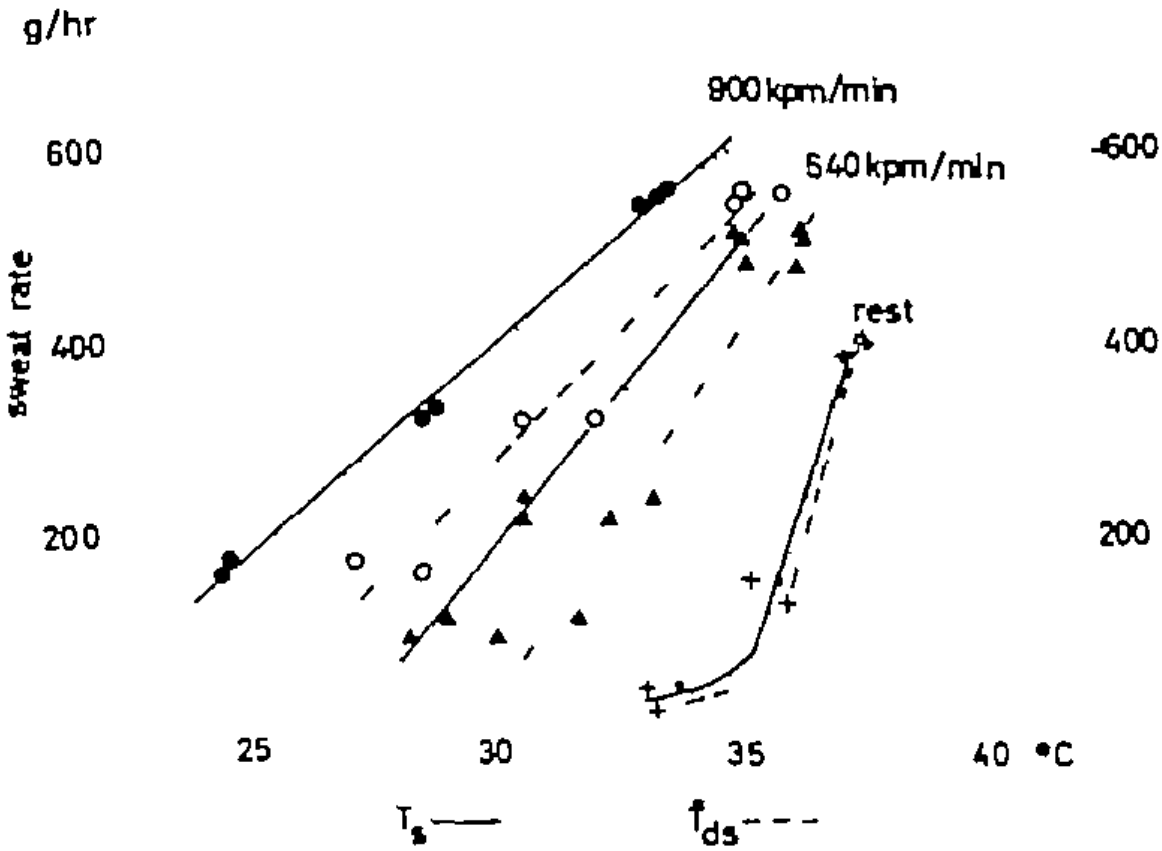


Fig 5

Steady state sweat rate plotted against mean skin temperature ($\bar{T}$ —— closed symbols) and average deep skin temperature (T_d - - - - open symbols). Subj. TD

3 metabolic levels { ○ 900 kpm/min ($\bar{T}$ = 10, 20 30°) ▲ △ 540 kpm/min ($\bar{T}$ = 20, 25 35) + rest ($\bar{T}$ = 30, 35 44)

Fig. 7 shows the sweat rates and the esophageal temperatures for the 3 metabolic levels in two subjects. $\dot{S}w$ for each condition varies from about 100 to about 600 g/hr with only small changes in internal temperature. The esophageal temperature (T_{es}) is increased in proportion to the metabolic rates. In all the rest experiments and also at the low work level for subject KHN the temperature is drifting towards higher values in the more stressful environments.

In Fig. 8 the average "steady state" sweat rate ($\dot{S}w$) is plotted against T_d The relationship varies with the metabolic rate.

The average skin surface temperature ($\bar{T}$) is linearly correlated with ambient temperature independent of the metabolic level. This relationship is shown in Fig. 9 and 10

The calculated heat loss by convection and radiation (C + R) is plotted against the difference between the skin surface temperature and the operative

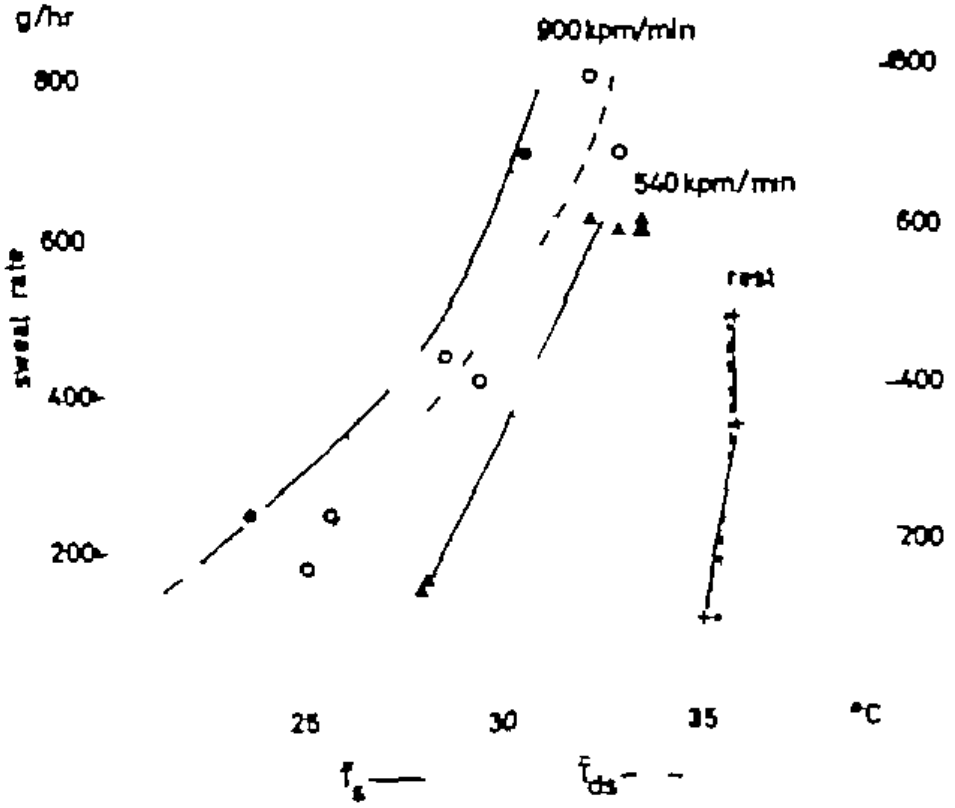


Fig. 6

Steady state sweat rate plotted against mean skin temperature ($\bar{T}$ ——, closed symbols) and average deep skin temperature (T_{ds} – – – – open symbols). Subj. KHN

3 metabolic levels { ○ 900 kpm/min ($\bar{T}$ = 10, 0, 30) ▲ △ 540 kpm/min ($\bar{T}$ = 20 and 35) + rest ($\bar{T}$ = 30, 35, 40°)

temperature ($\bar{T} - T_a$) in Fig. 11. The slope of this curve gives the combined heat transfer coefficient (h_c) for the two subjects. It amounts to 14.3 kcal/hr–m²– C for TD and 13.5 kcal/hr–m²– C for KHN.

When, as in Fig. 12, the heat loss by convection plus radiation (C + R) is plotted against $T_{(ds,\ a)}$ another nearly linear relationship results. The slope of these lines may be used to estimate the heat flow by conduction through the skin (see p. 33). A value of 12 kcal/hr–m²–mm– C for TD and 23 kcal/hr–m²–mm– C for KHN for heat conductivity can be estimated. (Average depth to measurements in skin TD: 3.6 mm, KHN: 2.4 mm).

With the assumption that all heat transported to the skin surface by the blood is given off by evaporation in the experimental conditions of this study and that the blood enters the skin at T_{ar} and leaves it at $T_{ar} - 2\ (T_{ar} - T_{ds})$ (see p. 35) the skin blood flow can be estimated. The skin circulation calculated in this way is plotted against skin surface temperatures in Fig. 13.

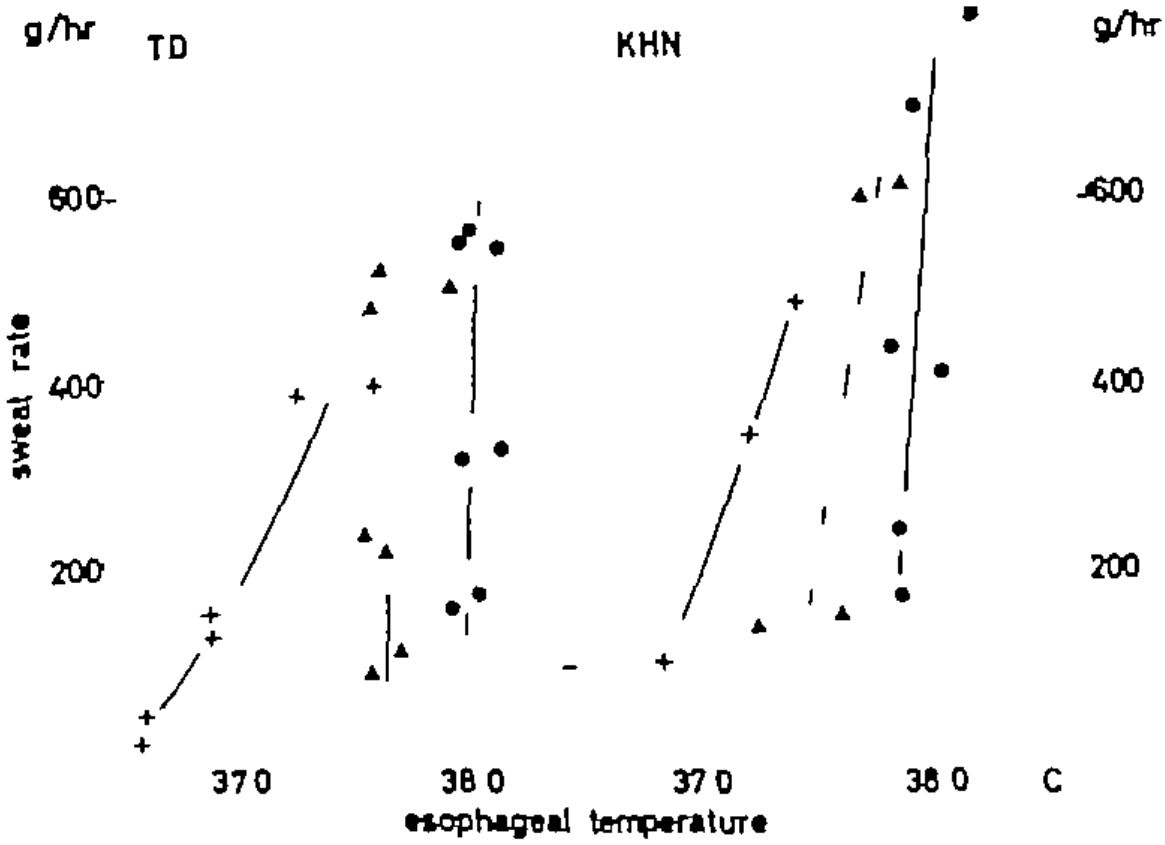


Fig 7

Steady state sweat rate in relation to deep esophageal temperature. 3 metabolic levels: 900 kpm/min 540 kpm/min ▲ and rest + Left: Subj. TD Right. Subj KHN

For comparison, the changes in skin blood flow expressed by the conductance of the peripheral tissues is shown in Fig. 14 These two expressions of skin blood flow are plotted against one another in Fig. 15 Finally in Fig. 16 the thermal resistance of the skin $R_s = \frac{T_{(d\quad)}}{H}$ viz. the temperature differential per unit heat flow through the skin, is plotted against surface skin temperature.

Discussion

The interesting theory of Kerslake (1955) is not supported by the present experimental data. Even when measurements were made in the level where temperatures were highest, i.e in the deepest layers of the subcutaneous tissues (chap II) the relationship between "deep skin temperature" and sweat rate was found to vary at the 3 metabolic levels (Fig. 5 and 6) In Kerslake's theory it was supposed that the thermal resistance, i.e. the temperature dif

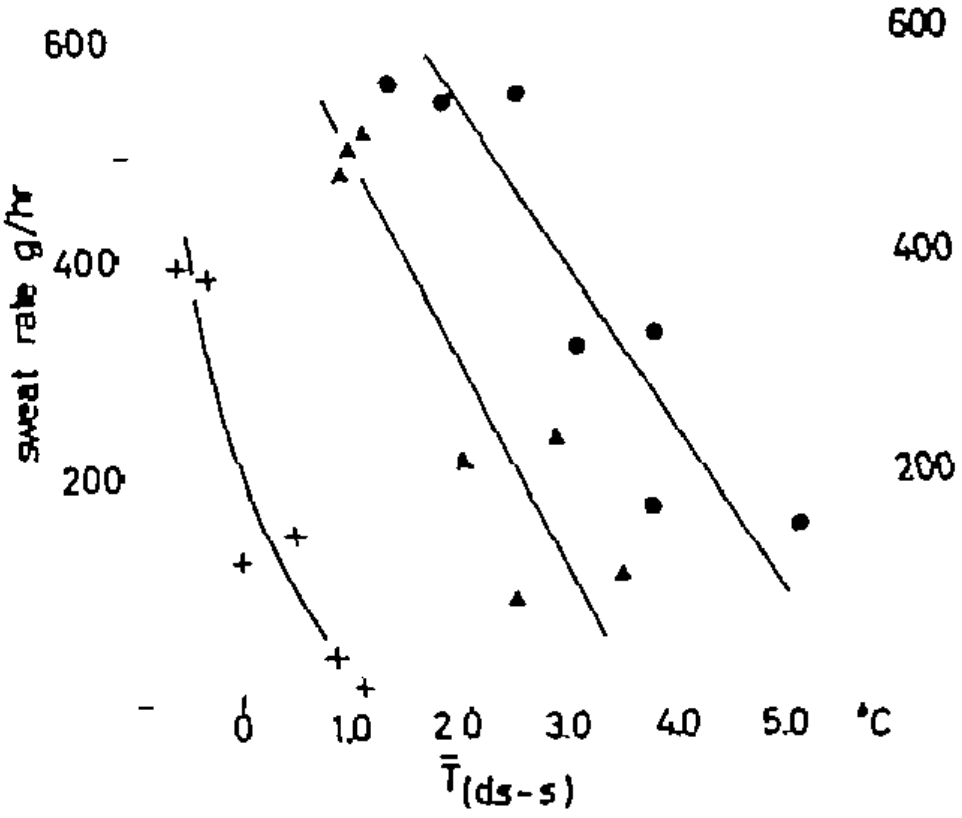


Fig 8.

Sweat rate versus $\bar{T}_{(ds-s)}$ at 3 metabolic levels: 900 kpm/min ● 540 kpm/min ▲ and rest + Subj. TD

ferential per unit heat flow between the hypothetical deep receptor and the surface, was constant. Thus, the temperature of the deep skin receptor would increase over the surface skin temperature as the product of the heat flow through the skin and thermal resistance. The present experiments (cf. Fig. 5) show that for the same rate of heat flow (same work rate) the total peripheral thermal resistance from the surface to a depth of half the skinfold thickness decreases with increasing surface temperature (Fig. 16). In Blockley's (1966) calculations of deep skin temperatures, a decrease in the peripheral thermal resistance with increases in surface skin temperature was presupposed. The thermal resistance calculated in the present experiments (Fig. 16) where T_{ds} is measured shows different values for the same $\bar{T}$ at the 3 metabolic levels. The maximum value of 0.012° C-hr/kcal, obtained by extrapolating the curves in Fig. 5 to surface skin temperatures equal to sweat rate zero (cf. Fig. 16) is identical for the 3 metabolic levels. The range is of the same order of magnitude as Kerslake's 0.013–0.017° C hr/kcal for different subjects and Blockley's 0.006–0.019° C-hr/kcal which increases with decreasing skin tem-

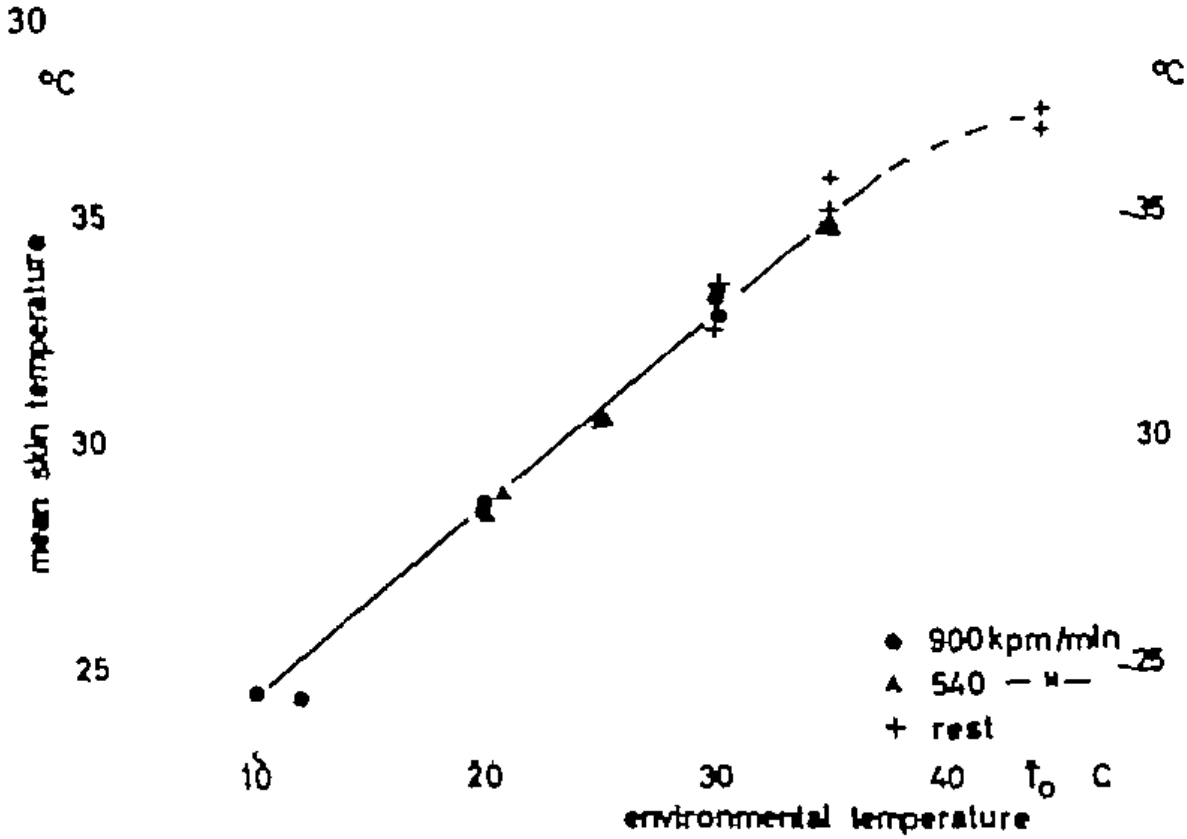


Fig 9

Mean skin temperature at various levels of environmental (operative) temperature, 3 metabolic levels. 900 kpm/min • 540 kpm/min ▲ rest + Subj. TD

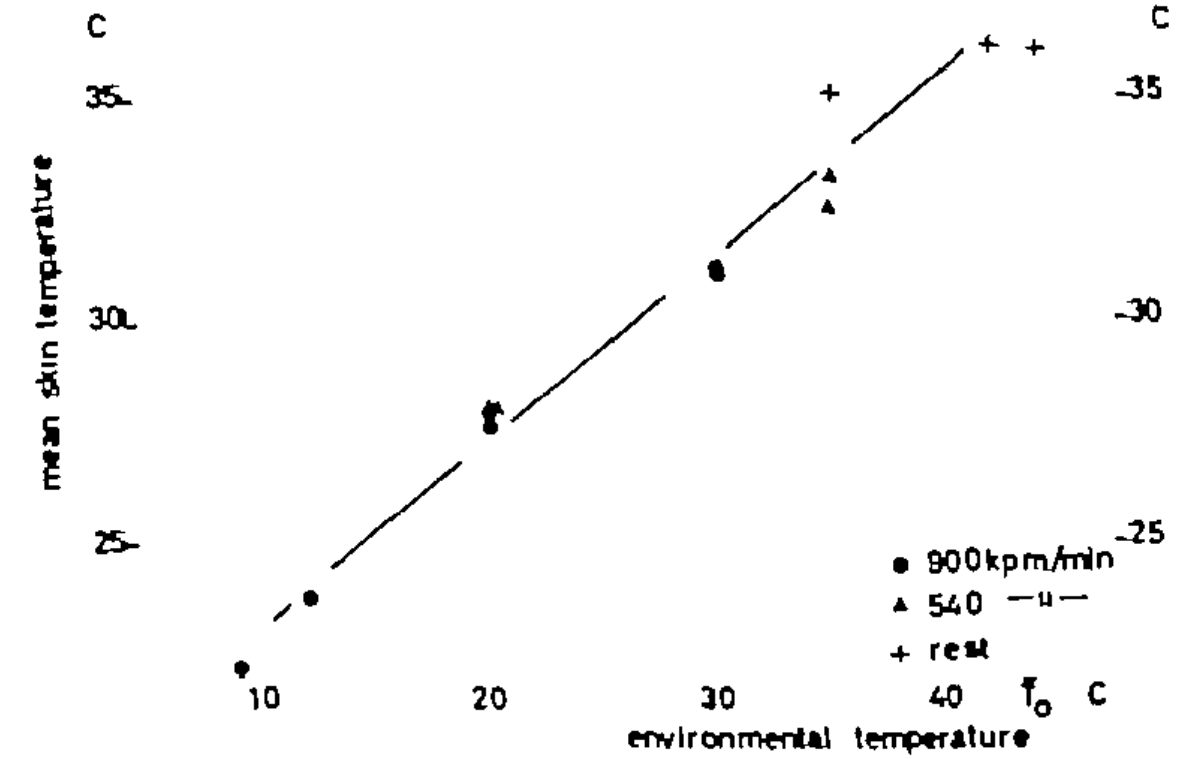


Fig 10

Mean skin temperature at various levels of environmental (operative) temperature, 3 metabolic levels: 900 kpm/min • 540 kpm/min ▲ rest + Subj. KHN

perature. However it is evident from the present experiments that no average deep temperature in tissues superficial to muscle and bone is equal in rest and work for the same rate of sweat secretion.

The mean temperature differential across the skin $T_{(d_s)}$ is different at the same sweat rate for the three metabolic levels (Fig. 8) At the first sight, this finding seems to rule out the assumption of *Bazett (1949 1951) that* temperature gradients in the skin supply information on heat production for the regulation of sweating. However the differential measured in the present study is believed to be the maximum difference existing between the surface and the cutis + subcutis. Although unlikely it is possible that the gradients in more superficial layers of the skin, e. g. in bloodless layers, are the same for equal sweat rates.

Neither of the differences $(T_{co} - T_s)$ $(T_{co} - T_{ds})$ nor $T_{(d_s)}$ shows the same relationship to sweat rate for the three metabolic levels. However the *possible interaction between core temperature (hypothalamic temperature)* and deep skin temperature or temperature gradients across the skin may be considered as stimulus for the regulation of sweat secretion and skin circulation. Multiple regression correlations for T_{co}, $\dot{T}$ and Sw *for* T_{co}, T_{ds} and Sw and for T_{co}, $T_{(d_s)}$ and Sw are presented in an appendix, p. 59

The mean skin surface temperature (T_s) was found to be a linear function of ambient temperature (Fig. 9 and 10) This correlation agrees with earlier findings (e. g. Winslow and Gagge 1941 Robinson 1949 B Nielsen and M. Nielsen 1965 a) In conditions in which the subject can attain thermal equilibrium with the environment, the skin temperature was found to be independent of the activity level and of the sweat rate (Fig. 9 and 10) The skin surface temperature is determined partly by the conductive heat flow and the thermal resistance between the thermoregulated core and the skin surface and partly by the relationship between the total heat flow from the core to *the skin surface and the amount of heat dissipated to the environment.*

The total heat flow from the *core* to the *skin surface* takes place as conduction and as convection due to blood flow

$$H_{cond} = k(T_{core} - T_s)$$
$$H_{conv} = \dot{Q} \times c \times (T_{art} - T_{venous})$$

where.

H_{cond} = conductive heat transfer in the peripheral tissues.
H_{conv} = convective heat transfer in the peripheral tissues.
k = conductive coefficient of the peripheral tissues.
$\dot{Q}$ = skin blood flow l/min.
c = specific heat of blood.

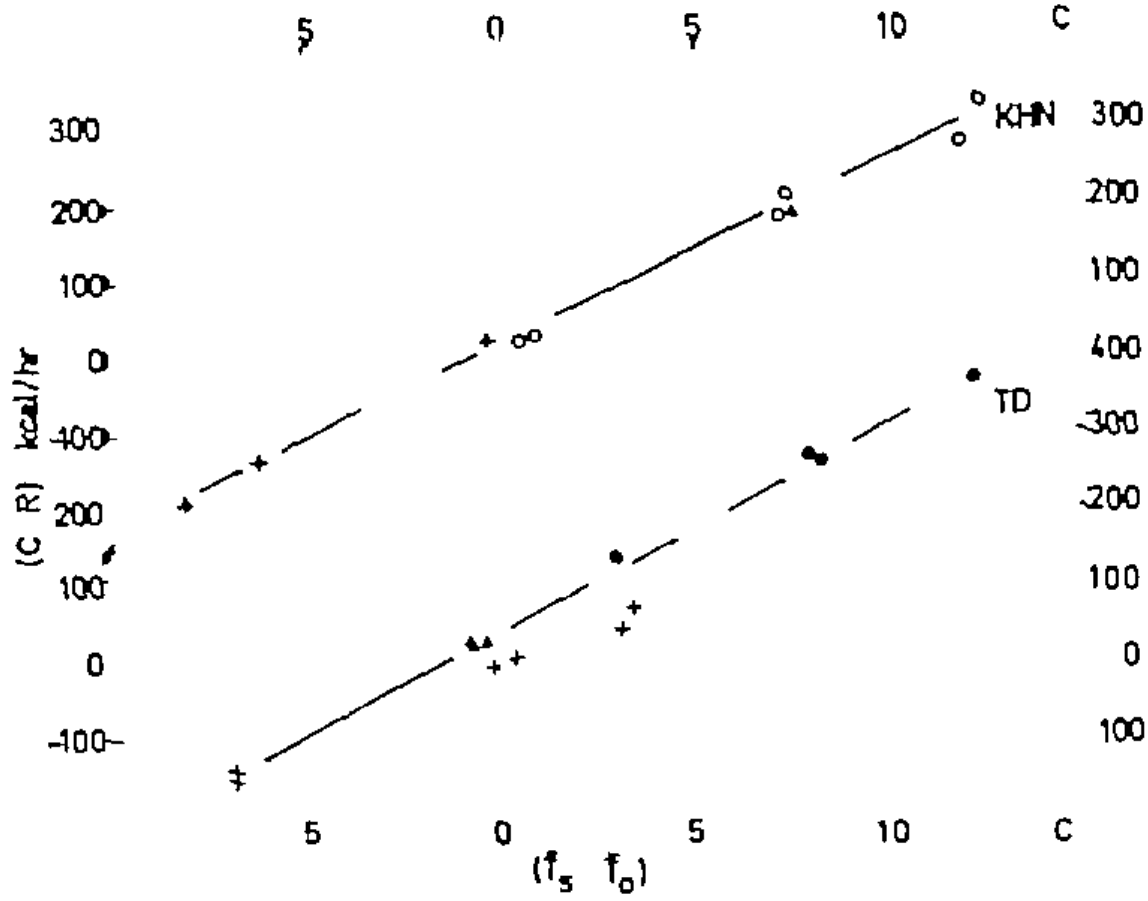


Fig 11

(C + R) heat loss plotted against $(\bar{T} - \bar{T}_a)$. Two subj: Above KHN below TD

		TD	KHN
3 metabolic levels	900 kpm/min	•	○
	540 kpm/min	▲	△
	rest	+	◇

The heat loss from the *surface* takes place by convection radiation and evaporation. In thermal equilibrium heat production = heat loss, and hence

$$k(T_{core} - \bar{T}) + \dot{Q} \times c \times (T_{art} - T_{venous}) = h_o(\bar{T} - T_a) + E_{sw}.$$

As found in the present study when $\bar{T}$ is a function of $\bar{T}$ independent of sweat rate and total heat production it follows that for a constant environmental temperature $C + R = h_o(\bar{T} - T_a)$ remains constant and $k(T_{core} - \bar{T}_s)$ is practically constant. Therefore, the amount of heat brought to the surface by the skin blood flow must be exactly counterbalanced by the evaporative heat loss. Variations in heat production result in changes in the amount of heat delivered to the skin by the blood (convective transfer). By the criterion of the constant skin temperature, this same amount of heat must be dissipated by evaporation of sweat.

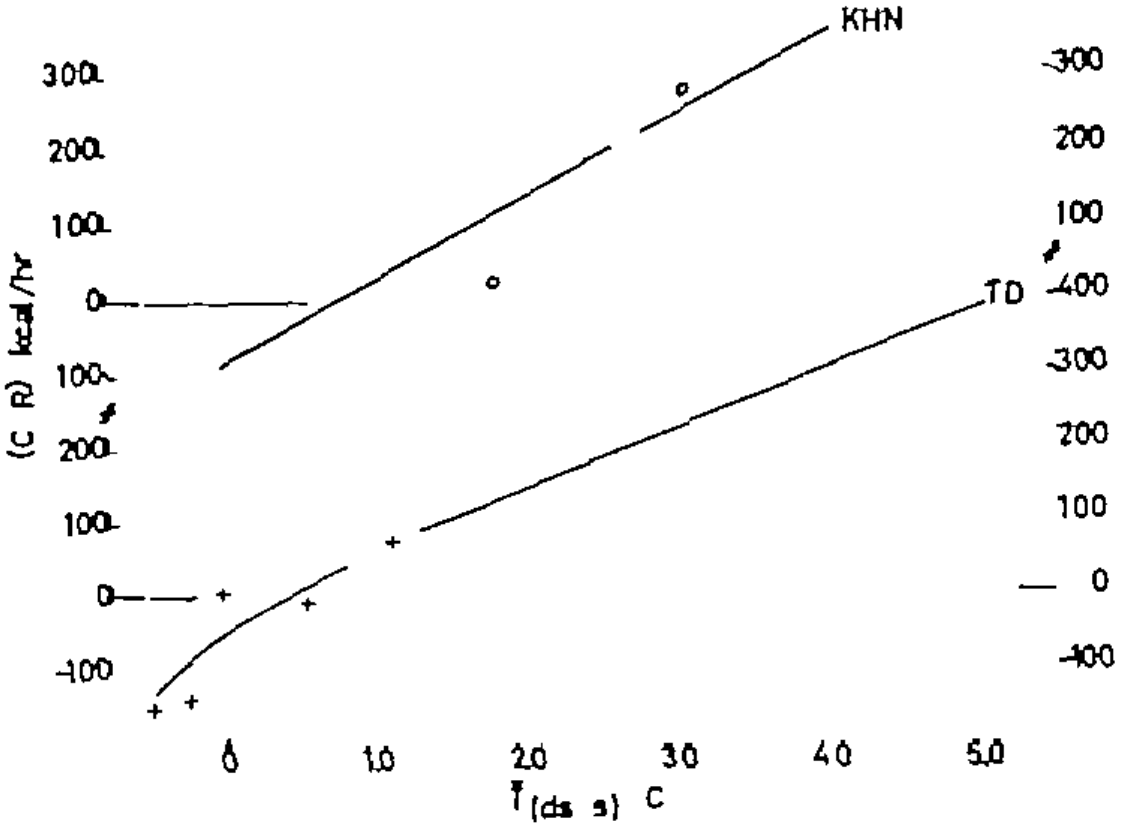


Fig 12.

(C + R) heat loss plotted against $T_{(dis\ s)}$ Two subj: Above: KHN below TD

		TD	KHN
3 metabolic levels	900 kpm/min	•	o
	540 kpm/min	▲	△
	rest	+	◇

How this coordination of convective heat transfer by the skin blood flow and sweat secretion is brought about is unknown. Bradykinin mechanisms may be involved in the fine adjustment of blood flow to the sweat gland activity (Fox et al 1958)

When the sweat rate is zero at the lower limit for thermal equilibrium, the skin blood flow is also very nearly zero (cf. Fig. 13 and 14) The total heat transfer to the skin then takes place as conduction across the skin ($H_{cond} = k_t(T_{sk} - T_s)$) and the heat is dissipated from the skin to the environment by convection plus radiation ($H_{conv} = h_c(T - T_s) = (C + R)$)* Since the skin surface temperature remains constant and independent of increases in heat production and sweat rate, it follows that increases in heat production

*Should be (C + R) + insensible perspiration, but the relative small value of the latter 10–20 kcal/hr may permit its omission in the following considerations.

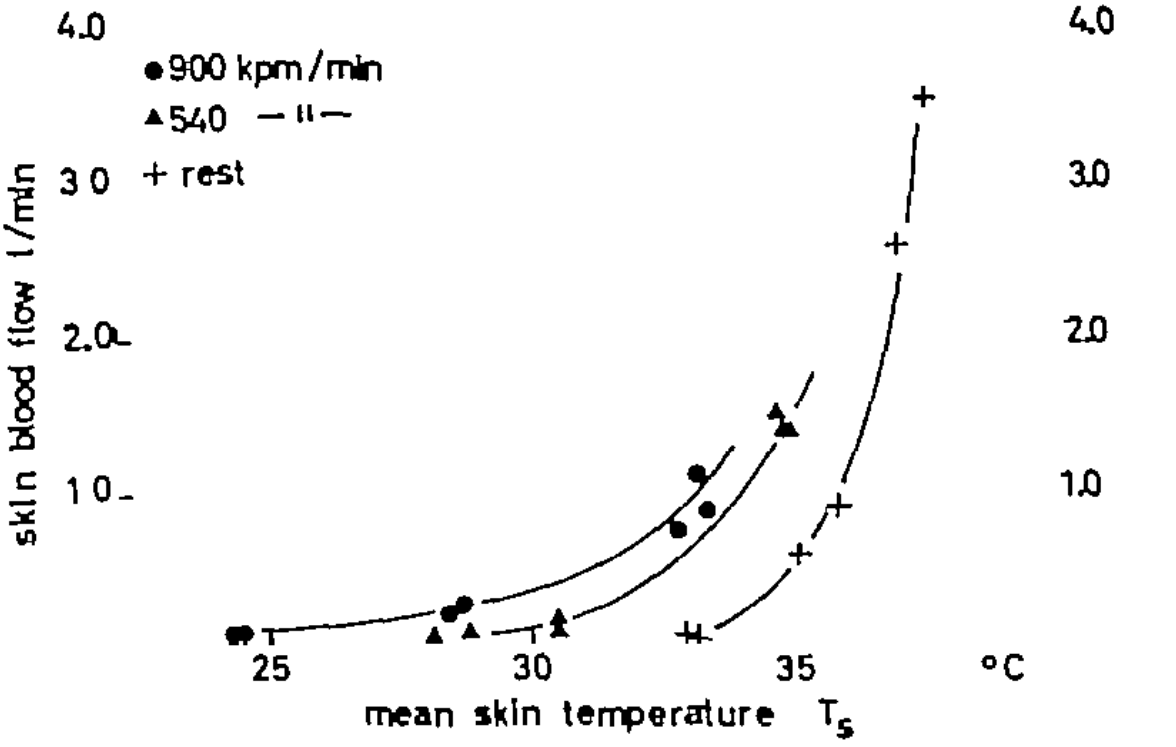


Fig 13

Skin blood flow l/min calculated from $\frac{E_{sw}}{2(T_{ss} - T_{sk}) \times 60 \times 1.0}$ versus mean skin skin surface temperature (T_s). 3 metabolic levels: 900 kpm/min • 540 kpm/min ▲ rest + Subj TD

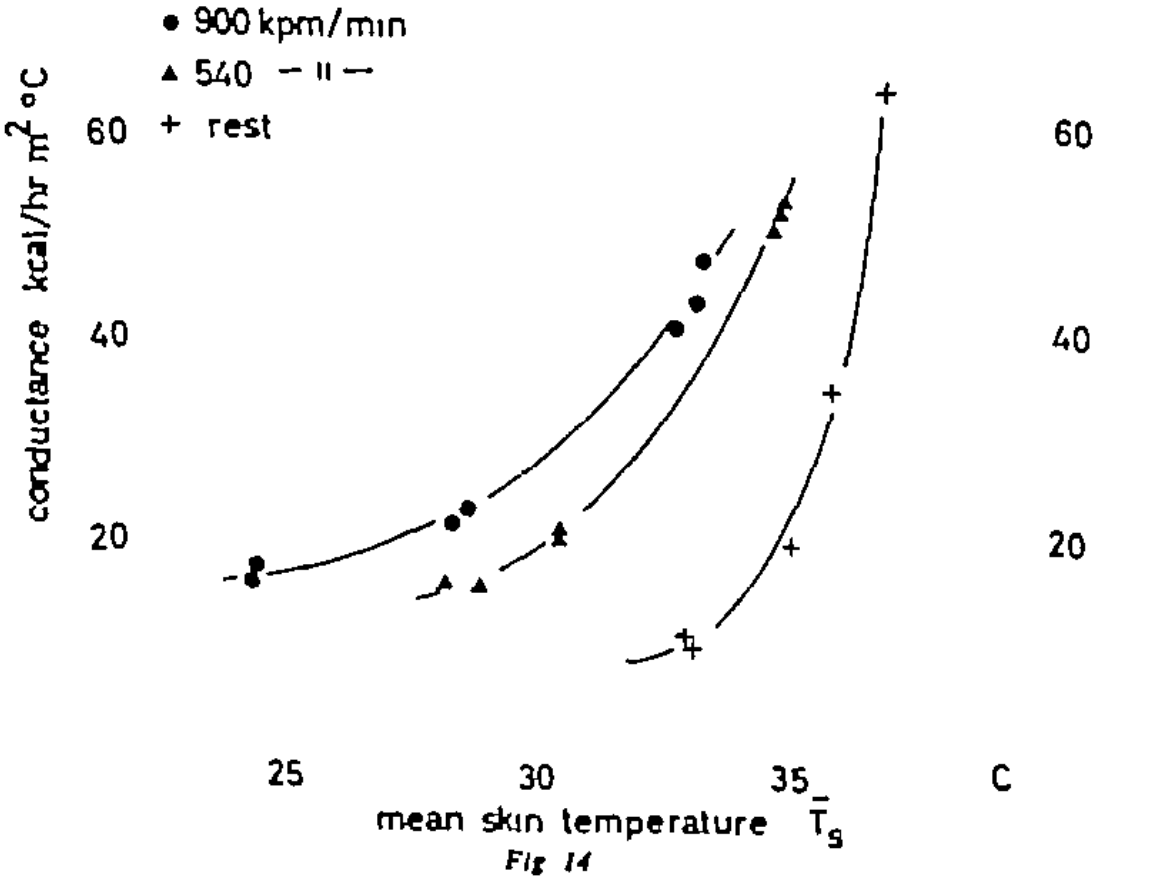


Fig 14

The conductance of the peripheral tissues plotted against mean skin surface temperature (T_s). 3 metabolic levels: 900 kpm/min • 540 kpm.min ▲ rest + Subj. TD

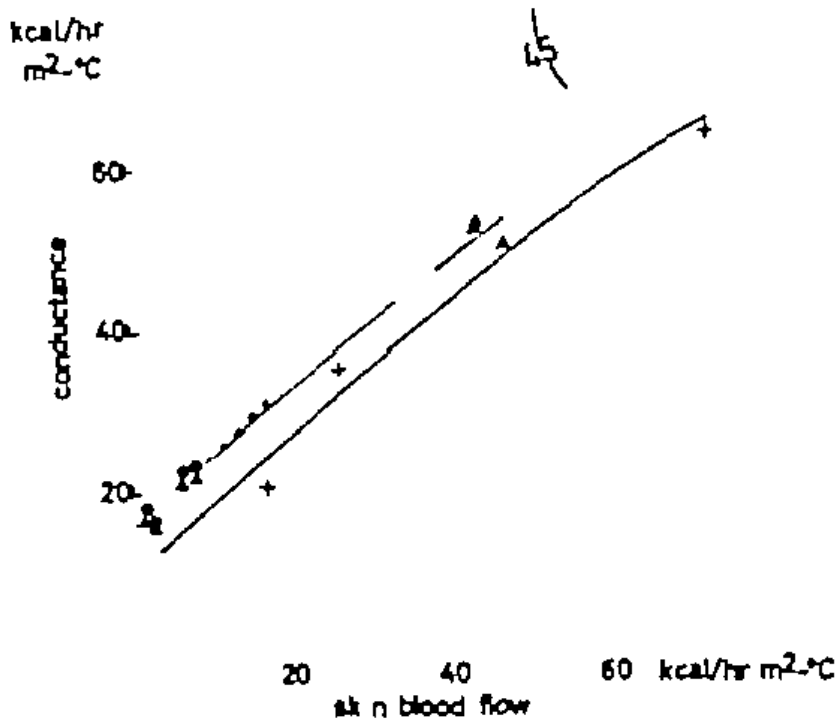


Fig 15

The calculated skin blood flow (Fig. 13) expressed in the same units as conductance, plotted against conductance (Fig. 14). ---- the 45 line of identity" for the two expressions of skin circulation. 3 metabolic levels: 900 kpm/min • 540 kpm/min ▲ rest + Subj. TD.

above the lower limit of equilibrium must cause an increase in convective heat transfer (skin blood flow) equal to the increase in evaporative heat loss, while the conductive heat transfer still equals the (C + R) heat loss.

If this explanation is sound, then a plot of (C + R) versus $T_{(sk\ s)}$ (Fig. 12) can be used to calculate the conductive heat transfer in the skin. The slope of the $(C + R)/T_{(sk\ s)}$ line is then the conductive heat transfer coefficient for the peripheral tissues. The values for 2 subjects are 41 kcal/hr–m²–° C (subj TD) and 52 kcal/hr–m²–° C (subj KHN)

The above mentioned conclusion that the amount of heat given off by evaporation is equal to the heat transferred to the skin by the blood flow also permits calculation of skin blood flow in such experiments in a new way cf. Fig. 13 It is assumed that the arterial blood entering the skin from the core has the same temperature as the aortic blood, which is considered to be equal to deep esophageal temperature (Cooper and Kenyon 1957) The venous blood leaving the skin has given off heat at the epithelial borderline so that its temperature at this stage is probably very close to T_s. Returning through the skin and subcutis the venous blood is rewarmed, receiving heat from the tissues and perhaps by countercurrent exchange with the arteries.

The temperature of the "shell" tissue must lie between the temperature of the arterial and venous blood, which runs into and out of the shell When measured in the layer nearest to the thermoregulated core (i.e. in a region very little influenced by heat conductance) the tissue temperature should be equal to the average between the arterial and the local venous blood temperature. It is further assumed that in heat equilibrium the deep skin temperature (T_{d_s}) as measured in the present experiments represents this tissue temperature, i.e. $T_{d_s} = \frac{T_{es} + T_{venous}}{2}$ Thus T_{venous} (the temperature of the blood which enters the core) becomes $2\ T_{d_s} - T_{es}$. The skin blood flow may then be calculated as

$$\dot{Q} = \frac{E_{sw}}{2(T_{es} - T_{d_s}) \times 1\,0 \times 60}$$ where:

$\dot{Q}$ = skin blood flow in l/min
E_{sw} = sweat heat loss kcal/hr*
T_{es} = esophageal temperature
T_d = mean deep skin temperature
1 0 = specific heat of blood in kcal/l–° C.

Results from subj TD are presented in Fig. 13

The very high calculated skin blood flow of 3.5 l/min during rest at 45° (T = 37°) is of the same order of magnitude as the "approximately 2 l/min–m²" reported by Robinson (1963) Horvath et al. (1964) and Koroxenidis et al. (1961)

Both during rest and in the two working conditions the heart rate increases with rising environmental temperature. This change from lowest to highest environmental temperature was 30 beats/min at rest while 15 beats/min in the work experiments. Assuming a constant stroke volume of 120 ml in exercise, this HR response would produce an increase in cardiac output ($\dot{Q}$) of (120 ml × 15) = 1 8 l/min during work, and, with a resting stroke volume of 80–100 ml, an increase in $\dot{Q}$ of (80–100 ml) × 30 = 2.4–3.0 l/min in the rest experiments. Experiments of Rowell et al. (1966 1967) however show that the increased skin circulation due to thermal stress superimposed on exercise is attained by a reduction in splanchnic and renal blood flow with a constant or even reduced $\dot{Q}$ due to a diminished stroke volume, in spite of the increased heart rate.

* In conditions where heat is *received* from the environment ($T > T_s$) the received external heat is subtracted from E_{sw}, since this heat is not transported to the skin surface by the blood.

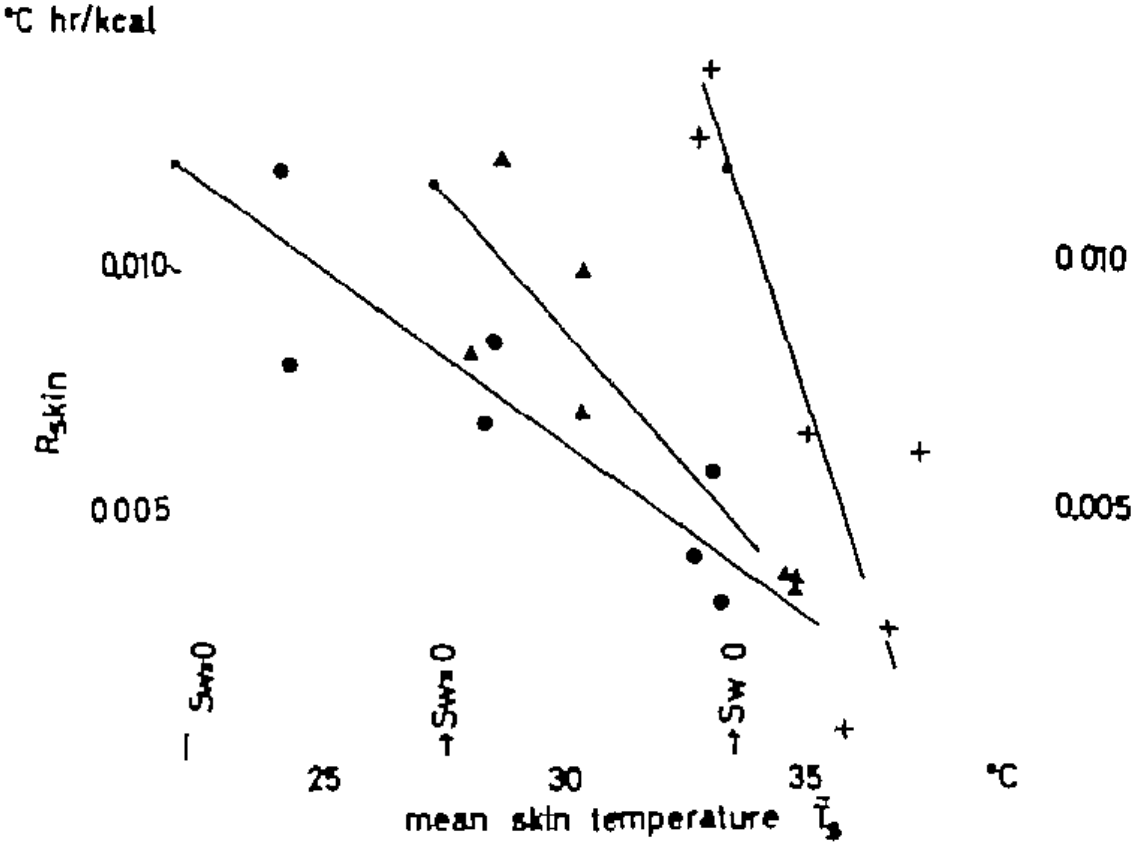


Fig 16

The thermal resistance of the skin, R_{skin}, at different values of mean skin surface temperature ($\bar{T}_s$). 3 metabolic levels: 900 kpm/min • 540 kpm/min ▲ rest + ↓ Sw = 0 denotes the $\bar{T}$ values, at which the sweat rate is zero (from left to right) at 900 kpm/min • 540 kpm/min ▲ and rest + Subj TD

A comparison of the calculated blood flow with the conductance of the peripheral tissues, Fig. 15 for the same experiments shows that the two calculated indexes of skin blood flow are remarkably similar The same is found for subj KHN The deviation from the "45° line of identity" at the higher values of skin blood flow may be the result, if the blood at very high flow rates is not cooled to skin temperature as presumed in the calculation of conductance.

The intercept on the conductance axis for skin blood flow zero is 15 kcal/hr–m² in the work experiments and 10 kcal/hr–m² for the rest experiments in Fig. 15

These minimum values of conductance are in good agreement with those reported in the literature (cf. Robinson 1963) The finding that the minimum value during work is greater than in rest also corresponds with earlier studies (e. g. Pugh and Edholm 1955) where the determinations were made in water In these studies the rectal temperature decreased much faster during work

than at rest in cold and cool water (cf also Keatinge 1961 and Craig and Dvorak 1968) This difference in rate of cooling is ascribed to cold vasodilation for the very cold conditions but no explanation seems to be given for the moderately cold 15–25° conditions. It may be suggested that the elevated values of minimum conductance during work are due to the higher ratio of "core" to "shell" This increased ratio favouring heat loss—must be the outcome of the increased heat production and blood flow in the working muscles.

In air, the temperature of the muscles during rest has been shown to fall to low values especially in cold environments (Buchtahl, Höake and Lindhard 1944 Saltin et al. 1968) Therefore, this same explanation may also be valid for the higher minimum conductance during work in the less uniform and less easily defined air environment.

Chapter IV

DEEP SKIN TEMPERATURE, MUSCLE TEMPERATURE AND THE REGULATION OF SWEATING DURING POSITIVE AND NEGATIVE WORK

When external work is performed the amount of heat liberated in the body is less than the total energy output. This is the case when a person performs positive work, e. g. walks or bicycles uphill. When walking or bicycling downhill, i. e. doing negative work, potential energy is converted to heat in the muscles, and the total heat liberation becomes greater than the metabolic energy liberation. In a recent study of thermoregulation during uphill and downhill walking (B Nielsen 1966) it was demonstrated that the core temperature increases to levels proportional to the metabolic energy liberation and not proportional to the heat production. The sweat secretion, however increased in proportion to the amount of heat liberated in the body in spite of differences in the level of core temperature.

Thermoreceptors placed at an appropriate depth below the surface could be stimulated in proportion to the heat flow through the skin and hence to the total heat production in the body (cf. p 11 and 22) A study of the deep skin temperature and the gradient across the skin during positive and negative work,—i. e. in conditions where, the ratio of total heat production to metabolic energy liberation (and body temperature) is varied,—might help to explain the correlation found between sweating and heat production.

Procedure

The subject, dressed in shorts and shoes, was fitted with the thermocouples and started to work at the fixed speed and inclination. During the walk/run experiments 2 big fans directed towards the subject's chest and back produced an air movement of 0 75 m/sec (chest) and 1–1.5 m/sec (shoulder) No fans were used in the bicycle experiments. Every 5 min the rectal temperature was measured and the heart rate (HR) was counted. After a preliminary warming up period of 25 min he stepped off the treadmill and sat down on the Krogh balance for the first weighing. This pause lasted $1^1/_2$–2 min. The subject returned to the treadmill and continued the work (walking

or bicycling) for the 45 min experimental period. During this period T_{re} and HR were measured every 5 min, while the deep skin and esophageal temperature were recorded continuously $\dot{V}$ was measured at the 30th, 37th and 42nd min and the wall temperature at the 30th and 42nd min

Expired air was collected in the Douglas bag at about the 40th min of work.

After the 45 min experimental period the subject stepped off again and sat down for the weighing as quickly as possible after stopping.

In experiments where the muscle temperature was measured, the thermocouple was inserted in the muscle as soon as the subject sat on the seat of the balance. After this the thermoneedle was withdrawn and the subject weighed. The weighing thus was delayed less than 2 min.

Three male subjects were used KHN (who also took part in the experiments reported in chapter II and III) and BJ bicycled uphill and downhill, while BI walked and ran on the treadmill. Their data are presented in the table below

Subj	Age yrs	Weight kg	Height cm	A_D m	Est. max. $\dot{V}O_2$ l/min	Number of exp.
KHN	21	65	181	1.83	4.20	16
BJ	22	72	185	1.94	2.50	25
BI	23	77	183	1.99	3.50	21

Cf. p. 25

Results

In Fig. 17 the time relationships of the measurements are shown for subj BI It appears that in the experimental period a temperature equilibrium is practically achieved Values obtained from the last 15 min of this period are those presented in the following graphs.

The oxygen uptake in relation to work intensity in uphill (positive) and downhill (negative) bicycling is presented in Fig 18 As shown earlier e. g. by Abbott, Bigland and Ritchie (1952) and Asmussen (1952) the oxygen consumption is only slightly increased with greater loads during negative work. The same relationships for walking are shown for another subject in Fig. 19

The heart rate was 15–25 beats/min higher in negative work than in positive work at the same oxygen uptake. The pulmonary ventilation was only slightly higher in negative work than in positive work at the same $\dot{V}_{O_2}$

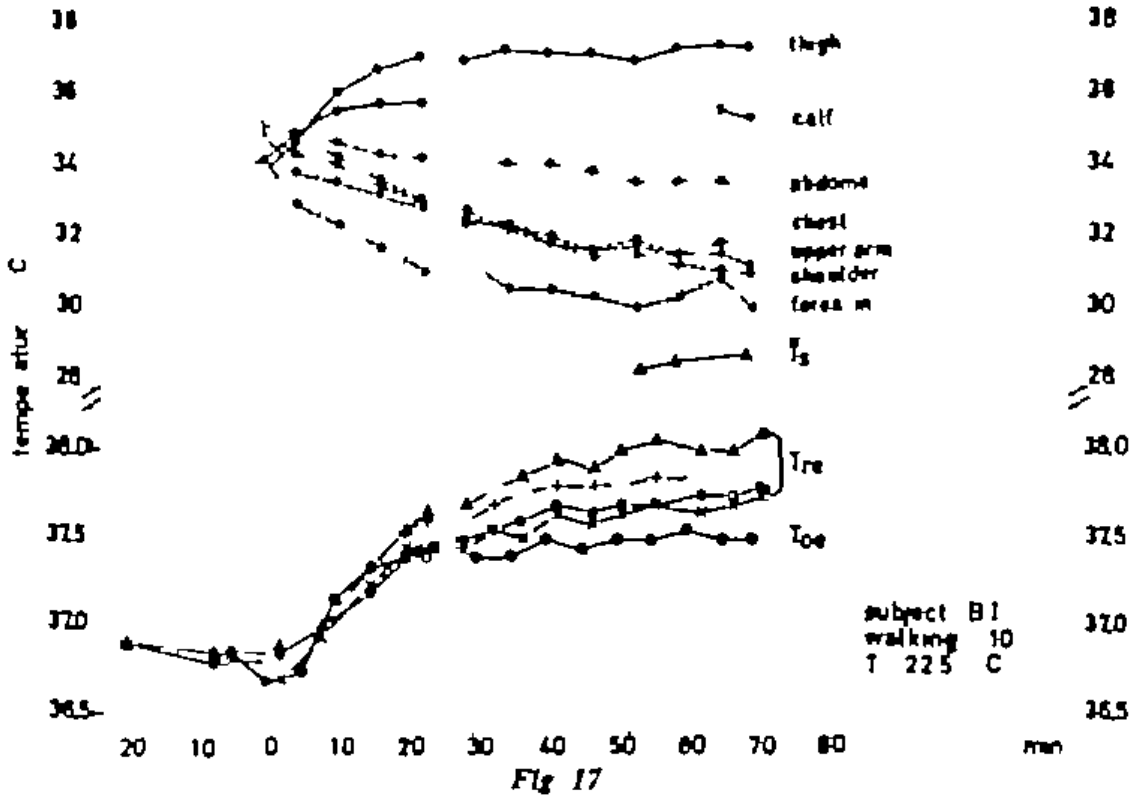


Fig 17

The time relations of the 7 single intracutaneous temperatures, $\bar{T}$ (▲), T_{re} at 4 depths (+ 27 cm, ○ 22 cm, × 17 am and △ 12 cm) and T_{oe} ( ) during the 25 min warming-up period and the 45 min experimental period. Pause for weighing between the 25th and 26.5th min. Subj. BI, downhill −10° speed 6.48 km/hr HR 116 $\dot{V}_{O_2}$ = 1.31 l/min, total weight loss 600 g/hr T_{re} at 73 min: 38.85 C.

In all 3 subjects the skin temperatures decreased with increasing metabolic rate. The relationships between T_s, T_{d-s}, T_{re} and metabolic energy liberation (M) and total heat production (H) are illustrated by the results from subject BJ in Fig. 20 and BI in Fig 21 The 3rd subject shows similar results.

The relationships between internal body temperature and M and H are shown in Fig. 22. This data reconfirms that the plateau level of esophageal temperature is a function of the metabolic energy liberation and not of the total heat production

In Fig. 23 (subject KHN) the temperature differential across the skin is plotted against M and H respectively In all subjects the relationship to H is nearly the same for uphill and downhill work

The slope $\frac{T_{(d-s)}}{H}$ has the dimensions of a thermal resistance, C–hr/kcal degrees temperature differential per unit heat flow The slope is 0 0045° C–hr/kcal for KHN in positive and 0 0068° C–hr/kcal in negative work For

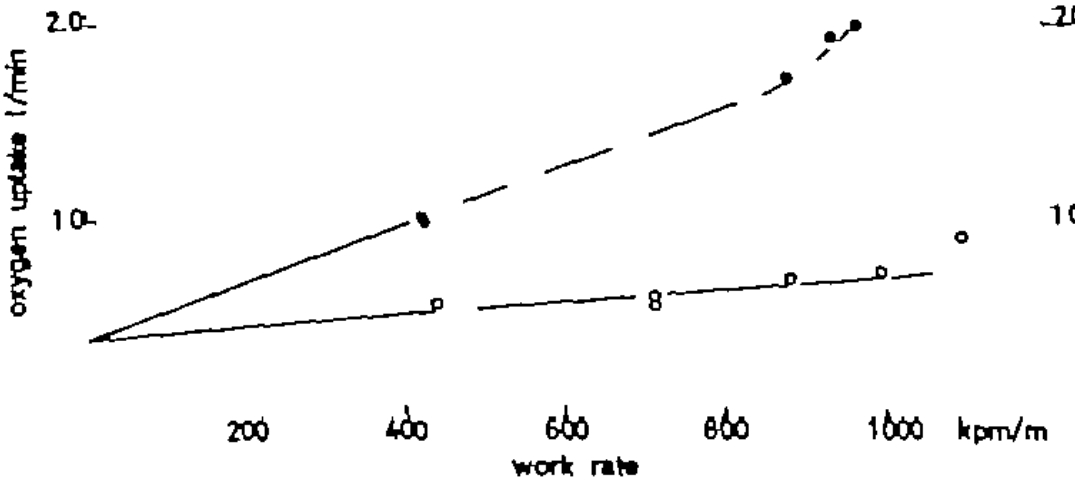


Fig 18

The oxygen uptake l/min at different work intensities. Subj. BJ
• bicycling uphill, 60 rev./min, positive work
o – downhill, 60 rev./min, negative work.

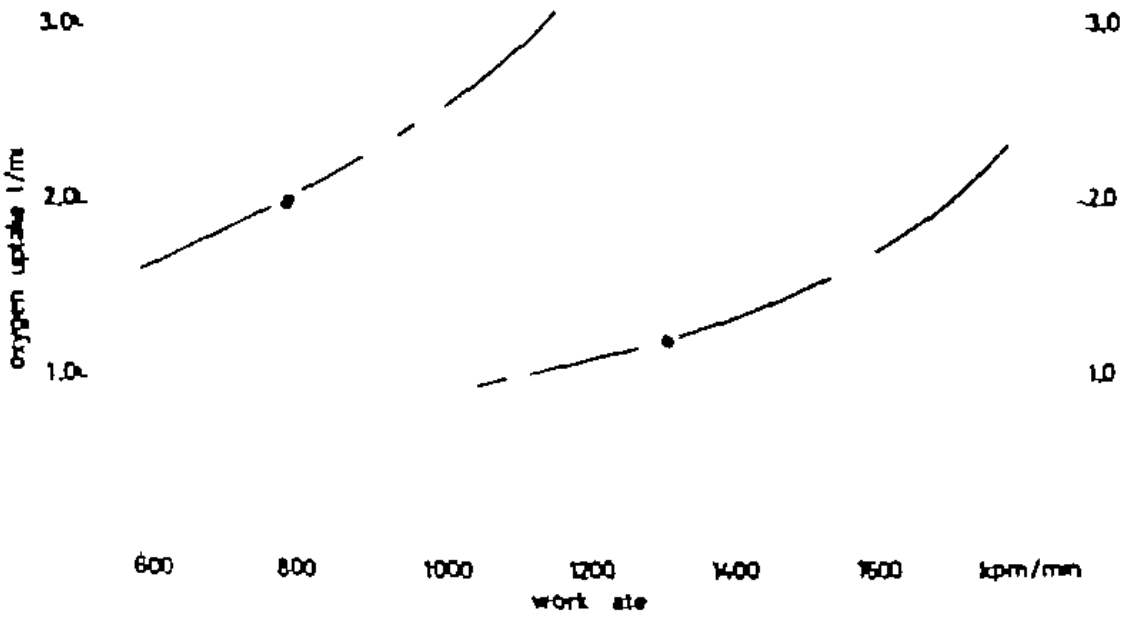


Fig 19

The oxygen uptake l/min at different work intensities. Subj. BL.
walking or running uphill +10 positive work
o – – downhill −10 negative work.

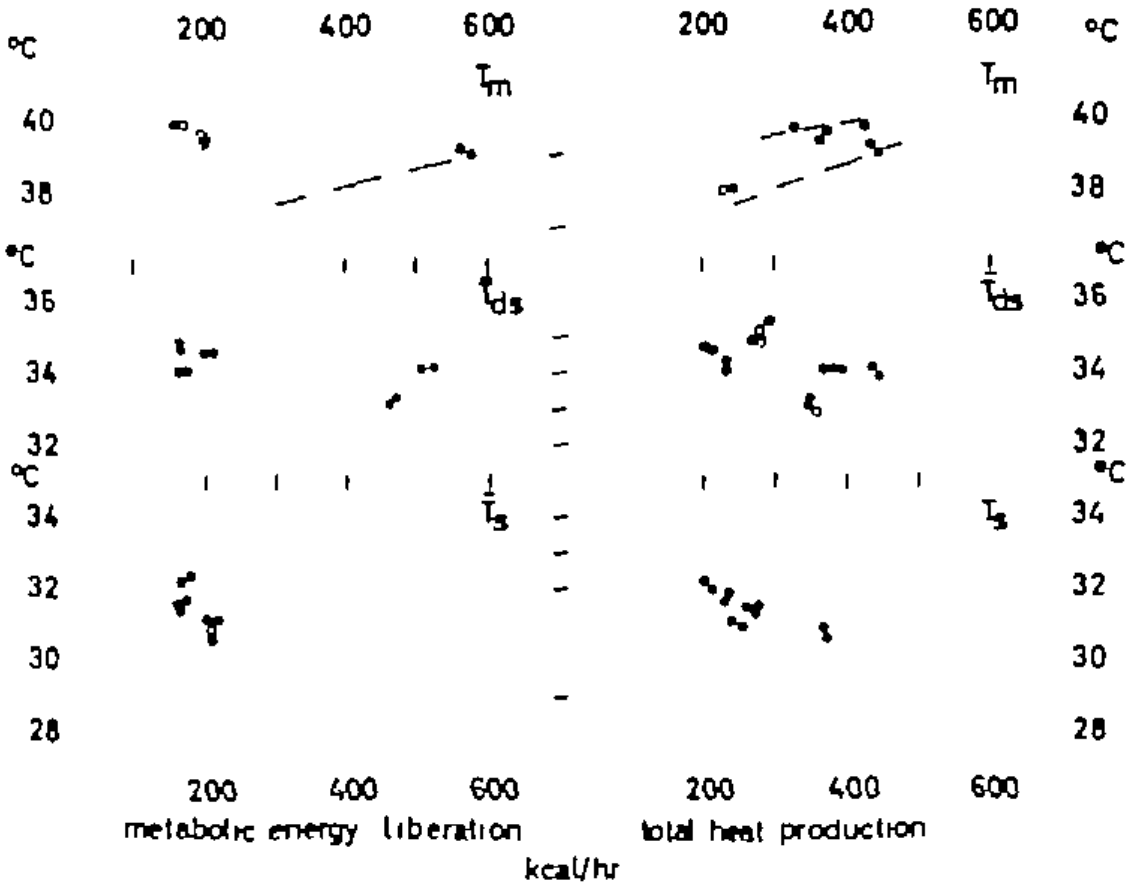


Fig 20

The temperature in vastus lateralis (T_m) average deep skin temperature (T_{ds}) and mean skin surface temperature (T_s) at different levels of metabolic energy liberation, (left) and different levels of total heat production, (right). Subj BJ Bicycling. Positive work • negative work ○ T 22.4

BJ it is 0 0086° C–hr/kcal for positive work and 0 0060° C–hr/kcal in negative work, and for BI 0 0095° C–hr/kcal for positive and 0 0050° C–hr/kcal in negative work. Their average ½ skinfold thicknesses, i. e. the skin layer across which this gradient is accumulated are: KHN 2.4 mm, BI. 4.4 mm, and BJ 5 9 mm. The correlations of $T_{d\ \ }$) with M are different for uphill and downhill work as seen in Fig. 23 The sweat rate (Fig. 24) also shows nearly the same relationship to H in both positive and negative work, while its relationship to M is different in the 2 cases. There are much higher sweat rates in negative work at the same metabolic energy liberation

In Fig. 25 sweat rate is plotted against esophageal temperature (T_{es}), average skin surface temperature (T_s) average deep skin temperature (T_{ds}) average temperature differential $T_{(ds\ s)}$ across the skin, and muscle temperature (T_m) At the same rate of sweat production, only core temperature (esophageal) and muscle temperature are different in positive and negative

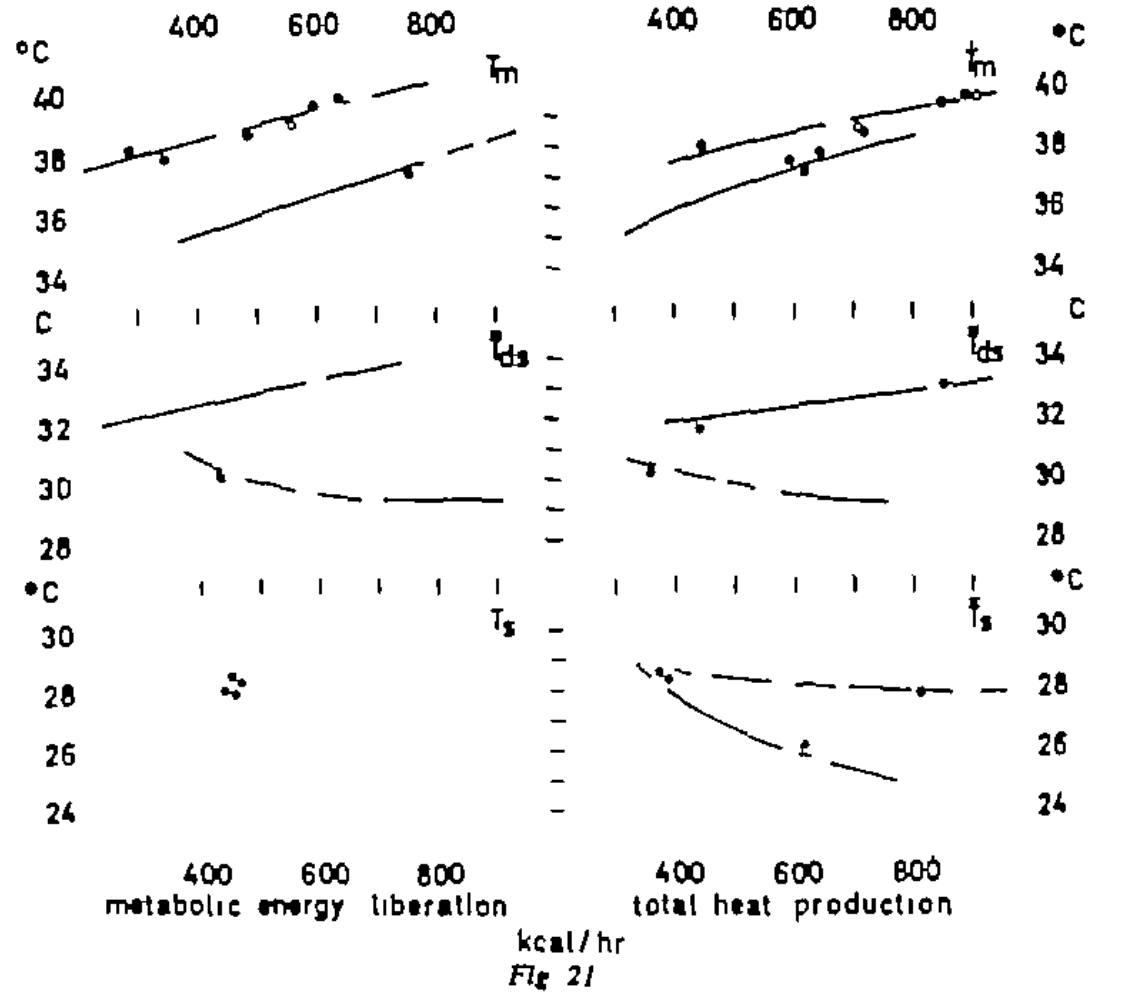


Fig 21

The temperature in vastus lateralis (T_m) average deep skin temperature (T_{ds}) and mean skin surface temperature (T_s) at different levels of metabolic energy liberation (left), and different levels of total heat production (right). Subj BI Walking. Positive work negative work ○ T 22.3

work Esophageal temperature is lower and muscle temperature higher in negative work (compared to positive work) In subject BI (Fig. 26) T and T_d as well are different for the same sweat rate in positive and negative work In all 3 subjects there is a positive correlation between sweat rate and the temperature differential across the skin with the values for positive and negative work scattering around the same line

Discussion

The procedure adopted in the present study was the same as that used in a recent study (B Nielsen 1966) of temperature and sweat regulation dur ing positive and negative work The main results from the earlier study were:

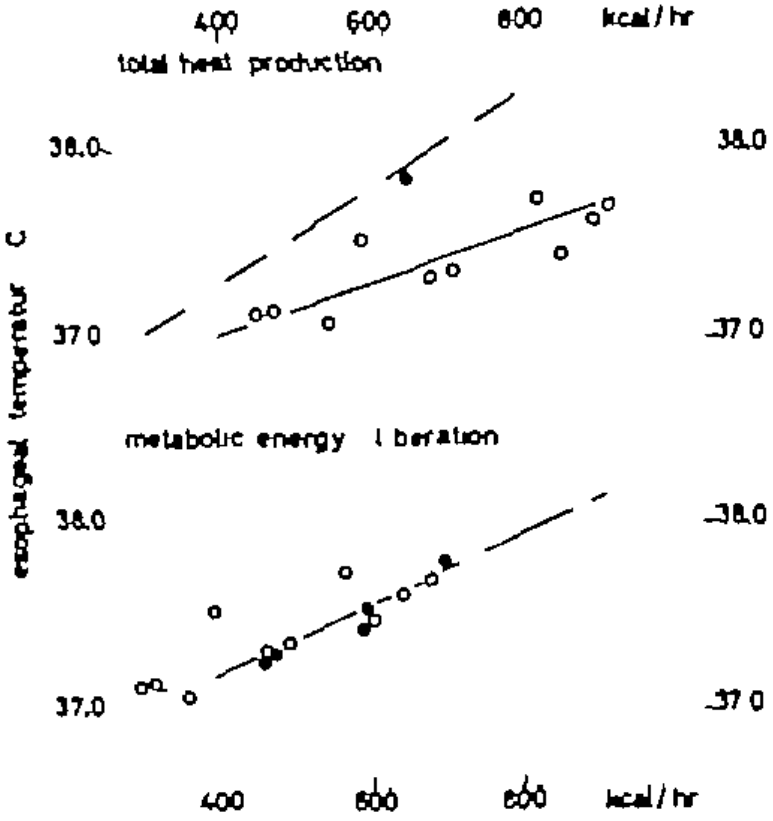


Fig 22

The correlation of esophageal temperature (T_{oe}) with total heat production (above) and with metabolic energy liberation (below). Subj. BI Walking. Positive work • negative work ○ T 22.3

1) that the level of internal body temperature is determined by the metabolic rate and 2) that the rate of sweat secretion varied in proportion to the total amount of heat liberated in the body in spite of differences in the level of internal body temperature. These findings were confirmed and will not be discussed at any length The experiments led to the question of how information on total heat production can be obtained by the temperature regulation centres. In the present study it was intended to measure temperatures in the deeper skin layers and in the muscles which might act as stimuli for thermal receptors at these sites. It must, however be remembered that the existence of such thermal receptors has not been histologically proven. (See chap. II)

One complicating factor in the present experiments is that, although the room temperature was the same in all experiments, the skin surface temper

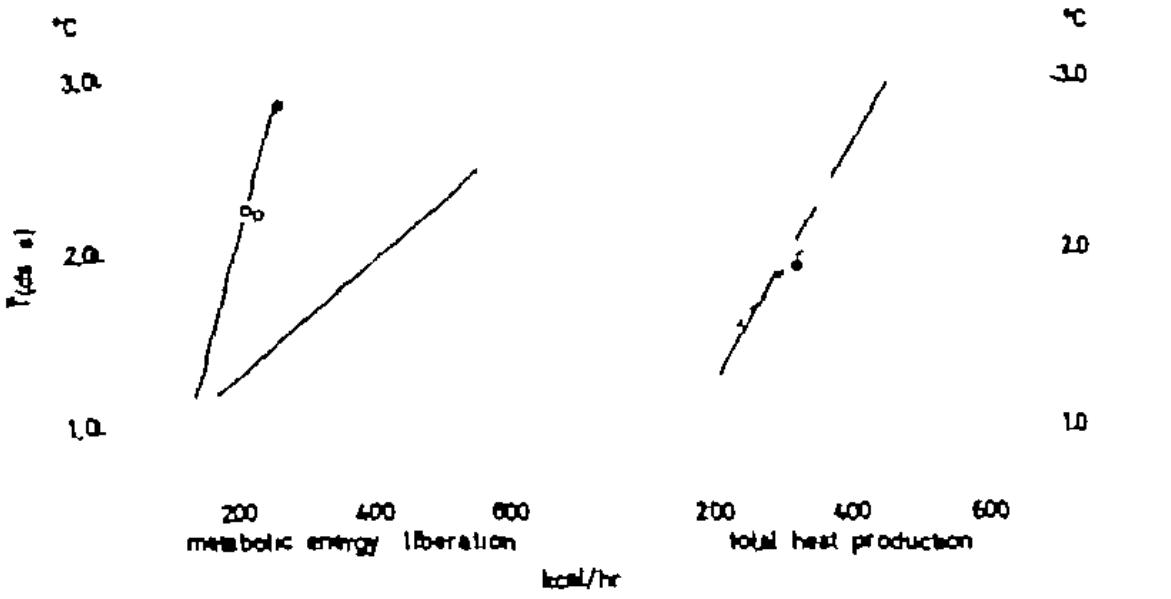


Fig 23

$T_{(d,\ a)}$ plotted against *metabolic energy liberation (left) and total heat production* (right). Subj. KHN Bicycling. Positive work • negative work ○ T 22.4

ature was not constant, but declined with increasing energy metabolism (Fig. 20 and 21) This must mean that, as the metabolic rate rises sweat production increases *more* than the heat transfer to the skin by the blood flow (cf. chap. III) Perhaps the *increasing demand for blood to the working muscles* with higher work intensities is met by a gradual reduction of the proportion of blood available for thermoregulatory purposes. Such a relationship of T to work load was not found in the experiments reported in chapter III and by B Nielsen and M Nielsen (1965 a) This discrepancy may exist because these earlier work experiments were performed in a sitting position working with the legs horizontal as opposed to the nearly vertical position when walking or bicycling on an ordinary bicycle as in the present series of experiments. In the sitting (legs horizontal) position the heart rate was found to be lower for the same work load, presumably due to less pooling of blood in the legs with a consequently better diastolic filling of the heart (M Nielsen and Fridrichsen 1939) Cardiac output ($\dot{Q}$) is less for the same oxygen uptake if blood is pooled in the legs (e.g. Asmussen and Christensen 1939 Bevegård, Holmgren and Jonsson 1960 Bevegård et al. 1966 Bevegård and Shephard 1967) This reduction in $\dot{Q}$ may cause a redistribution of the peripheral circulation favouring muscle blood flow with a concomittant reduction in skin-blood flow As seen in Fig. 20 and 21 the skin surface temperature (T_s) in these subjects decreases about 2° when the metabolic energy liberation is almost tripled. A similar although smaller decrease of $T_{d,}$ is also observed.

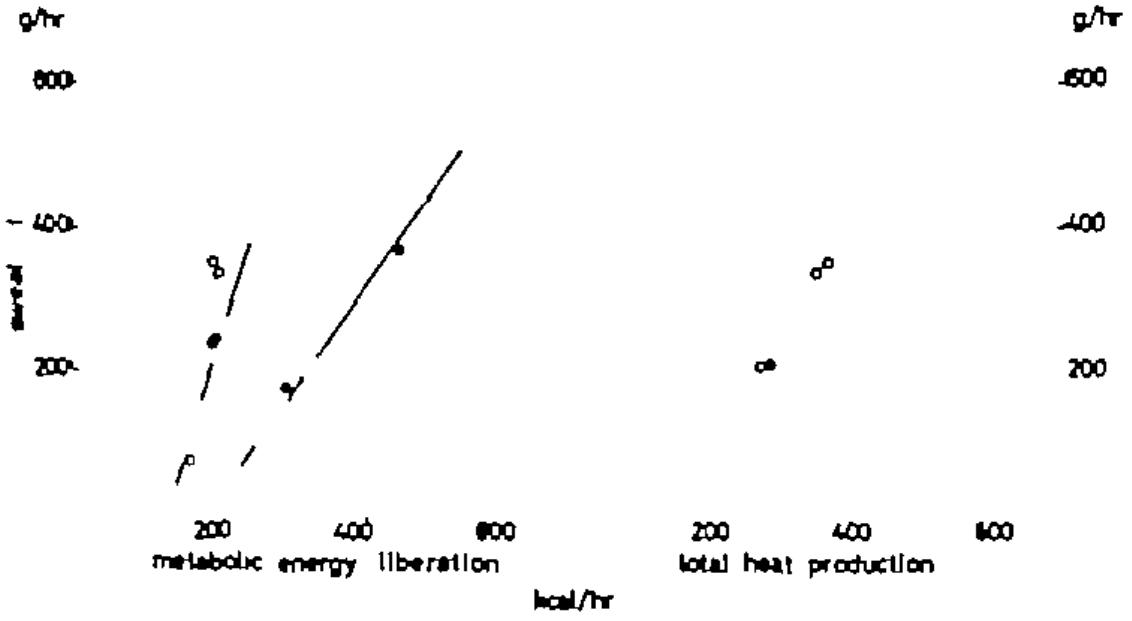


Fig 24

Sweat rate plotted against metabolic energy liberation (left) and total heat production (right). Subj. BJ Bicycling. Positive work • negative work ○ T 22.4

Values of T for positive and negative work, however fall on the same line. The small differences (1–2½°) in skin surface temperature between the positive and negative work experiments when compared at the same rate of *total heat production* (Fig. 20 and 21) are probably the reason for the slightly lower sweat rate at comparable heat productions in the downhill experiments (Fig. 24) The increments in (C + R) heat loss as calculated from $h_c \times (T - T_a)$ are apparently exactly compensated by a decrease in sweating

At the same rate of total heat production the deep skin temperature (T_{ds}) also is *higher* in the negative work experiments. Therefore, the correlation of sweat rate with total heat production Fig. 24 can hardly be explained as a result of stimulation of deep skin temperature receptors alone (Kerslake 1955 cf. p. 11 and 22)

The deep-to-surface temperature differential, $T_{(ds\ -\ s)}$, seems to be closely correlated with total heat production (Fig. 23) and is nearly the same in positive and negative work. As Fig. 25 and 26 show the sweat rate is positively correlated with $T_{(ds\ -\ s)}$ the values for positive and negative work scattering around the same line. In other words, $T_{(ds\ -\ s)}$ might be assumed to be an index of the stimulus to the sweating mechanisms. However this assumption conflicts with the results from chapter III which showed that an *increase* in $T_{(ds\ -\ s)}$ i. e. when the surface skin temperature decreased during exposures to colder environments, is accompanied by a *decrease in sweat rate*

The muscle temperature, which has been considered a factor in the sweat

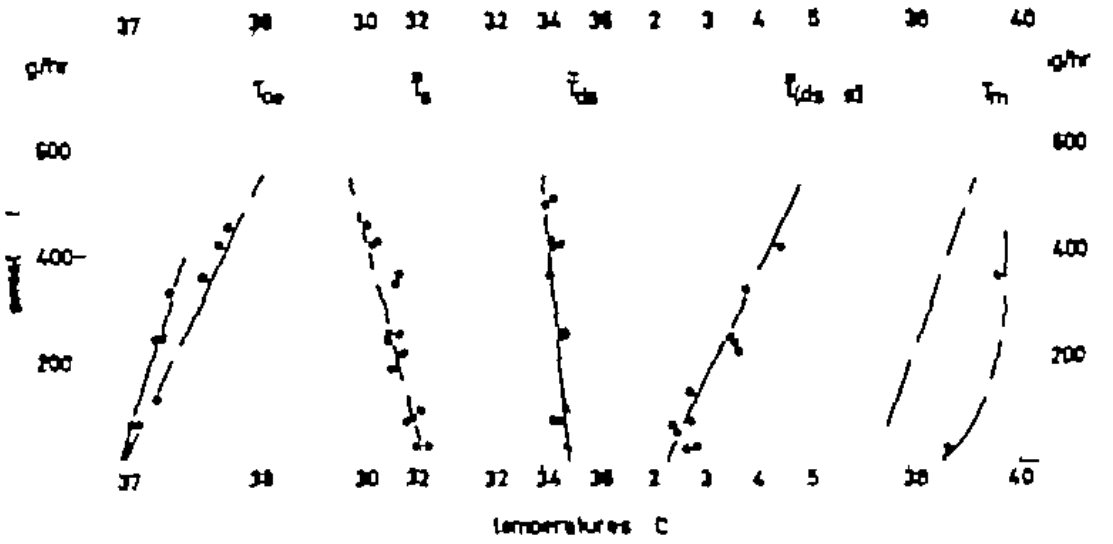


Fig 25

Sweat rate versus esophageal temperature (T_{oe}), mean skin surface temperature (T_s), average deep skin temperature (T_{ds}), $T_{(ds\ \ )}$ and the temperature in vastus lateralis (T_m). Subj BJ Bicycling. Positive work • negative work ○ T 22.4



Fig 26.

Sweat rate versus esophageal temperature (T_{oe}), mean skin surface temperature (T_s), average deep skin temperature (T_{ds}), $T_{(ds\ \ )}$ and the temperature in vastus lateralis (T_m). Subj. BL Walking. Positive work negative work ○ T 22.3

regulation by several authors (Robinson et al. 1965 Stolwijk and Hardy 1966 a, Saltin and Hermansen 1966 Saltin, Gagge and Stolwijk (1968)) was different for uphill and downhill work, compared at both the same rate of metabolic energy liberation and at the same total heat production (Fig. 20 and 21) The data confirms the findings of Saltin et al. (1968) that the local muscle temperature is the net result of the regional heat balance i. e. roughly the balance between the amount of heat liberated in the muscle and the heat transported by the blood flow However in the present experiments the rectal temperature (or T_{es}) and the muscle temperature do not differ by a constant value as in Saltin et al. s experiments. In negative work the muscle temperature was much higher than in positive work at identical core temperatures (i. e. metabolic rates, Fig. 22) cf. Fig. 20 and 21 It must therefore be concluded that muscle temperature cannot be the stimulus that correlates sweat rate with total heat production, since it differs for the same rate of total heat production in positive and negative work (Fig. 20 and 21) In the appendix p 59 a statistical treatment of the data is presented.

Chapter V

GENERAL DISCUSSION

The thermoregulatory system of the human body allows it to maintain thermal equilibrium within wide limits of external and internal heat loads. In thermal equilibrium of rest or exercise, the heat gains and heat losses are balanced by adjustments in skin circulation and sweat secretion. These adjustments are supposed to be monitored by central and peripheral drives. But it is not yet known where and how the information necessary for the regulated interplay of these drives are gathered.

Most investigators discuss the possibility of some interaction between two factors, core temperature and skin surface temperature, for the regulation of sweating and skin blood flow (e. g Robinson 1949 Belding and Hertig 1962, Cooper et al. 1964 Beamont and Bullard 1965 B Nielsen and M Nielsen 1965 a and b Wyndham 1965 Hardy and Stolwijk 1966 Stolwijk and Hardy 1966 b Wurster McCook and Randall 1966, Stolwijk, Saltin and Gagge 1968) The results of the experiments with positive and negative work (B Nielsen 1966 and chapter IV) however cannot be explained unless an additional factor correlated with the rate of heat production is also included. In the following paragraphs the regulation of the core temperature, the skin temperature, and the regulation of heat dissipation will be discussed separately

The core temperature. M. Nielsen demonstrated in 1938 that the increase in core temperature during work within wide limits is independent of the external conditions for heat loss and concluded that the increase is not due to a failure of the heat dissipating mechanisms but that it is a controlled rise determined by the metabolic rate. This finding has since been confirmed by Robinson (1949) Eichna et al. (1950) Wyndham et al. (1952) Lind (1963 a, b)

However the value of the rectal temperature as index of the regulated body temperature has been disputed (cf reviews by Grant 1951 and Hardy 1961) Mead and Bonmarito (1949) for instance, found that the rectal temperature measured simultaneously at different depths under various conditions of warming or cooling of the body showed considerable differences. In 1962 therefore, B Nielsen and M Nielsen studied the rectal temperature measured

in four depths and compared it with the temperature measured in the esophagus. It was found that during work only small and unsystematic differences existed at different depths in the rectum The temperature gradient along the esophagus was considerable, but measured just above the diaphragm it was uninfluenced by changes in external conditions. During legwork the deep esophageal temperature, T_{es}, was 0.2–0 4° lower than T_{re}, while during arm-work they were equal. It was therefore concluded that T_{re} is elevated due to heating of a large part of the pelvic cavity by venous blood returning from the working muscles. Benzinger (1959 1960) claimed that tympanic temperature, T_{ty} was the best index of core temperature, since it was thought to mirror the hypothalmic temperature. B Nielsen and M. Nielsen (1965 a) found T_{ty} to be 0 4–0 6° C lower than T_{re} and also lower than T_{es}. But all three core temperatures reached plateau values during work independent of the environmental conditions inside a wide range.

In other words during work the temperature of the whole body core increases to new levels, the height of which is a function of the work load. The studies of B Nielsen (1966) and chapter IV further show that the increase in core temperature is independent of the heat liberation connected with the working condition and is only correlated with the metabolic rate. This is a direct confirmation of the therory of M Nielsen (1938) that the increase in temperature during work is a regulated rise.

I. Astrand (1960) and Saltin and Hermansen (1966) demonstrated that the increase in body temperature is proportional, not to the absolute values of metabolic rate, but to the relative work load, i e. to the percentage of the individual's maximum aerobic capacity This finding also agrees with the results of Clasing and Laumann (1968) who in 20 trained and 20 untrained subjects working with the same heart rate of 130 beats per min obtained exactly the same rectal temperature, in spite of widely different work loads and metabolic rates.

How this regulation of the core temperature to higher plateau values during work is brought about is still unknown The mechanical events in the muscles do not seem to influence the level of body temperature attained during work, since variations in the speed of running or in the forces produced by the muscles, for instance in positive and negative work had no effect on the relationship of core temperature level to metabolic rate.

Irradiation of impulses from the motor cortex to the temperature regulating centre in the brain has been proposed as a possible "work factor" responsible for some of the functional changes occurring during work, e. g. to the respiratory centre (Krogh and Lindhard 1913 Asmussen et al 1943 1965) and to the sweat regulation centre (Robinson 1949 Beaumont

and Bullard 1963) However Kitzing and Bleichert (1965) using partial blocking of the neuromuscular system with curare during exercise, found no change in deep esophageal temperature with this procedure, which is presumed to increase the cortical activity together with the subjective feeling of strain Also, when work performed with a small muscle group the arms, was compared to leg work, the esophageal temperature level was found to be the same function of the metabolic energy liberation in spite of the very different neuromuscular conditions that must exist in the two kinds of exercise (B Nielsen 1968)

Since direct cortical or mechanoreceptor impulses do not seem to be important work factors regulating the deep temperature in exercise, it may be thought that chemical factors are involved in the controlled increase in body temperature during work It is generally accepted that the increase in temperature which occurs during a fever is produced by the action of chemical compounds, "pyrogens" These substances have recently been shown to act on quite well defined areas in the anterior hypothalamus (Cooper et al. 1967) the same areas as those involved in thermoregulation. Cabanac, Stolwijk and Hardy (1968) showed that pyrogens inhibit the activity in "warm" neurons and increase that in "cold" neurons in the rabbit's brain stem. In 1964 Feldberg and Myers suggested that thermoregulation is mediated through the release of certain amines, serotonin and noradrenaline in the hypothalamus. These amines, which are found in high concentrations in the hypothalamic area, are perhaps released continuously and the body temperature may thus be determined by the balance between these substances. They further proposed that the actions of pyrogens and antipyretics may be explained as an interference with this system Since this "chemical" theory was forwarded, a large number of studies of the action of these amines in different species have been published (Cooper et al 1965 Bligh 1966 a, Feldberg, Hellon and Myers 1966 Andersen Jobin and Olsson 1966 Cunningham, Stolwijk, Murakami and Hardy 1967 Findlay and Robertshaw 1967 Banerjee et al 1968) showing rather divergent results, interspecific differences, and reverse effects of larger and smaller doses of the same substances (cf the reviews of Cooper 1966 and Bligh 1966 b) Myers and Sharpe s (1968) recent experiments with rhesus monkeys, on the other hand, seem to show unambiguously that the natural occuring changes in the concentration of these substances can evoke thermoregulatory reactions Cooling a donor monkey and transfusing perfusate from the anterior hypothalamus to a corresponding site of a recipient monkey produced fever in the latter Conversely heating the donor produced a temperature fall in the recipient.

It still remains a question whether these experiments only show that the

transmittor substances from terminals of the neurons involved in thermoregulation are effective when placed in the appropriate area, or whether they give evidence for the "chemical" temperature regulation theory i. e. that the concentration of these substances in the hypothalamus actually determines the body temperature. If Feldberg and Myers (1964) are correct it is possible that the temperature elevation during work also can be effected through the interference of some chemical agent, liberated in proportion to the aerobic processes in the muscles. Interacting with serotonin and noradrenalin or interfering with their release in the hypothalamic area, this agent would have an action similar to that ascribed to the pyrogens in fever This hypothetical agent is not simply a pyrogen because acetylsalisylates have no effect on the "work fever" (Downey and Darling 1962) The combined effects of work and fever on body temperature seem to be additive (Fox and Macpherson 1954 Macpherson 1959 Grimby 1962)

How the plateau values of core temperature are related to the individual s maximum aerobic capacity (I Astrand 1960 Saltin et al. 1966) also remains unclear It may be that these phenomena are related to circulatory functions. For instance the total blood volume, maximum stroke volume, and maximum cardiac output shows a high positive correlation to the individual's maximum aerobic capacity (P -O Astrand 1952 Astrand et al 1964 Holmgren and Astrand 1966) and all of these change with training. A greater core-to-surface temperature differential, as that found when the same work is performed by an untrained subject, will increase the conductive heat transfer to the environment and consequently decrease the demand for skin blood flow thus leaving more blood for the working muscles. When on the other hand, submaximal work is performed in a hot environment, the increased demand for skin blood flow causes a reduction in muscle blood flow so that the work becomes more anaerobic (Williams et al. 1962, Klausen et al. 1967) These problems need further investigation including a systematic study of core and skin temperatures, cardiac output, heart rate, and peripheral blood flow and their relation to physical performance capacity

There has been speculations upon the reasons for the regulated elevation of core temperature Since the demonstration (M Nielsen 1938) that the rise is not due to a failure of the heat dissipating mechanisms, it has been suggested, that the increment in body temperature might serve to increase the body's capacity for work. Asmussen and Bøje (1945) demonstrated that the ability to perform a short maximal work was improved considerably when the core temperature was elevated, either by "warming up" exercise or by passive heating.

The increase in temperature may also serve other purposes, Hammel

(1968) for instance, assumed that the increase was necessary for activating the thermal regulator because the inputs from the peripheral receptors alone would be insufficient. The same view was held by Bradbury Fox, Goldsmith and Hampton (1964)

The discussed regulated plateau values of core temperature during work are independent of the environmental conditions as long as thermal equilibrium with the environment can be maintained. When the environmental stress becomes greater than what can be compensated for by the heat dissipation mechanisms, the core temperature increases (Robinson 1949 Lind 1962, 1963 a, b, Leithead and Lind 1964 Wyndham et al. 1966) The range of the temperature independent zone varies with the subject s state of training and acclimatization.

Conversely in extremely cold environments the core temperature may decrease during work It has been found that the metabolic rate remains almost unchanged during light work in a cold environment, even when the core temperature decreases (Kitzing 1966 Kitzing, Kutta and Bleichert 1966 1968) It would seem that shivering in the non working muscles is insufficient to increase the metabolic rate enough to maintain the normal work temperature.

The skin surface temperature. The temperature of the different areas of the body surface in a warm environment is relatively uniform while in colder environments the more distal parts have lower surface temperatures than the proximal areas, such as the trunk and the head. (Hardy and DuBois 1938 b Stolwijk Saltin and Gagge 1968) The average surface temperature i.e. the single measurements weighted according to the area they represent (Hardy and DuBois 1938 a) shows a linear correlation with the ambient temperature. This relationship was independent of the metabolic rate, internal temperature, and rate of sweating (Winslow and Gagge 1941 Robinson 1949 B Nielsen and M. Nielsen 1965 a, Snellen 1966, Stolwijk Saltin and Gagge 1968 Mitchell et al. 1968 chap III) In other types of work (cf chap IV) $\bar{T}$ decreases slightly with increasing metabolic rate. Therefore, heat exchange by convection and radiation to the environment is also nearly independent of metabolic rate, sweat rate, and core temperature, and dependent predominantly on the ambient temperature.

The skin temperature is the result of a balance between the heat *supplied* to the surface from the body core by conduction and by convection due to blood flow and the heat *lost* from the surface to the environment. For environmental conditions above the "zone of vasomotor regulation" (Hardy and Söderström 1938) and for the metabolic levels studied in the present investigation (chap III) there is a very close coupling of the changes in skin

blood flow and in sweat rate. This interrelation occurs in such a way that the amount of heat transported by the blood to the skin surface equals the amount of heat dissipated through the evaporation of sweat. In other words, the skin surface temperature at thermal equilibrium probably depends on the temperature fall across the skin due to the conductive heat transfer from the thermoregulated constant core to the environment through the thermal resist ance of the peripheral insulating tissues. In other types of work (bicycling with the legs down instead of horizontal) the above mentioned interrelation of sweating and skin blood flow is slightly altered. Perhaps the upright position causes a redistribution of the circulation favoring the working muscles at the expense of the skin blood flow The decrease in T with increasing metabolic rate (1/2° C per 100 kcal/hr) could then be explained

The control of heat dissipation The concept of a regulated deep body temperature implies as stressed by Hammel in his review (1968) that "the regulated temperature" is checked against some reference. When a difference from the desired level occurs, a response tending to reduce the deviation is elicited. This response would mean a change in heat dissipation, brought about through changes of skin blood flow and sweat rate. The sensory inputs necessary to elicit the response that controls this balance have been the subject of a great number of papers (conf. reviews by v Euler 1961 Hardy 1961 Bligh 1966 Hammel 1968) and a complete survey of the literature will not be attempted here.

As mentioned, most authors agree that there is an interaction between central and peripheral sensory inputs. (Robinson 1949 Belding and Hertig 1962, Robinson et al. 1965 Wyndham 1965 Hardy and Stolwijk 1966, Stolwijk and Hardy 1966 a and b, Hammel 1968) Benzinger (1959 1960) and Benzinger et al. (1963) maintain that only hypothalamic temperature (expressed by T_{hy}) is of importance for the regulation of heat dissipation, although a certain inhibition from peripheral receptors may interfere at lower skin temperatures. However in experiments with a wider range of work rates and environmental temperatures B Nielsen and M Nielsen (1965 a) were not able to confirm Benzinger's hypothesis.

The interaction between the peripheral and central drives is often described in terms of *control theory* (Hardy 1961 1965) with *proportional control* as the best model for the dominant part of the thermoregulatory responses.

The variables in the equations are usually 1) hypothalamic temperature assumed to be the regulated temperature (for the discussion as to which temperature is the regulated temperature, cf. Hammel 1968) which is compared to some sort of intrinsic reference, and 2) the average skin surface tempera-

ture, which is compared to a "set temperature" for the skin (Stolwijk and Hardy 1966 a) The proportionality constants and the combinations of the two terms vary In some models the core and skin terms are added, (Stolwijk and Hardy 1966 b, Stolwijk, Saltin and Gagge 1968) and in others they are multiplied by one another (Stolwijk and Hardy 1966 a, Hardy and Stolwijk 1966)

According to Hammel et al. (1963) Jackson and Hammel (1963) Hellstrøm and Hammel (1967) and Hammel (1968) the "set point" of the controlling system, defined as the hypothalamic temperature at response zero, may be changed by a variety of factors such as skin temperature, state of awakeness, exercise etc. Their conclusions result from observations that the actual hypothalamic temperature in dog and monkey is more or less unchanged between situations where panting or shivering are elicitied. The controlling system thus is assumed to operate by adjusting the set point temperature so that the error signal is achieved with minimal changes in the hypothalamic temperature. However Jackson and Hammel's (1963) work experiments may just as well be interpreted by postulating, that during work an extra stimulus is added to those already existing, since no point on their curves relating panting to hypothalamic temperature during work was found to fall to the left of the "set point" of the corresponding curve for the resting condition. Hammel's theory also fails to explain how and why the body temperature during work increases to the *same plateau value* independent of ambient temperature.

Hammel's concept of temperature regulation is an attempt to answer the most difficult problem in the other models of sweat regulation, i. e. how to combine the thermoregulatory responses for working and resting conditions. For all "comparable conditions" of rest and work, the sweat rate is always much higher during work (Robinson 1949 1962, Minard and Copman 1963) A hypothetical "work factor" has therefore been introduced in many control models in order to explain the regulation of sweating. This "work factor" may be thermal, e. g. muscle or venous temperature, (Robinson et al 1965 Stolwijk and Hardy 1966 a, Saltin, Gagge and Stolwijk 1968) or it may be non-thermal, e. g. a neuromuscular factor (Robinson 1949 Beaumont and Bullard 1963)

Experiments with passive heating through diathermia (B Nielsen and M Nielsen 1965 b) and treadmill experiments with positive and negative work (B. Nielsen 1966 and chapter IV) showed that the sweat rate and skin blood flow varies in proportion to the summated rate of heat produced and induced in the body For a given skin temperature the sweat rate and conductance varied proportionally to changes in the heat production in spite of

widely different levels of internal body temperatures (T_{re}, T_{es}) and neuromuscular conditions.

The possibility of thermal receptors, stimulated by the rate of heat flow in the deeper parts of the skin below a thermal resistance (as proposed by Bazett 1951 and Kerslake 1955) is evaluated in chapter III and IV of the present study. It seems that neither "average deep skin temperature" nor muscle temperature can be the sole stimulus to the sweating mechanisms. However one cannot exclude the possibility that the stimulus to the sweating mechanism may consist of an interaction of the inputs from the skin surface, deep skin receptors and muscle temperature receptors with a central temperature information (cf. appendix p. 59). Such additional effect of for instance muscle temperature is suggested by Robinson et al. (1965), Stolwijk and Hardy (1966 a), Saltin, Gagge and Stolwijk (1968). Perhaps the only way of evaluating the summated thermal inputs to the thermoregulatory centre is to measure the changes in average body temperature calorimetrically as proposed by Snellen (1966).

Since the heat balance equation provides the best prediction of the sweat rate in stable conditions of both rest and work, a simple thermostatic control could be responsible for the sweat rate in these conditions. It may justly be presumed that, like the rectal, esophageal and tympanic temperatures, the temperature of the hypothalamic centres for thermoregulation also changes to new levels in proportion to the oxygen uptake. It may then be assumed that in stable conditions of rest or exercise the hypothalamic temperature, determined by the metabolic rate, is used as reference temperature for an on–off control of sweating. Any disturbance of the thermal equilibrium would eventually produce a change in temperature in the hypothalamus itself.

It can be argued that since no change in core temperature (T_{re}, T_{es} and T_{ty}) can be demonstrated during work at 5, 20 and 30° ambient temperature in man (B. Nielsen and M. Nielsen 1962, 1965 a) or during positive or negative work at the same metabolic energy liberation (B. Nielsen 1966 and chapter IV) such a control mechanism would need an even greater sensitivity to changes in temperature than that found in experiments with direct measurement and manipulations of hypothalamic temperatures (cf. v. Euler and Söderberg 1958, Jackson and Hammel 1963, v. Euler 1964, Hammel et al. 1963, Hellström and Hammel 1967). However the "gain" of the hypothalamic control system may be underestimated in such experiments. If there exist extra-hypothalamic receptors for deep body temperature (as demonstrated by for instance Hallwachs, Thauer and Usinger 1961, Hallwachs, Hupfer and Thauer 1961, Simon, Rautenberg and Thauer 1964, Thauer 1964, Simon, Rautenberg and Jessen 1965, Jessen et al. 1967, Jessen 1967

Bligh 1963 Brück and Wünneberg 1966) which can influence the hypothalamic centre, then, in experiments where the heat dissipation is increased by an artificial increase of the local hypothalamic temperature, the "deep thermoreceptors" elsewhere in the body would register a temperature fall and, through a negative feedback would tend to counteract the stimulus from the hypothalamic thermoreceptors (cf. Hammel 1968 p 692) Even if the hypothalamus is not as highly sensitive to its own temperature as presumed above, the small temperature fluctuations which must occur under natural conditions, due to slight deviations between heat production and heat dissipation, could provide the stimulus to the sweat regulation. Through the *dynamic* responses from hypothalamic and extracerebral thermal receptors in the core and perhaps also from the skin (Hensel and Boman 1960) the sweat rate might be adjusted, even though no deviation in deep temperature for a constant oxygen uptake can be detected. This theory implies, as mentioned above, an adjustment in core temperature corresponding to the oxygen uptake, which is not explained by the theory

The stimulus to the sweating mechanism in transient states appears to be at least partly non thermal. The onset and cessation of sweating when work is started and stopped seem to take place before any thermal changes have time to occur Beaumont and Bullard (1963) report that sweating is increased within 1.5 sec after the beginning of work Furthermore, the rate of increase in sweating at the start of work was found to be proportional to the work load. (Beaumont and Stier 1965)

The only temperatures that have a time constant at the onset of work of the same order of magnitude as sweating are the temperature in the working muscles (Robinson et al. 1965 Saltin, Gagge and Sto wijk 1968) and the temperature of the blood in the femoral vein draining the working muscles (Robinson et al. 1965)

Appendix

The "steady state" values of Sw T_{oe}, T_{re}, T_{dre}, $T_{(dt\ \ re)}$ and T_{re} from the experiments in chapter III and IV was tested in the following models:

1 $Sw = f(T_{oe}, T_{re})$
2 $Sw = f(T_{oe}, T_{dre})$
3 $Sw = f(T_{oe}, T_{(dt\ \ re)})$
4 $Sw = f(T_{oe}, T_{re})$
5 $Sw = f(T_{oe}, T_{re}, T_{dre}, T_{(dt\ \ re)}\ T_{re})$

Model 5 in Table I is the model which gives the best linear fit to the experimental values in the sense that it is chosen as to minimize the emperical realdual standard deviations SE_1

Assuming that the relationships are linear $Sw/100 = c + a_1(T_{oe} - 30) + a_2(T_{re} - 30) + a_3(T_{dre} - 30) + a_4T_{(dt\ \ re)} + a_5(T_{re} - 30) + \varepsilon$ (ε is the random fluctuation component) the estimates for the multiple regression parameters c, a_1, a_2 a_3, a_4 and a_5 for four subjects were computed. The values are presented in Table I

The statistical analyses show that for the values from the experiments in chapter III with 3 metabolic levels and various ambient temperatures the sweat rate can be satisfactory explained by model 1 the multiple correlation coefficient being 0 95 and SE_1 0 63 for TD and R = 0 95 and SE_1 = 0 81 for KHN Table II shows that the influence of the other measured temperature factors are insignificant; indicating that no relevant information on sweat rate is contained in these variables. Correspoodingly the best description, model 5 for the experiments in chapter III turns out to be equal to model 1 It is further confirmed that model 1 is insufficient in predicting the sweat rate for BI and BJ in the positive–negative work experiments, chapter IV (R = 0 84 and 0 72 and SE_1 0 83 and 1 69) Table II shows significant influence of at least one of the variables, T_{dre}, $T_{(dt\ \ re)}$ and T_{re}

Model 2 is almost equivalent to 1 in expressing the sweat rate for the experiments in chapter III and for subject KHN and BJ in chapter IV while for subject BI this model provides a better prediction.

The predictions of sweating from model 3 are not essentially superior to those obtained with model 2. All in all the experiments from chapter III are described better with model 2 and those from chapter IV better by model 3

Model 4 explains only 76 % of the variations in Sw in subj BJ while in BI T_{es} and T_m both play a significant role the two of them together having a R value of 0 85 and SE_1 1.28

As mentioned the fifth model is selected as the best in predicting Sw Table II shows that in the uphill–downhill experiments (chap. IV) at least one of the three variables T_{ds}, $T_{(ds\ )}$ or T_m has a significant influence on the description of the sweat rate, but for each of the 3 subjects a different variable is the most important (Table I model 5)

The conclusion to be drawn from the statistical analyses is that the changes in T_{es} and $\dot{T}$ (model 1) do not explain sufficiently the variations in sweat rate in all the studied conditions Extra information may be obtained by including T_{ds}, $T_{(ds\ )}$ and T_m No one of these last mentioned temperature factors can be singled out as being the most important, due to the individual variation. This finding may mean that the sweat rate is determined by the summated input from thermal receptors in all parts of the body and that the prediction of Sw is improved the more temperature factors we measure. Or perhaps, the information held in T_{ds}, $T_{(ds\ )}$ and T_m only reflects changes in some other factor e. g rate of heat production which is not included in the present analysis.

The computed multiple regression parameters c, a_1 a_2, a_3 , and a_5 and their standard deviations, SE, computed from the xperiments described in chap. III (p. 24) and chap. IV (p. 39) R = multiple correlation coefficient. SE_1 = standard deviation of the residuals from the estimated model, measured in (1/100) g/hr n = number of experiments. Four subjects: TD KHN BJ and BI.

	Subj.	Data from chap.	Constant c	SE	T_{ss} a_1	SE	T a_2	SE	T_d a_3	SE	$T_{(d_1)}$ a_4	SE	T_m a_5	SE	R	SE_1	n
Model 1	TD	III	−25.85	2.59	3 73	0.34	0.40	0.04							0.95	0.63	20
	KHN	III	−43.26	5.67	6.22	0.74	0.44	0.06							0.95	0.81	12
	KHN	III +IV	−35 43	5.99	5 11	0.79	0.33	0.09							0.83	1.28	22
	KHN	IV	−8.08	6.21	1.60	0.79	−1.02	0.33							0.93	0.70	10
	BJ	IV	−13.64	5 68	2.26	0.74	−0.59	0.30							0.84	0.83	21
	BI	IV	−47 05	13.66	7.20	1 93	0.61	0.43							0.72	1 69	21
Model 2	TD	III	−23.74	2.47	3.32	0.32			0.49	0 05					0.95	0.63	20
	KHN	III	−40.71	5.80	5 79	0.76			0.51	0.08					0.95	0.85	12
	KHN	III +IV	−34.68	6.22	4 94	0.81			0.35	0.11					0.82	1.33	22
	KHN	IV	−14.17	7 89	2.46	0 95			−0.57	0.39					0.87	0.94	10
	BJ	IV	−19.38	5.86	3.06	0.66			−0.21	0.43					0 79	0.95	19
	BI	*IV*	*−51.95*	*6.55*	7.52	0.86			0.61	0.18					0.93	0.93	14
Model 3	TD	III	−26.57	3.90	4.18	0.54					−1 00	0.17			0.89	0.91	20
	KHN	III	−47.21	8.86	7 13	1.21					−1.64	0.38			0.89	1 18	12
	KHN	III +IV	−34.58	6.24	5.33	0.87					−1.22	0.39			0.81	1.34	22
	KHN	IV	−17 46	5 40	2.18	0.85					1 91	0.89			0.90	0.83	10
	BJ	IV	−5.81	5 10	0.29	0.45					1 76	0.86			0.90	0.68	19
	BI	IV	−29 13	9.37	4 14	1.37					0.79	0.34			0 91	1 08	14
Model 4	BI	IV	1 2	9.97	−.77	0.72							0.34	0.32	0.76	0.97	15
	BI	IV	−4 18	2	3.64	1.06							0.97	0.23	0.85	1..8	18
Model 5	[illegible]	III	[illegible]	[illegible]	[illegible]	[illegible]	0.40	0.04	0		0		0		0.95	0.63	40
	[illegible]	III	[illegible]	[illegible]	[illegible]	[illegible]	0.44	0.06	0		0		0		0.95	0.81	12
	[illegible]	III + IV	[illegible]	[illegible]	[illegible]	[illegible]	0.33	0.09	0		0		0		0.83	1.28	22
	[illegible]	IV	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	0.19	0		1.68	0.44	0		0.98	0.40	10
	[illegible]	IV	[illegible]	[illegible]	[illegible]	[illegible]	−1 44	0..4	0		0		0.88	0.19	0.95	0.50	15
	[illegible]	IV	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	0.59	0.74	0.30	0		0		0.94	0.96	14

Table II

Test of hypothesis a = 0, rejected on the 5 % significant level if the computed F value exceeds the 95 % fractile in the F-distribution.

Model	Subj.	Data	Degrees of freedom numerator f_1	Degrees of freedom denominator f_2		95 % fractile in the F-distribution $F_{0.95}(f_1\ f_2)$		Computed F-value		
				T_d $T_{(ds\ )}$	T_m	T_{ds} $T_{(ds\ s)}$	T_m	T_{ds}	$T_{(ds\ s)}$	T_m
1	TD	III	1	16	–	4 49	–	0.74	0.99	–
	KHN	III	1	9	–	5 12	–	0.01	0.08	–
	KHN	III + IV	1	19	–	4.38	–	0.59	0.65	–
	KHN	IV	1	7	–	5.59	–	7.21	14.92	–
	BJ	IV	1	16	12	4.49	4 75	5 77	8.17	21 71
	BI	IV	1	10	14	4.96	4.60	6 16	4.82	12.94

Summary

Two theories for the regulation of sweating (Bazett 1951 and Kerslake 1955) are based on the idea that temperature receptors placed at a certain depth in the skin are stimulated by heat, both of *external* origin, i. e. environmental temperature and *internal* origin, i. e. the degree of muscular activity which is proportional to the heat production To evaluate these theories on an experimental basis, a method is worked out by which the temperature at different depths in the skin and subcutaneous tissues can be measured.—In *Chapter I* p. 13 a survey of the applied methods is presented.

In *Chapter II* (p 18) a series of experiments is described in which the temperature is measured at 4 depths between the skin surface and a depth corresponding to "1/2 skinfold thickness" (i. e. at approx. 1 2, 4 and 6 mm s depth) Experiments at various external temperatures in rest and during work showed that the temperature generally rose with increasing depth in the tissues. Differences in temperature between the surface and "1/2 skinfold thickness" of up to 8 were found. The differential across the skin was *larger* when the environmental temperature was *lower* and widened further with increasing heat production. It was therefore decided to measure an average of the highest possible temperatures in the studied tissue layers viz. "average deep skin temperature (T_{d_a}) measured at "1/2 skinfold thickness at upper arm, forearm, chest, abdomen, shoulder thigh and calf. Changes in T_{d_a} with different conditions of rest and work would be of interest for the discussion of the two above mentioned theories.

In *Chapter III* (p 24) therefore, the variations in T_{d_a} with different environmental temperatures are investigated. Results from experiments during rest and at 2 work intensities, 540 kpm/min and 900 kmp/min, are presented. At identical sweat rates in rest and work, the same average temperature could not be found at any depth superficial to muscle and bone. This means that *the sweat secretion cannot be regulated solely by the stimulation of thermal receptors in the deep layers of the skin*

The average skin surface temperature (T_s) was found to be determined by the environmental temperature, independent of the rate of sweating and heat production and identical for rest and work. From this finding it is concluded

that *the amount of heat transported to the skin surface by the skin blood flow must be exactly equal to the amount of heat which is given off by the evaporation of sweat* The heat lost by convection and radiation is transported to the skin by conduction through the peripheral tissues It is therefore assumed that the skin blood flow may be calculated from T_{d_s}, esophageal temperature (T_{oe}) and sweat heat loss (p 35) The calculated values agree with the variations in skin circulation expressed as "conductance"

In *Chapter IV* (p 39) T_{d_s} and the temperature in the thigh muscle, m. vastus lateralis, (T_m) are studied during bicycling and walking, or running on a treadmill By having the subject work uphill (positive work) or downhill (negative work) the proportion of metabolic energy liberation (M) to total heat production (H) can be varied For positive work we have H = M − external work (W) while for negative work H = M + W The possible correlations of sweat rate with various measured temperatures (T_{oe}, T_{re}, $T_{d_{re}}$, $T_{(d_s\ s)}$ and T_m) are discussed. The only parameter with which the sweat rate had the same positive correlation in both positive and negative work was $T_{(d_s\ s)}$ This temperature difference might therefore be assumed to be an index of the stimulus to the heat dissipation mechanisms. However this idea must be discarded in the light of the relationships between sweat rate, T_d and T_{oe} found in chapter III. The changes in muscle temperature with changes in total heat production are different for positive and negative work so *muscle temperature cannot be the stimulus that correlates sweat rate with heat production*

In *Chapter V* the general discussion (p. 50) deals with the presently available information on temperature regulation during muscular exercise. *The deep body temperature* reaches steady state values proportional to the metabolic rates, independently of the temperature of the environment within wide limits, and independently of the heat production in the body These temperature levels are not a function of the absolute values of metabolic rate (oxygen uptake, V_{o_2}) but are proportional to the relative demands on the organism (to % of V_{o_2} max) How this adjustment takes place is unknown. Various theories for the setting of body temperature, e. g. during fever are mentioned.

It may be concluded that *the average skin surface temperature* (p 54) is determined predominantly by the environmental temperature. $\bar{T}$ seems to be nearly independent of work intensity rate of heat production and core temperature in conditions where thermal equilibrium with the environment can be attained. This independence exists because the heat flow to the skin surface via skin blood flow is linked with the heat dissipation by sweat evaporation in some unknown way

Thirdly *the regulation of heat dissipation* is discussed (p 55) Within the temperature zone where thermal equilibrium can be reached, the heat loss by sweat evaporation in rest and work equals the total heat production less the heat loss due to convection and radiation in spite of differences in the levels of deep body temperature. Various theories for the interaction between deep and superficial temperatures in the regulation of sweating are mentioned. None of these theories seem to explain the observed correlation of sweat rate with heat production during steady state of rest and work It is suggested that *the sweat secretion in these stable conditions is regulated from the thermal balance* Any disturbance in thermal equilibrium would produce temperature changes somewhere in the body (hypothalamus, skin etc.) from where thermal receptors, perhaps through their strong "dynamic" responses, might feed information back to the thermoregulatory centre. Reflex adjustment of sweat rate would then reestablish the thermal equilibrium.

In the appendix (p. 59) a statistical treatment of the data is presented. Multiple correlation equations have been calculated for the correlations of the sweat secretion with the measured temperature factors.

Dansk Resumé

En række tidligere publicerede undersøgelser over reguleringen af legemstemperaturen og svedsekretionen under muskelarbejde havde som resultat, at temperaturreguleringscentret må kunne registrere den varmeproduktion, som finder sted i organismen. Svedsekretionen og blodgennemstrømningen i huden ændres proportionalt hermed, uafhængigt af den dybe legemstemperatur og uafhængigt af de mekaniske forhold i musklerne.

To teorier for reguleringen af svedsekretionen (Bazett 1951 og Kerslake 1955) bygger på den idé, at termoreceptorer beliggende i en vis dybde i huden skulle kunne stimuleres af varmepåvirkning dels udefra (ydertemperaturen) – og dels indefra (ved muskelarbejde) og derfor påvirkes proportionalt med varmeproduktionen. For at prøve disse teorier experimentelt, er der udarbejdet en teknik, der gør det muligt, med termoelementer at måle temperaturen i forskellige dybder i huden og underhuden. – *Første kapitel* (s. 13) giver en kortfattet oversigt over de anvendte metoder.

I *andet kapitel* (s. 18) beskrives en række forsøg, hvor der foretages temperaturmålinger i 4 dybder mellem hudoverfladen og en dybde svarende til $^1/_2$ hudfolds tykkelse (dvs. i ca. 1, 2, 4 og 6 mm's dybde). En række forsøg ved forskellig ydertemperatur dels i hvile, dels under muskelarbejde viste at temperaturen steg indefter i huden. Temperaturforskelle på op til 8° mellem overfladen og $^1/_2$ hudfolds dybden blev målt. Gradienten over huden blev *større* jo lavere ydertemperaturen var og desuden jo større varmeproduktionen var. Det konkluderes, at under hensyn til at man endnu ikke histologisk har kunnet påvise placeringen af termoreceptorerne i huden vil en undersøgelse af temperaturen målt så dybt som muligt i huden dvs. i $^1/_2$ hudfolds dybde kunne være af interesse til belysning af de ovenfor omtalte teorier for reguleringen af svedsekretionen. T_{d_m}, den gennemsnitlige d hudtemperatur bestemmes derfor som gennemsnittet af temperaturen i $^1/_2$ hudfols dybde på overarm, underarm, bryst, abdomen, skulder og lår.

I *tredie kapitel* (s. 24) er undersøgt, hvorledes T_{d_m} forholder sig ved forskellige ydertemperaturer dels under siddende hvile, dels ved to

tensiteter 540 kpm/min og 900 kpm/min Det konkluderes – at under forhold hvor svedsekretionen er ens – er der ingen steder i de væv der ligger mere perifert end muskler og knogler hvor man har samme T_{d_s} i hvile og under arbejde. Dvs. *svedsekretionen kan ikke styres alene udfra påvirkning af termoreceptorer i hudens dybere lag*

Den gennemsnitlige hud-overflade temperatur (T) fandtes, i overensstemmelse med tidligere undersøgelser at være bestemt af omgivelsernes temperatur og uafhængig af svedproduktionen, varmeproduktionen og af, om forsøgspersonen var i hvile eller arbejdede. Heraf kan sluttes, at *den varmemængde der med blodstrømmen transporteres til hudens overflade nøjagtigt svarer til den ved svedfordampning afgivne* mens den ved konvektion og stråling afgivne varmemængde transporteres fra det indre af kroppen til hud overfladen ved ledning gennem de perifere væv Det antages derfor at hudens blodstrøm kan beregnes udfra T_{d_s}, oesophagus temperaturen (T_{oe}) og svedfordampningen (s. 35) De beregnede værdier er overensstemmende med de variationer i blodstrøm, der kan beregnes som »conductance«

I *fjerde kapitel* (s. 39) undersøges T_{d_s} og desuden temperaturen i lårmusklen m. *vastus lateralis* (T_m) – under gang og løb og ved cykling – dels opad, (positivt arbejde) og dels nedad bakke (negativt arbejde) – på et løbebånd. Herved kan forholdet mellem energistofskiftet (M) og varmeproduktion (H) varieres. Ved positivt arbejde har man, at H = M – ydre mekanisk arbejde (W) og ved negativt arbejde, H = M + W Svedproduktionens mulige afhængighed af forskellige temperaturfaktorer (T_{oe}, T_m, T_{d_s}, $T_{(d_s\ oe)}$ og T_m) diskuteres. Kun til $T_{(d_s\ \ )}$ udviser svedproduktionen den samme positive afhængighed både for positivt og negativt arbejde. $T_{(d_s\ \ )}$ kunne derfor tænkes at være et udtryk for stimulus til varmeafgivelsescentret. Men det må afvises i betragtning af de relationer mellem svedproduktionen, $T_{(d_s\ \ )}$ og T_{oe}, som fandtes ved forsøgene i 3 kapitel. *Heller ikke muskeltemperaturens ændringer med variationer i varmeproduktionen kan accepteres som udtryk for det stimulus der tilpasser svedsekretionen efter varmeproduktionen.*

I *femte kapitel* den generelle diskussion (s. 50) opsummeres de til dato opnåede resultater for termoreguleringen under muskelarbejde. Det fastslås at *den dybe legemstemperatur* (s. 50) indstilles efter stofskifteintensiteten, ilt optagelsen i organismen, mens den inden for vide grænser er uafhængig af omgivelsernes temperatur og varmefrigørelsen i organismen Desuden er det temperaturniveau som organismen opnår under arbejde proportionalt, ikke med den absolutte iltoptagelse men med den relative belastning af organismen (% af den maximale aerobe kapacitet) Hvordan denne omstilling finder sted vides ikke. Forskellige teorier om indstillingen af legemstemperaturen, bl. a. under feber omtales.

Om *huditemperaturen* (s. 54) kan man konkludere, at den hovedsagelig er afhængig af omgivelsernes temperatur men er ved visse arbejdsformer uafhængig af arbejdsintensitet, varmeproduktion og den dybe legemstemperatur i de ydertemperatur intervaller hvor varmebalance kan opnås. Dette skyldes, at varmetransporten med blodet til hudoverfladen, på ukendt vis, er koblet meget nøje til varmeafgivelsen ved svedsekretionen Til sidst diskuteres *reguleringen af svedsekretionen* (s. 55) Indenfor reguleringsområdet har svedsekretionen både i hvile og under muskelarbejde en størrelse svarende til den totale varmeproduktion minus den ved konvektion og stråling afgivne varmemængde, trods forskelle i dyb legemstemperatur Forskellige teorier for regulering af svedsekretionen udfra samspil mellem temperaturreceptorer forskellige steder i kroppens indre og i huden omtales. Ingen af disse teorier synes at kunne forklare den fundne afhængighed mellem svedsekretion og varmeproduktion *Det foreslås at svedsekretionen under »stationære tilstande« i hvile og arbejde reguleres udfra varmebalancen i organismen* Hvis varmebalancen forstyrres, medfører det straks temperaturændringer i kroppen. Herved stimuleres termoreceptorer i hypothalamus eller andre steder i kroppens indre og evt. i huden Det meget kraftige »dynamiske response« på selv små temperaturændringer kan måske reflektorisk, via varmeafgivelsescentret, ændre svedsekretionen så varmebalancen genoprettes.

I appendix et (s. 59) præsenteres en statistisk behandling af talmaterialet. Der er beregnet multiple korrelations-ligninger for svedsekretionens afhængighed af de forskellige målte temperaturfaktorer

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In the following text these reports are referred to with the above letter code (ref. *A—E*).

Preface

This investigation was carried out in 1964—1968 at the Institute of Physiology University of Aarhus, and at the Institute of Medical Physiology B University of Copenhagen. I thank the members of these institutions, particularly the directors, Professor J C. Skou, M.D., D M Sc., and Professor Poul Kruhoffer M.D D.M.Sc., for ideal working conditions.

I am greatly indebted to: Professor Skou for rousing my interest in research and for basic instruction in scientific methods, Professor Kruhoffer for post-graduate training in physiology as well as for constructive help, inspiration and encouragement throughout the present investigations L. James Brown for revision of the English Drs E. Boye, A. B Craig Jr., N Juel Christensen, I Dahn, E. H. Lanphier, N A. Lassen, O I Nissen, N Nørrø, H. Rahn, K. Tonnesen, L. Vanggaard, H. Westling, and H. Wetterqvist for help with various problems Engineer Jacob Fredelund Nielsen, H Christiansen, E. Hansen and John Jonson for help with electronic and mechanical problems Mrs. Jytte Zeppelin, Miss Grete Nielsen, and Miss Hanne Ring for skilled technical assistance Mrs. Inge Jakobsen and my wife Eva, for never tiring secretarial assistance.

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Contents

PAGE

INTRODUCTION 7

PART I METHODS

CHAPTER

1 Methods used in respiratory physiology 10
a. Alveolar air sampling 10
b. Scholander's micro-analyzer 11
c. Electrodes 11
d. Gas volumes 12
e. Intrapulmonic pressure 14

2. Methods used in circulatory physiology 17

A. Electrocardiography 17

B Direct measurement of the blood pressure 19

C. Measurement of the cardiac output 21

D Plethysmographic measurement of flow 22
Historical comments 22
Plethysmographic methods employed 26
WATER PLETHYSMOGRAPHY 26
CALCULATION OF BLOOD FLOW 33
AIR PLETHYSMOGRAPHY 33
STRAIN GAUGE PLETHYSMOGRAPHY 36

E Auxiliary equipment 39

PART II. PROCEDURES RESULTS AND DISCUSSION

3 Breath-holding and decompression sickness 43
Critical N_2 tensions 49
N_2 saturation degree 51
Practical application 51
Conclusions 52

PAGE

4. Carbon dioxide retention and hypoxia as a cause of loss of consciousness and drowning 53
Composition of alveolar air 54
Net change of alveolar gases 57
Between and after dives 58
Breath-holding above water 59
Comparison between breath-holding in air and in water 61

5 Cardiac rhythm 63
Procedure 64
Results and discussion 66
Cause of apneic bradycardia and diving bradycardia 69
Cardiac arrhythmia and other ECG abnormalities 72
Sudden death 72

6. Cardiac output 73
Results 75
Discussion 78

7 Blood pressure and limb blood flow 83
Material and procedures 83
A) Preliminary experiments 83
B) Supplementary experiments 85
1 Control experiments 85
2. Standard experiments 86
3 Comparison 86
4. Blood flow and pressure 86
5 Muscle and skin vessels 87
6. Venoconstriction 88
Results and comments 88
Cause of the limb blood flow reduction 92
The efferent path of the reflex arc 94

SUMMARY AND CONCLUSIONS 101

RESUMÉ OG KONKLUSIONER 103

REFERENCES 105

SUBJECT INDEX 111

SYMBOLS 115

Introduction

While serving as a medical officer in the Navy in 1964 I was placed in charge of Danish crews undergoing submarine escape training in a tank (Fig 1). On my arrival at the training school, which is at the Norwegian Naval Base in Haakonsvern at Bergen, a Norwegian confrere told me about 4 recent, serious accidents that had occurred in the tank after routine breath-hold diving and sometimes after free escape from a chamber simulating a sunken submarine some of the divers had experienced nausea, dizziness, eructation, fatigue, pain and even paralysis. No satisfactory explanation could be offered for these complications, and to regard them as manifestations of decompression sickness would have been incompatible with the general consensus of opinion, according to which 'Decompression sickness is virtually impossible for the skin diver because he cannot submerge deep enough or remain long enough under water to take up a troublesome amount of nitrogen—unless he has access to a supply of air at depth" (Lanphier in Submarine Medicine Practice 1956—p 259). Neither could the complications be ascribed forthwith to so-called "diving bradycardia of man (*e.g.*, Scholander 1962, Wolf 1965), because it was not known whether such bradycardia can become severe enough to arrest cardiac activity and cause sudden death. After a personal interview with the men who had had these complications, I suspected that decompression sickness might after all be the culprit.

In the light of the considerations set forth above the present investigations were started in an attempt to elucidate the basis of:

loss of consciousness and drowning in association with breath-hold diving

the bradycardia observed among human breath-hold divers and

the alterations in limb blood flow cardiac output and blood pressure occurring during breath-holding under various experimental conditions.

BREATH HOLD DIVING IN A 20 M DEEP SUBMARINE ESCAPE TRAINING TANK

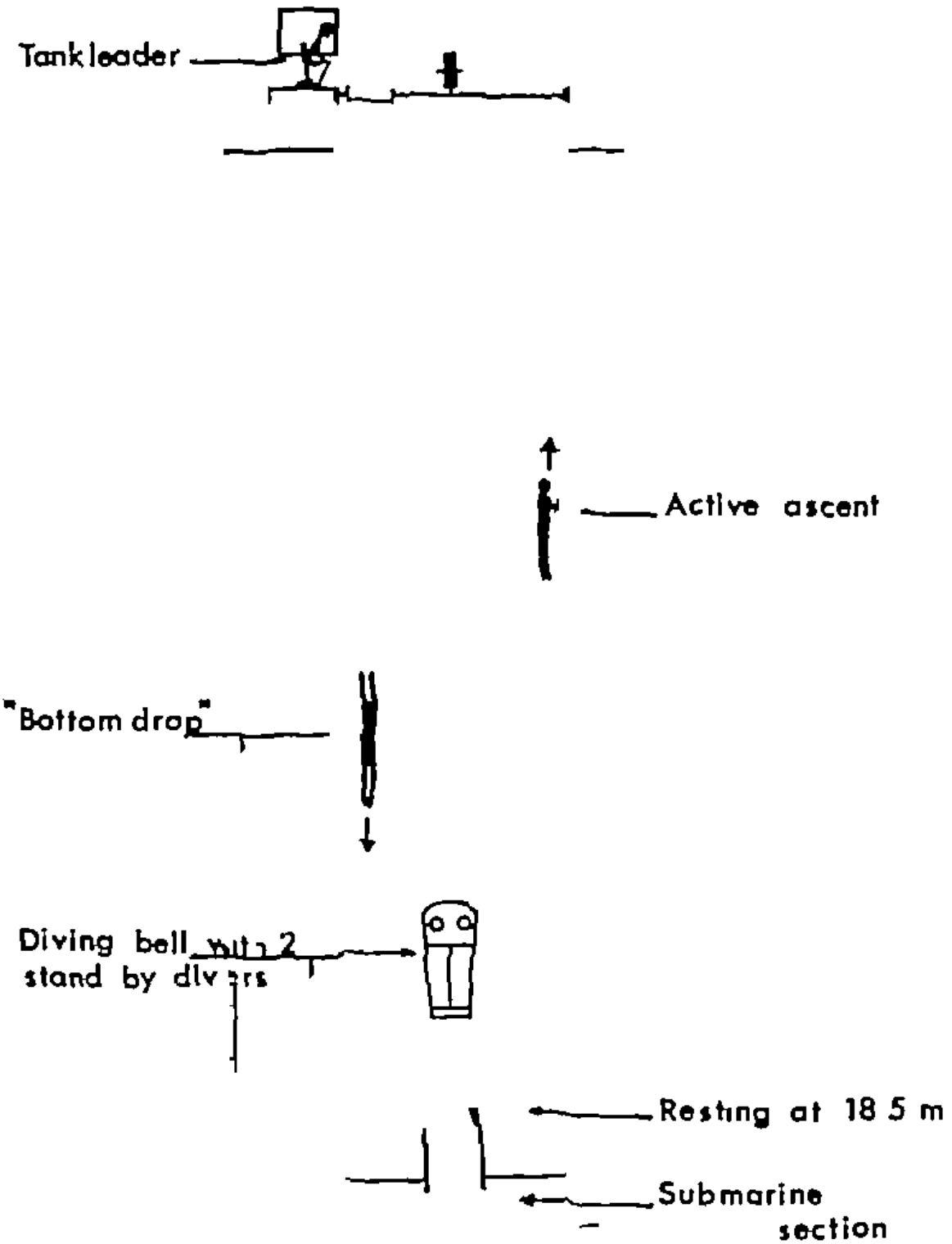


Fig. 1 Principal features of breath-hold diving in a submarine-escape training tank.

PART I

METHODS

CHAPTER 1

Methods used in respiratory physiology

2. ALVEOLAR AIR SAMPLING

Samples of expiratory air, after a "Haldane cough" were delivered to the apparatus shown in Fig 2 The apparatus consisted of a brass T-tube (22 mm I.d.) The left arm was fitted with a detachable mouthpiece the right, with a 1000 ml breathing bag (A). In the vertical part of the T was a rubber stopper, penetrated by a piece of rigid polyvinylchloride (PVC) tubing carrying a football bladder for the alveolar sample (B) The dead space of the apparatus was 43 ml.

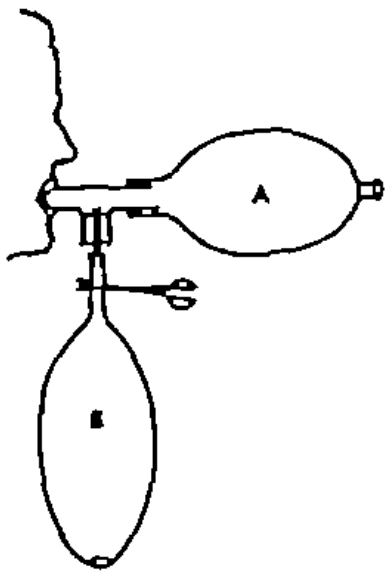


Fig 2. Equipment for obtaining samples of alveolar air A, breathing bag for dead space wash out B football bladder for collecting sample of expired air

When the subject expired into this apparatus, with or without the mouthpiece, the breathing bag (A) expanded and when it was full, the final part of the expired air approximately one liter filled B With this device it was also possible to obtain expiratory air samples during breath-hold diving in a training tank (Fig 1) During deep dives the total lung volume decreased roughly proportionally to the rise in ambient pressure, with consequent compression of the gases in the lungs. A breathing bag of only 500 ml instead of the 1000 ml was therefore used to wash out the dead space of the subject. The subject put the apparatus to his mouth while he was totally immersed in water the apparatus thus had practically no dead space. In such experiments it was not necessary to respire the contents of the bag A, because after delivery of the sample at the bottom of the tank, the subject swam immediately to a diving bell at approximately the same depth (Fig 1).

The samples of alveolar air were analyzed as soon as possible, and mostly within one hour. Analysis, at various intervals, of aliquots of a given air sample showed a fall of 1—3 mm in the carbon dioxide tension (P_{CO_2}) in one hour, but no alteration of the P_{O_2} level.

b SCHOLANDER'S MICRO-ANALYZER

The composition of the calibration gases was checked with a Scholander micro-analyzer. Duplicate analysis showed agreement within ± 0.02 % for O_2 and for CO_2. The micro-analyzer was also used for analysis of alveolar air samples and for samples from Douglas bags, when the number of samples to be analyzed was small. When the number was large, electrodes were used (see c.). The micro-analyzer was built by the author and differed somewhat from Scholander's (1947) The vernier of the micrometer had divisions of 0 001 mm, instead of only 0 01 mm. A Van Liew (1961) adapter was used, but the two cannulae were glued together so that the adapter consisted of a single unit and was therefore easier to handle.

c. ELECTRODES

Humidified samples were analyzed simultaneously in a polarographic Clark oxygen cell and a Severinghaus P_{CO_2} electrode. The two electrodes were thermostatically controlled (37°C) and connected to a pH meter with a gas monitor (Radiometer) Before each series of measurements two point (O_2 10 and 18 % CO_2 3 and 8 %, approximately) gas calibration was

repeated until stability was achieved. As a rule, only one sample was examined between two consecutive calibrations. Readings were made at exactly 60 (P_{O_2}) and 120 (P_{CO_2}) seconds after the introduction of the gas or the blood sample. The flow was kept at a fixed rate of one or two bubbles per second. The measuring precision was determined by treating the sets of calibration gas readings before (D_1) and after (D_2) the analysis of a given sample as duplicate samples. The SD was calculated from $\sqrt{(D_1-D_2)^2/2n}$. The mean difference between duplicate samples was 0.07 and 0.1 mm, SD $\pm$0.38 and 0.65 mm, n=56 and 62, for P_{O_2} and P_{CO_2}, respectively.

In preliminary experiments a medical gas analyzer (Beckman) with a CO_2 pick up and a physiological gas analyzer (Beckman) with a P_{O_2} electrode, were used. The values indicated by these meters were traced on a U.V. recorder (ABEM, type 'Ultralette'. Fig. 7).

d. GAS VOLUMES

Volumes of expiratory air were delivered to conventional Douglas bags and measured with two dry low resistance gas meters. Inspired and expired volumes were measured by having the test subjects breathe through an air-tight Ruben valve, directing the inspired air through one gas meter and the expired air through another. When 50 liters of air was sucked or blown through two gas meters the recordings differed by $\pm$50 ml at most ($\pm$1 %).—Also vital capacities (VC) were measured with the dry gas meters. The values found in a given subject were reproducible with an accuracy of $\pm$0.1 liter.—The residual volumes (RV) were calculated from the VC and the ratio between RV and VC given by Schaefer (1955). In one of the subjects the RV found with the helium dilution method and a Godart expirograph was 1.4 liters (STPD) which agreed well with the calculated value of 1.45 liters. In many respects the Godart expirograph resembles the light weight spirometer designed by Bernstein & Mendel (1953).

The air-tightness of various respiratory valves (von Döbeln, Ruben, Dräger werke Lübeck) was examined. The Dräger valve is generally used in connection with a self-contained underwater breathing apparatus. During the examination the inspiratory side of a valve was closed and connected to a transducer (Statham, type BB). Instead of being connected to a mouthpiece the valve was connected to a source of compressed air with an intermediate safety outlet. A constant flow of air passed through the valve and out at the expiratory side of the valve. In this system a significant pressure increase on the inspiratory side of the valve indicates a back flow *i.e.*, failure of the valve. The air passing through the valve was given

a statical pressure of 250 mm H_2O on the input side. In 6 von Döbeln valves, one Dräger valve, and two Ruben valves examined, a similar pressure developed at the inspiratory side and thereby indicated leakage. Five of the 6 Dräger valves examined showed no leakage. In another test of the air tightness of the 14 valves (Table 1) the expiratory side of the valve was sealed with a rubber stopper, penetrated by a tube connected to a water manometer and a transducer. The inspiratory side was immersed 1 cm in a water bath. A test subject expired into the valve with creation of the pressures shown in Table 1 before air bubbles escaped through the water bath. This procedure failed to produce any backflow in the 6 Dräger valves over the pressure range of zero to 400 mm H_2O (Table 1). These findings show that none of the types of valves examined are always tight, but that the Dräger valve is the most acceptable one. On the other hand, the dimensions of the Dräger valve—although better than the other two types—are suitable only for low and moderate ventilation, and the Dräger valve should be provided with a septum also in the cylindrical part in order to eliminate the dead space.

Commercially available (Wilhelmsen, Ltd., Aarhus) thin-walled, flexible plastic tubes (*i.d.* 25.4—31.8—38.1—44.5—50.8 mm), with an intramural PVC-spiral and a smooth internal surface, proved better than the conventional, antistatic rubber tubes used for anesthesia, when gas meters, valves, Douglas bags, and other units were to be connected.

Rubber Douglas bags are heavy and require the use of an expensive stopcock. A serious disadvantage of these bags is that if a partial pressure

Table 1 Tightness of respiratory valves.

Identification numbers of valves tested	Valves		
	von Döbeln	Ruben	Dräger
	70	40	—
	250	200	—
3	5		—
4	60		—
5	40		—
6	90		—

The above figures are mm H_2O pressure measured at the expiratory side of the valve. No leakage of the Dräger valves was demonstrated by this procedure (—). Only two Ruben valves were tested.

difference across the wall is allowed to operate for any appreciable time, gas molecules will diffuse through the wall. According to Carlowitch (1963) the permeability coefficient for gas molecules in certain polymers is 133×10^{-9} ml (STPD) $\times$ cm^{-2} area $\times$ mm^{-1} thickness $\times$ cm^{-1} Hg pressure difference $\times$ sec^{-1} at 298°K for CO_2 in rubber, a value 1330 times higher than that for CO_2 in polyethylene-terephtalate. The polyethylene compound is 50 times more permeable to CO_2 than polyvinylidene chloride, but the later cannot be used directly for bags because the two layers will adhere to one another. It was therefore decided to use polyethylene (PE), and suitable PE-bags were procured (Danish Plast Industry Haarlev) The bags were provided with a long neck so that they could be closed simply with a clamping screw and a stopcock could be avoided. Such bags were filled with air and lead plates were placed on them for days without any consequent measurable reduction in the volume of their contents. A rubber Douglas bag and a PE-bag of similar size and shape, were filled with similar portions of one and the same sample of expiratory air. Duplicate samples from each bag were then removed immediately and again two hours later The samples were analyzed in the micro-analyzer described above. The CO_2 concentration fell from 3 84 % to 3.46 % in the rubber Douglas bag (0 8 mm thick) and to 3 69 % in the PE-bag (the wall of which was 0.4 mm thick). The O_2 concentration increased from 16.43 % to 16.69 % in the rubber Douglas bag but was essentially unchanged in the PE-bag (16.43 %) —The PE-bags can be completely emptied by passing them through a mangle or simply by flattening them out. Talcum was not used to prevent adhesion of the walls of the bag.

e. INTRAPULMONIC PRESSURES

During breath-holding the intra-oral pressure was measured with a water manometer (Knowles et al. 1959). Both intra-oral pressure and, in water the pressure at the mid-axillary line were recorded via Statham transducers, on the U V recorder (Fig 7) The subjects were instructed to hold the glottis open during the breath-holds (usually 60 seconds) and to maintain a steady level of intraoral pressure by watching the water manometer or by voluntary relaxation of the respiratory muscles This procedure was used in most breath-holds in this study and the breath was usually held at the end of a deep inspiration (mean lung volume: 90 % of VC). The fact that the intra-oral (intra-pulmonic) pressure in such cases was often only slightly positive (0 to +5 mm Hg) indicates that the subjects were

apparently unable to relax completely Goggles enabled the subject to watch the manometer even during total immersion of the head when lying supine in water. Each subject has his own fairly characteristic pressure during relaxation.

The pressure measured at the mouth during breath-holding with the glottis open was taken as representative of that in the airways (Knowles et al. 1959) especially since the use of oesophageal balloons is inconvenient and of limited value (Mead & Milic Emili 1964).

CHAPTER 2

Methods used in circulatory physiology

A ELECTROCARDIOGRAPHY

In most of the experiments standard leads (one electrode on each limb) were used. These electrodes were used also when the limb was immersed in water. To insulate the electrodes when in the water a circular stainless steel plate was cemented into a piece of solid nylon and the insulated electrode lead was connected to the plate. The electrodes were protected from the water by placing them on the skin and holding them there by means of a rubber band. A shielded single conductor cable connected the subject with a recorder. In the preliminary experiments a three or six channel recorder (Elema-Schonander mingographs, types 51 or 81) was used. The simple device just described, could be placed on the limbs within 2 minutes, and the recordings obtained were just as good as those obtainable by more complicated systems, which required about one hour to secure the position of the electrodes before water immersion.

Electrocardiographs are equipped with a differential, high input impedance amplifier. When recording ECG with the electrodes underneath the water surface, the water provides an external shunt (with an assumed resistance of 500 ohms) between the sites of the body lying close to the electrodes. This shunt will reduce the voltage available for the electrocardiograph, creating artifacts and noise. These problems have been solved by using a recorder, with an input resistance much smaller than the water shunt resistance, and with a high current sensitivity namely the already available U V recorder (Type ABEM). For further details, see footnote.

FOOTNOTE:

The ECG potential differences are assumed to be produced by constant voltage equivalent generator with an electromotive force (E) equal to the voltage measured between two standard lead-off areas, with high input impedance recorder. The R wave in lead II, between the right arm and the left foot of a supine subject resting on land, was found to correspond to an electromotive force of approximately 1 mv (Fig. 3 A). If the subject is then immersed in water and still connected to the conventional ECG apparatus, water—being good conductor—will affect the potential difference prevailing between the lead-off areas (Fig. 3 B). Since R_{H_2O} is much smaller than 10 Kohms, the voltage available for the recorder device will be small fraction of E. The R wave will then be so small that it will be masked by noise, and it will not be possible to measure the heart rate. The ECG was therefore recorded with a mirror galvanometer with low input resistance (50 ohms) while the subject was immersed in water (Fig. 3 C). Since R_{H_2O} is

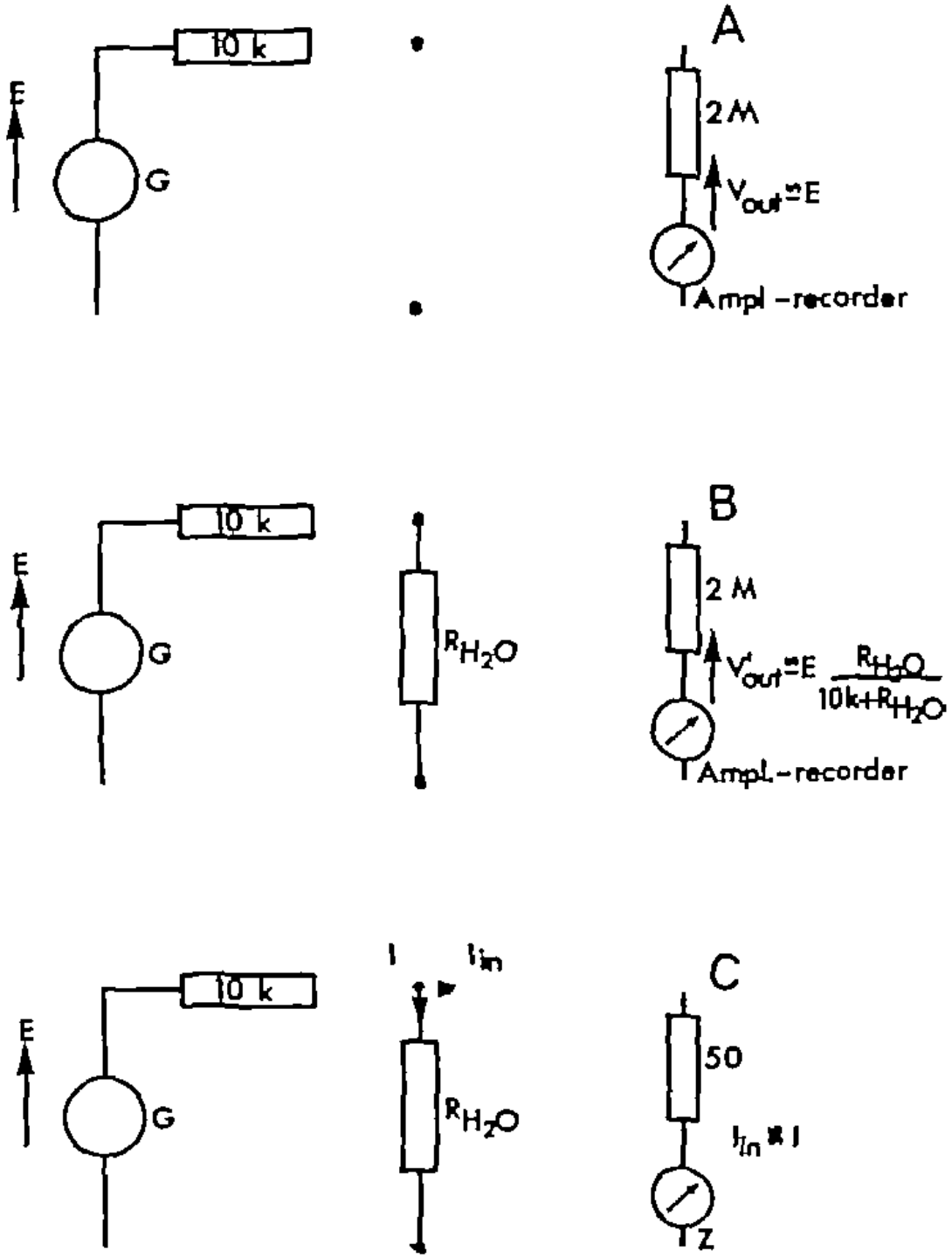


Fig. 3 ECG recording. The sum of the resistances of the two electrodes and the body

B DIRECT MEASUREMENT OF THE BLOOD PRESSURE

In the first part of the work Elema—Schonander pressure transducers (capacitance principle) were used together with mingograph type 81 During the final part of the work Statham transducers (strain gauge principle) were used with properly selected mirror galvanometers and the U V recorder. Usually the transducer model P 23 AA was used for arterial pressures, and P 23 BB for venous pressures. However when only a small volume displacement could be tolerated, the small model P 23 Dd was used for measuring arterial and central venous pressures. In the arrangement shown in Fig 4 the units are connected with sterilized tubes. Between the bottle of saline and the manometers was a so-called air tube a 60 cm long polyethylene tube with a 20 cm cannula. The transducer tube was 75 cm long, it had a 5 cm cannula, and Y-pieces varying in number with the number of transducers used at the same time, and it connects the bottle with the transducers. The blood pressure transducer was placed with its central axis in the same horizontal plane as the surface of the water in the manometer bottle with heparinized saline (Fig 4). The manometers were fastened to the wall one was a water manometer for calibration of the venous pressure transducers the other, a mercury manometer for calibrating arterial pressure transducers. They were connected to the bag of a sphygmomanometer in such a way that it was possible to create static pressures of 0, 5 10 20, 30 40, 50 100, 150, 200, and 250 mm Hg above the surface of the saline in the bottle, with only one air tube' Thus, only the bottle with sterilized saline the transducer tube' and the air tube' had to be

1 time as great as the internal resistance of the galvanometer, it is obvious that the major part of the current supplied from G will reach the recording device. If the ECG recordings are to be of conventional shape, current amplifier must be introduced between the electrodes and the critically damped galvanometer

is assumed to be 0 Kohms, G denotes a constant voltage equivalent generator I, the current from G I_{in}, the fraction of I flowing through the galvanometer; V_{out} and V'_{out}, the voltage available for the recording device All resistances are given in ohms.

A Conventional ECG apparatus with an internal resistance of Mohms, recording from a subject in air.

B Conventional ECG apparatus, recording from a subject lying up to neck in water (the resistance, R_{H_2O} is assumed to be 500 ohms).

C The (underdamped) galvanometer (Z type) system with low input resistance (50 ohms).

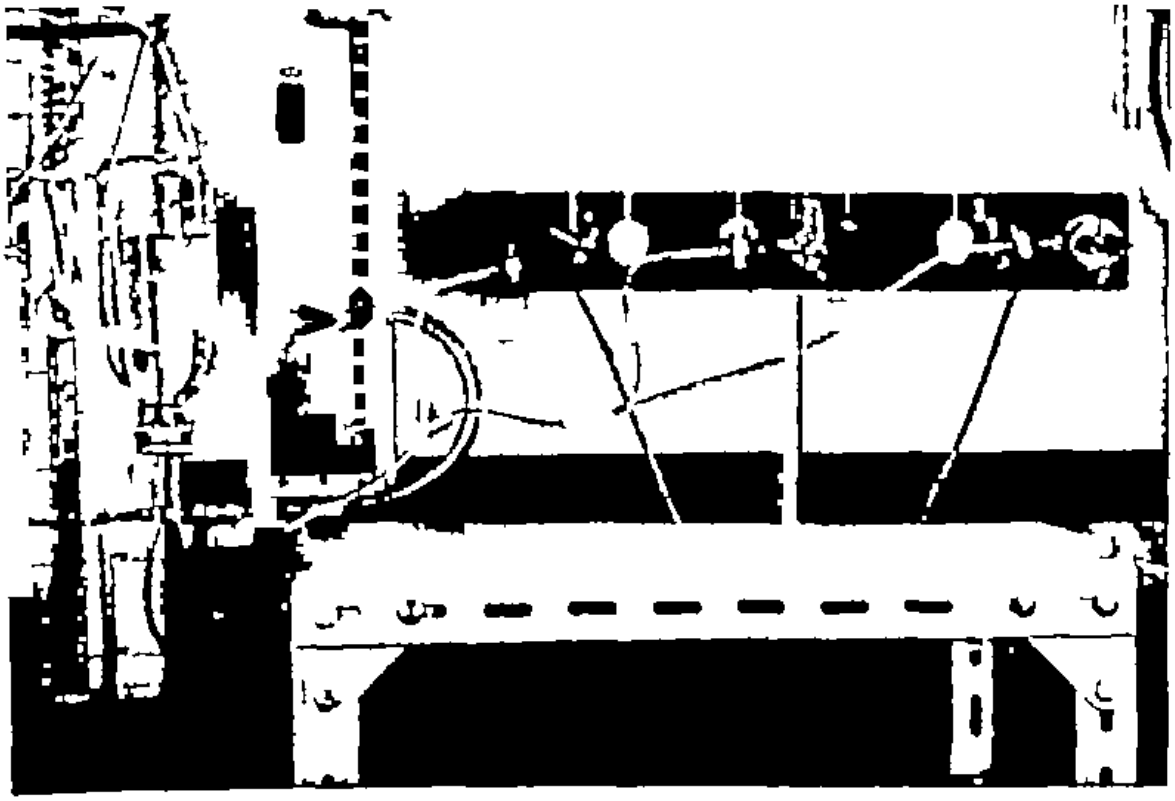


Fig. 4. Photograph of the pressure recording system.

changed from day to day (everything else remained unchanged) The transducers and stop-cocks were sterilized for 30 minutes in 60 % alcohol with 1 ‰ detergicide immediately before use, and flushed with sterilized saline. *Only heparinized saline was used (1250 I.U in 500 ml)* Polyethylene catheters (o d. 1 6 mm, i.d.. o 9 mm, length 6—50 cm) were placed in the brachial artery the basilic vein, and peripheral veins by the Seldinger technique (1953) "Intracat (Bardic) catheters were often used for recording the central and the peripheral venous pressure. Only Luer Lock fittings were used. A syringe was mounted on a stopcock on the transducer and up to 5 ml heparinized saline was used to flush the catheter in the course of the experiment (at most two hours)

Statham transducers of type P 23 BB and Dd with 6 cm long PE-catheters were examined regarding their response to a transient change in pressure. A "square wave generator" (Tybjærg Hansen 1949) was used. If the bore of the pist n and piston rod of the syringe is wide enough, this procedure is preferential to the other procedures described in the literature (*i.e.*, sucking with the mouth and after a short time, removing the tongue from the tube – exploding a condom etc.) With the "square wave generator' it

is possible to produce many pressure changes within a short time. The mirror of the galvanometer was placed at right angles to the direction in which the paper was fed. With the usual input voltage to the transducers Dd, a galvanometer deflection of 25 cm (30 cm broad paper) corresponded to 300 mm Hg. The examination was performed at the usual speed (12.15 cm/min) of the paper and at a speed of 245 cm/min. The transducer-catheter system responded so fast that no measurable time difference was found.

Tightness of the transducer-catheter system was checked with a pressure of 200 mm Hg immediately before recording of the biological pressure.

The volume displacement per unit of pressure increase (which is the reciprocal value of Tybjærg Hansen's (1949), "modulus of volume elasticity"), was determined with a calibrated capillary tube from August Krogh's latest microtonometer. According to the manufacturers, transducer Dd itself, has a volume displacement of 0.02×10^{-3} cm³ per 100 mm pressure increase (*i.e.*, a modulus of volume elasticity of 6.67×10^{9} dyn × cm^{-5}). This volume displacement is less than that of the optimal transducer studied by Tybjærg Hansen (thickness of membrane 35×10^{-4} cm, modulus of volume elasticity 3.4 to 4.5×10^{9} dyn × cm^{-5}, volume displacement 0.09 to 0.12×10^{-3} cm³/100 mm Hg). Our Dd transducer with the 6 cm catheter described above, had a volume displacement of 0.05×10^{-3} cm³/100 mm Hg (*i.e.*, a modulus of volume elasticity of 2.67×10^{9} dyn × cm^{-5}), which is thus very satisfactory.

In view of the above examination, and the fact that all the mirror galvanometers were critically damped, the peak systolic and the diastolic pressures recorded were presumably true measurements of those prevailing at the tip of the catheter. The "physiological reactance" (Fry 1960) is not dealt with here, because owing to the small volume displacement of the transducer-catheter system this factor must be of minimal importance compared with other pressure recording systems.

C. MEASUREMENT OF THE CARDIAC OUTPUT

With the use of the Seldinger technique (1953) under local anesthesia, a polyethylene catheter was introduced into the basilic vein, and the catheter tip was advanced to an intrathoracic position. Another catheter was introduced into the brachial artery. The indicator dilution technique used for determination of the cardiac output was essentially that described by Stewart—Henriques and Hamilton (Stewart 1897, Henriques 1913, Hamilton et al. 1948), but with a colourless and quickly eliminated indicator (bromsuphalein) in order to avoid possible discoloration of the skin by the frequent,

successive determinations. This indicator was first used for this purpose by Wassén (1956) who compared it with Evans Blue, and found no systematic difference between the cardiac output as measured with the two substances. In the present work Wassén's modification was employed, except for the injection technique described below All indicator dilution curves satisfied the extrapolation criteria of Dow (1956). At least 5 determinations were made to ascertain the slope of the descending limb of the curves. The $\dot{Q}$ was measured by the injection, through the catheter in the vein, of 3.0 ml of a bromsulphalein solution (50 mg/ml) from a cylinder mounted on a syringe with 20 ml physiological saline. The bromsulphalein and the saline was injected as rapidly as possible. This took 3 to 5 seconds, which implies that the 3 ml dye bolus was injected in 0 6—0 7 seconds. Blood samples were obtained from the arterial catheter every second for 30 successive seconds after the beginning of the injection. After the end of this period, arterial blood samples were obtained for gas and acid base analysis These samples were analyzed simultaneously in the above mentioned polarographic oxygen cell, in Severinghaus P_{CO_2} electrode and in a micro-electrode chain (Radiometer).

D PLETHYSMOGRAPHIC MEASUREMENT OF THE LIMB BLOOD FLOW

It might not be out of place to give a brief outline of previous studies in the field to facilitate understanding of the present investigation.

Historical comments

The first to use a plethysmograph was Francis Glisson (1677) who placed a musculous limb in a rigid walled chamber and utilized the principle that any swelling of the limb would displace the fluid from the rigid chamber to a system for volume measurements. In 1898 a Danish scientist measured spontaneous changes in volume of the forearm with a water filled plethysmograph (Lehmann 1898).

Venous occlusion plethysmography introduced by Brodie (1907), and modified by Hewlett & Van Zwaluwenburg (1909) provides a method for estimating the arterial inflow in organs and limbs. In recent years several improvements have been made. Lewis & Grant (1925) introduced the segmental plethysmograph during their famous work on reactive hyperæmia. Krogh, Landis & Turner (1932) described the pressure plethysmograph and important features of the plethysmographic method in general How important it is to keep the local temperature in the plethysmo-

graph constant is evident from Freeman's results (1935). On occlusion of the brachial veins the venous reservoirs in the hand fill quicker than those in the forearm, and blood passes from the distal hand veins into the forearm veins and thereby contributes to the increase in volume of the forearm (Grant & Pearson 1938). Arterial occlusion at the wrist was therefore introduced (Grant & Pearson 1938). Repeated resting blood inflow recordings over a period of half an hour were the same whether the circulation to the hand was continuously or only intermittently arrested (Grant & Pearson 1938). Kerslake (1949) produced evidence of a vasomotor reflex upon an arterial occlusion by a cuff around the wrist. This response was however short-lived and disappeared 90 seconds after the application of the occlusion (Hyman & Winsor 1961).

Greenfield & Patterson (1954) re-investigated the validity of the method of venous occlusion plethysmography. The apparent rate of blood inflow to the forearm (*i.e.*, the slope of the ascending plethysmographic line during the first seconds of venous occlusion) was found to be unchanged, even in the presence of a venous back-pressure sufficient to increase the resting forearm volume by anything up to 2 % (Greenfield & Patterson 1954). Since the increase in the volume of the forearm during ordinary venous occlusion plethysmography is less than 1 % there is no evidence for Gaskell & Burton's (1953) assumption that venous distention during the venous occlusion period causes a change in the apparent rate of blood flow.

Holling & Verel (1957) found a reduction in brachial arterial pressure and forearm blood flow when the arm was raised above heart level, the reduction in flow being greater than what would be expected if blood flow and the brachial arterial pressure varied linearly with one another. Some investigators measured the blood flow in a dependent limb (Gaskell & Burton 1953) or in one where the veins were distended for some other reason (Yamada & Burton 1954) but the validity of the method is questionable under these conditions (Greenfield 1960).

As far as forearm- and hand-plethysmography is concerned, a 45° abduction of the shoulder joint with a 130—150° flexion of the elbow joint was found to be the position inhibiting the arterial inflow to the smallest extent (Graf 1964 b).

Wallace (1958) found no significant difference between the apparent rate of blood flow to the forearm measured with a water pressure of 9—16 cm acting upon the forearm, and the flow obtained with a water head of 16—26 cm. According to Hyman et al. (1963) progressive increments in the pressure of an air plethysmographic cuff, decrease the total forearm blood flow this decrease probably occurring mainly in the intracutaneous

circulation against which the cuff exerts the greatest pressure. Graf & Rosell (1964 a) corroborated the above results and claimed that pressures less than 100 mm water do not measurably influence the arterial inflow rate. A decrease in forearm blood flow with increasing external pressure has also been found with the use of a water plethysmograph (Fewings & Whelan 1966).

Barcroft & Edholm (1943 1946) found that the water into which a forearm was to be immersed for plethysmography should preferably be 34 C, thus maintaining the local temperature of the skin practically equal to that of the adequately dressed forearm. The water plethysmograph acts as a thermostat on the skin and is generally maintained at 34 C in forearm plethysmography

Dohn (1956) has designed an air plethysmograph with several practical advantages. It consists of a 6 cm broad rubber cuff. Changes in the volume of the limb are assumed to cause proportional pressure changes in the air in the cuff, and the pressure changes are measured with a sensitive transducer. Though the compression of the air in the cuff in association with 10 seconds venous occlusion is not isothermic, the principle is acceptable in practice. But during an experiment the temperature within the rubber cuff slowly rises to reach a level between room and skin temperature. This rise is accompanied by a gradual increase in resting blood flow Thus comparison of blood flow values recorded with an air plethysmograph at different laboratories is warranted only if the local temperature beneath the cuff, the body temperature and environmental temperature are maintained at standardized levels, provided effects of such alterations are not the object of study

Graf (1964 a, b) carefully studied the accuracy of the venous occlusion method for blood flow measurements using Dohn plethysmographs. Graf (1964 a) mentions seven drawbacks of distal arterial occlusion during forearm blood flow measurements. Closure of the radial and ulnar arteries by the wrist cuff seems to produce such a change in the forearm vessels that it can hardly be regarded *a priori* as a natural system. As will later be apparent, the size of the apparent blood flow is not influenced by distal arterial occlusion. However prolonged arterial occlusion is unpleasant enough to warrant a search for another solution. Graf (1964 a) recommends simultaneous inflation of the distal occlusion and the proximal collecting cuff to the same venous occlusion pressure, but this produces a serious artefact due to displacement of tissue by the occlusion cuffs This makes it difficult to determine the initial slope. Therefore another procedure was developed (see later)

Venous occlusion plethysmography gives results in good agreement with those obtained by calorimetry (Cooper et al. 1949). Conrad & Green (1961) found that the plethysmograph and the electromagnetic flowmeter give corresponding values for a given flow

The results of venous occlusion plethysmography cannot of course be compared directly with those obtained by methods measuring a variable not linearly related to the overall blood flow *e.g.*, methods using thermal skin needles or disc oscillometry and accumulation or clearance of an indicator.

Plethysmography has been used not only for measurements of volume and blood flow but also in the assessment of capillary permeability (Krogh, Landis & Turner 1932) pulse wave velocity (Simonson & Nakagawa 1960) and venous tone and compliance (Wood et al. 1956)

Besides the above displacement plethysmographs, where a fluid (water or air) is displaced by the expanding tissue, also electronic plethysmographs have been used. Here only strain gauge plethysmography will be considered. —Several so-called "plethysmographs such as the "photo-plethysmograph" do not deserve the name since these devices do not measure the rate of volume increment or related variables at all (Hyman & Winsor 1961) —In 1953 Whitney introduced a new method for recording the relative change in girth of human limbs by means of a mercury in-rubber *strain-gauge*. According to this method, a narrow and very elastic rubber tube filled with mercury is wrapped round the limb and changes in its length are deduced from changes in the electric resistance. Assuming the limb to be a truncated cone and assuming equal reduction in thickness per unit length, the relative change in the limb girth will vary linearly with that in its volume.

Although venous occlusion plethysmography has survived major attacks through the years, and though it is still regarded as the best available method for measuring blood flow in an intact limb it is still questionable whether the venous occlusion is complete in all situations. The so-called blood flow may be the best approximation, but it is not necessarily the true blood flow especially not during dynamic muscular exercise, where the venous muscle pump can probably easily press out blood from under neath the venous occlusion cuff. However the resting blood flow as measured with a reasonably accurate venous occlusion plethysmograph, may not be far from the true blood flow

Other aspects of the history of plethysmography have been reviewed by Abrahamson (1944), Hyman & Winsor (1961), and Mune (1967).

Plethysmographic methods employed

Water plethysmography

In the course of the present work, the water plethysmographs were improved. The main improvements were 1) The use of a larger water surface and omission of the expansion chamber, 2) A minimal external water pressure on the limb segment, 3) Tailor made sleeves of extra thin rubber dam, 4) A stiff wide bore tube between the plethysmograph and the recorder

1 *Large water surface* The plexiglas plethysmograph consisted of a plethysmograph chamber proper, surrounded by a thermostatically controlled water jacket (Fig 5) The size of the water surface was an essential point in the construction of the chamber Dahn (1964) connected the plethysmograph chamber to an expansion chamber with a water surface area of 88 cm^2 and conclusively showed the advantage of the arrangement. The advantage is that a certain volume increase will only increase the height of the water column to a minimal extent (see 2). Therefore Dahn's principle was used in the present work, but the expansion chamber proved both unnecessary and undesirable. The water surface of the plethysmograph chambers of the present system was 140—200 cm^2 The transmission loss to the surroundings (*i.e.*, the difference between the true increase in limb volume and that recorded) was found minimal at all external water pressures used probably because the sleeves were made to measure and fitted well The transmission loss was determined as the difference (here mostly zero) between the recorder calibration with air and the water calibration (see later)

2 *Small external water pressure* The influence of different, external water pressures on the limb segment was studied. The blood flow was measured at 4 7 and 12 cm of water (measured from the axis of the limb segment). The upper part of the forearm segment was at the level of the sternal angle, and measurements showed no significant difference between the average resting forearm blood flow in this position, and in positions where the arm was lowered 3 cm or lifted 2 cm from this position. The position of the arm was as comfortable as possible (Graf 1964 b). The results are shown in Table 2. It is seen that the rate of relative volume increment was the same at 4 as at 7 cm of water whereas at 12 cm H_2O it decreased significantly Therefore, high water pressures, which have been used in numerous investigations on record, should be avoided. In the present system a water column of 4 or 7 cm was used throughout, and owing to the large water surface the rise in water pressure during venous occlusion was only 1—2 mm of water from the 4—7 cm level.

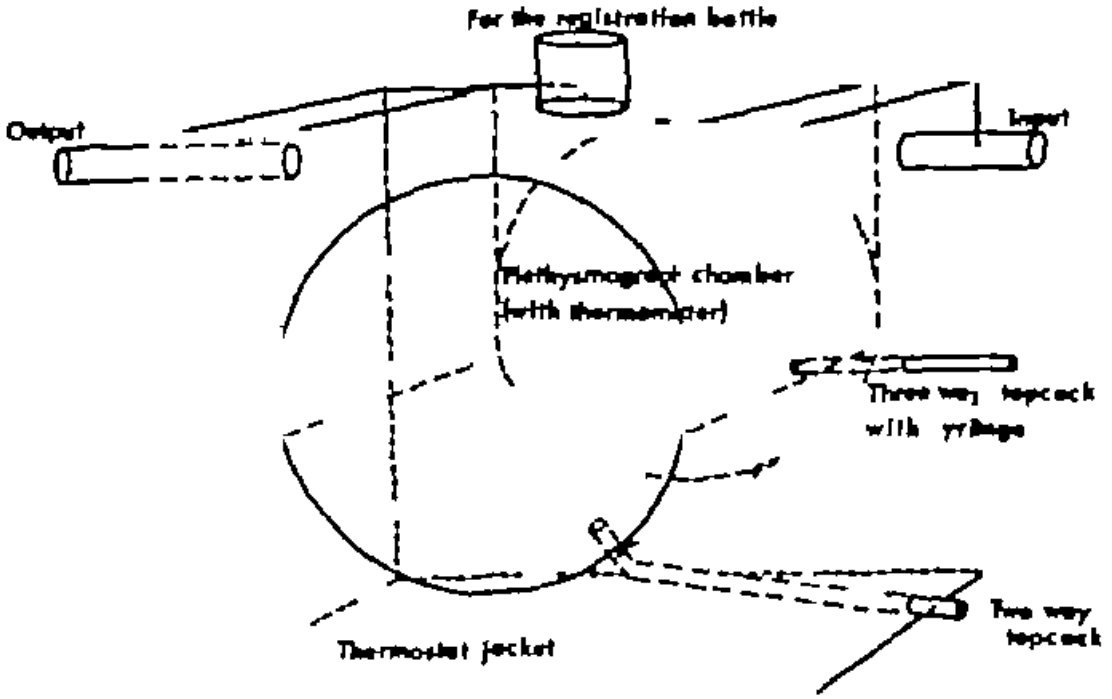


Fig 5 The plexiglas plethysmograph. Input—output for the water from the Heto thermostat to the thermostat jacket. The tube with the two-way stop cock is used to empty the chamber after an experiment. The three way stop cock with syringe is used for calibration. The registration bottle is connected to the plethysmograph chamber t the wide tube bove. The wide tube carries mark used when measuring the volume of the limb segment.

5 *Tailor made sleeves* The tailor made sleeve was cut out of extra thin hygienic dam (0.10—0.17 mm thick) and at each end it was fixed with latex glue to 4 mm thick, extra stiff rubber plates (Fig 6). The apertures

Table 1 Effect of the hydrostatic pressure.

Water pressure[1]	Percentage change in apparent blood flow[2]	Number of observations
4 cm	100 ± 2.4 %	32
7 cm	00 ± 1.6 %	34
cm	8 ± 3.2 %	33

The water pressure resting on the forearm segment is measured from the water surface to the mid axis of the forearm.

Mean values ± SE. The apparent resting blood flow was found essentially identical at 4 and 7 cm water pressure (this flow is called 100 %).

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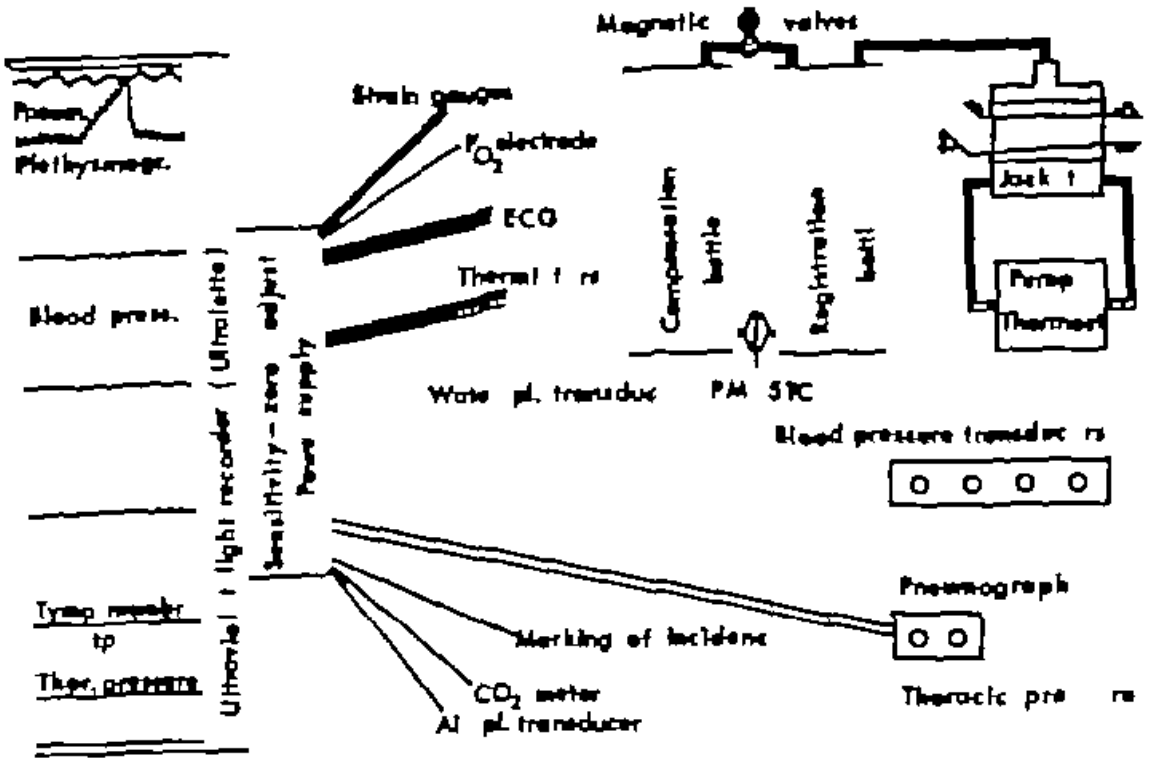


Fig 7 Block diagram of the main equipment used. Pressure equilibration between the bottles and the environment was performed with the magnetic valves.

4. *Wide bore tubes* The plethysmograph chamber communicates through a wide and rigid PVC tube (i.d., 8 mm e.d., 14 mm) with a 10 liter registration bottle (Fig 7). The wide bore tube allows a fast transmission of the response to the transducer membrane. The rate of ascent of a single cardiac oscillation recorded with this system was compared with the rate obtained with a perfectly rigid system, where the only elastic element was the membrane of a transducer (Statham, type BB) placed directly on the plexiglas plethysmograph. The PVC tube delayed the transmission only slightly and all the recordings of the duration of the ascending phase of a cardiac oscillation were less than one third of the lowest recordings of the "inclination time" obtained by Mune (1967) with a forefoot plethysmograph, in which narrow elastic rubber tubes were used

Measurement of volume The volume of the limb segment in the plethysmograph chamber was measured by filling the chamber up to a mark on the tube leading from the chamber, while the limb was still in the chamber The individual sleeve snugly fitted the segment of limb whose blood flow was to be measured. The water was led out and its volume was measured

accurately This volume was subtracted from the total water volume of the chamber measured several times without the extremity (the ends of the chamber were sealed with stiff rubber plates) A series of measurements in quick succession on the same arm, without change of position, gave the following values 535—535—525—530—533—538 ml, with a mean value of 533 ml. Thus the relative error of a single measurement of the volume was approximately ±1 to 2 % as compared with ±7 % when using the air plethysmograph (Dohn 1956).

The recording system The change of volume in a system consisting of a certain amount of gas molecules is measured with a transducer as the relative change in pressure, a principle which has been used for different purposes and for many years.

The differential form of the first law of thermodynamics can be applied to gases ($dQ=dU+PdV$) Here dU is the change in energy of the system, PdV is the energy exchange of the system in the form of work and dQ is the energy exchange in forms other than work (Fermi 1956). Slight compression or expansion implies only a small energy exchange in the form of work (PdV) and if the system is thermally insulated to prevent heat exchange with the surroundings ($dQ=0$) the internal energy of the gas will not change essentially according to the above equation. The compression or expansion of air molecules (air considered as an ideal gas) must thus be isothermal (*i.e.*, occurring at constant temperature) and reversible (*i.e.*, carried out at a hypothetically infinitesimally low rate) in order to satisfy Boyle-Mariotte's law of a hyperbolic relation between volume and pressure.

These requirements cannot, of course, be completely satisfied in practice, but the recording system illustrated in Fig 7 has proved to approach the ideal (Greenfield 1964) The two bottles and the transducer are enclosed in Rock Wool, in order to prevent thermal exchange with the surroundings ($dQ=0$) Excess water in the bottles increases the heat capacity of the system and thus further reduces the sensitivity to heat exchange with the environment In addition the bottles were filled with crumpled aluminum foil. This proved to have the advantageous effect of minimizing distortion of the calibration records observed. During recording the gas volume is reduced by 1—5 ml from 20,000 *i.e.* a 1/4 per mille pressure increase from the ambient pressure in the compensation bottle. The large size of the gas volume in the registration bottle makes the calibration practically independent of the volume of the limb segment and water in the plethysmograph chamber The use of the compensation bottle renders the system independent of fluctuations of barometric pressure, and largely

independent of fluctuations of temperature. Air temperature variations in the laboratory were minimal during application. The small pressure changes were measured with a Statham differential pressure transducer (PM 5TC pressure range ±7.8 mm Hg) connected to a sensitive mirror galvanometer recording on the U V recorder

The proximal occlusion cuff (6 cm wide) was inflated to pressures of 40 to 70 mm Hg, depending upon the individual (Munc 1967) Usually 50 to 60 mm Hg is optimal in normal subjects. The proximal cuff should be placed around the lowest part of the upper arm and as close as possible to the plethysmograph, but it is important that it is placed on the *upper* arm in order to close the veins as effectively as possible, as shown by Graf (1964 b)

The distal occlusion cuff (6 cm wide) was placed 2 cm distal to the lower border of the plethysmographs used in this study

The effect of variation of the pressure in the distal occlusion cuff was systematically studied with a water plethysmograph. The rate of relative volume increase by proximal, venous occlusion (60 mm Hg) was measured 1) without distal occlusion, 2) with continuous distal, arterial occlusion (270 mm Hg) for 10 to 20 minutes, 3) with intermittent, distal, venous occlusion (60 mm Hg) placed 2 seconds prior to inflation of the proximal cuff and kept until both cuffs were deflated (simultaneously). The results

Table 3 Different types of distal occlusion.

Procedure	Rate of relative volume increment FU	No. of observ
1) Without occlusion	7.6 ± 0.4	15
2) Arterial occlusion	4.9 ± 0.1	30
3) Intermittent, venous occlusion:	4.9 ± 0.2	0
3) Intermittent, venous occlusion	3 ± 0.06	15
4) Simultaneous distal and prox. occlusion.	3.1 ± 0.13	29

5) Constant distal venous occlusion (mean of 4 determinations the prox. occlusion period is indicated in sec, from the commencement of distal venous occlusion):

Sec	5—15	35—55	45—55	65—75	85—95	105—115
FU	2.5	2.9	3.6	3.9	4.1	3.9

The above figures are mean values in FU (*i.e.* ml blood per 100 ml forearm and min) ± SE. The order of the different procedures was changed in a random fashion. The figures of the upper and lower part of the Table are from two different subjects.

are given in the upper part of Table 3 In another subject procedure 3) was compared with procedures 4) 10 seconds of venous occlusion simultaneously by the proximal and the distal cuff (60 mm Hg), and 5) constant, distal venous occlusion (60 mm Hg) for 2 minutes. The results are given in the lower part of Table 3 In this study proximal venous occlusion was applied 3 times each minute, namely between 5 and 15 25 and 35 and 45 and 55 seconds of each minute, except in procedure 3), where the proximal cuff was inflated at the 7th 27th, and 47th second for 8 seconds Without wrist occlusion, the rate of the relative volume increment was high and was generally attributed to the increased rate of filling of the veins underneath the plethysmograph from the veins of the hand which normally have a large blood flow The agreement between the results obtained with procedures 2) and 3), and in the other subject, 3) and 4), indicates that the result does not vary with the type of occlusion. Thus, arterial wrist occlusion, introduced by Grant & Pearson (1938) and simultaneous application of distal and proximal venous pressure, recommended by Graf (1964 b), give corresponding values. We mostly used distal venous occlusion shortly before application of the proximal collecting pressure, as suggested by Professor Kruhoffer because it allows more precise determination of the initial slope (an artifact is avoided), and because procedure 2) may be unpleasant for the test subjects. Procedures 1) and 5) cannot, of course, be used for blood flow measurements, because the influence of the part distal to the segment plethysmograph is unknown and variable.

Pressure source Since compressed air from a central compressor system was available in the laboratory a simple inflatory–deflatory system was designed for securing venous-occlusion and venous-collecting pressure. All rubber tubes connected with the cuffs had an internal diameter of 9 mm allowing practically instantaneous inflation and deflation by the above system, consisting of a plastic bottle (15 liters) and a membrane manometer. The inflation and deflation was controlled by three magnetic valves timed to act automatically at an intermediate interval of 2 seconds

Calibration of the water plethysmograph The *recording system* was calibrated by injection of a known quantity of air with a well greased glass syringe into the system and measuring the galvanometric deflection.—In the daily routine work the *plethysmograph* was calibrated by injection of a known quantity of water into the water compartment (Fig 5) With the isothermic recording system described above, these procedures cause identical galvanometric deflections per ml volume reduction (*i.e.* no transmission loss—as mentioned previously). The deflection upon volume reduction corresponded to that upon volume increase, but occasionally slight hysteresis

was seen. The calibration was performed during breath-holding and with arterial occlusion by the proximal cuff.

If plethysmographs with thick sleeves are used, the equipment must, of course, be calibrated by injection of heparinized saline into the veins of the limb segment in the plethysmograph, (*i.e.*, 'limb calibration as recommended by Dahn 1964). We occasionally advanced a catheter from a hand vein into a vein located in the middle of the plethysmograph and in the axis of the limb and calibrated by injection of saline. *Limb calibration* is comparable to plethysmographic calibration in the water compartment, only if the sleeve fits well. During such calibrations the veins must be occluded at the upper and lower border of the plethysmograph during the injection.

With the above 3 procedures we found almost the same mean galvanometer deflection per unit of volume of air, of water and of heparinized saline (0.510—0.506—0.501 cm/ml, with 10 observations in each group)

Calculation of blood flow

During measurement of the flow the base line of the plethysmogram was essentially stable. Occasionally when base line drift was observed, no measurements were made until the cause, usually leakage, had been found and eliminated. The blood flow was defined as the tangent of the angle (v) between the base line and the initial slope during venous collection. The angle was measured with a protractor provided with a string (Dahn 1965). The blood flow was expressed in flow units (FU=ml blood/100 ml limb volume × min). The relevant constants (deflection on calibration, D cm/ml speed of recording paper S cm/min limb volume, V ml) were pooled to form a constant, C $100 \times S/D \times V$ as suggested by Dahn (1965), and $C \times \operatorname{tg} v$ gave the blood flow This method of calculation was used for air and water plethysmography

Air plethysmography

Dohn rubber plethysmographs and a transducer (Tybjerg Hansen 1949) with amplifier were kindly placed at our disposal by Dr. N A. Larsen, Department of Clinical Physiology Bispebjerg Hospital, Copenhagen. The Dohn plethysmogram was also recorded on the U V Recorder, with a suitable mirror galvanometer.

Recordings by the Dohn plethysmograph were compared with those by the water plethysmograph described above. Resting blood flow and flow at the end of 4 minutes arterial occlusion were recorded simultaneously

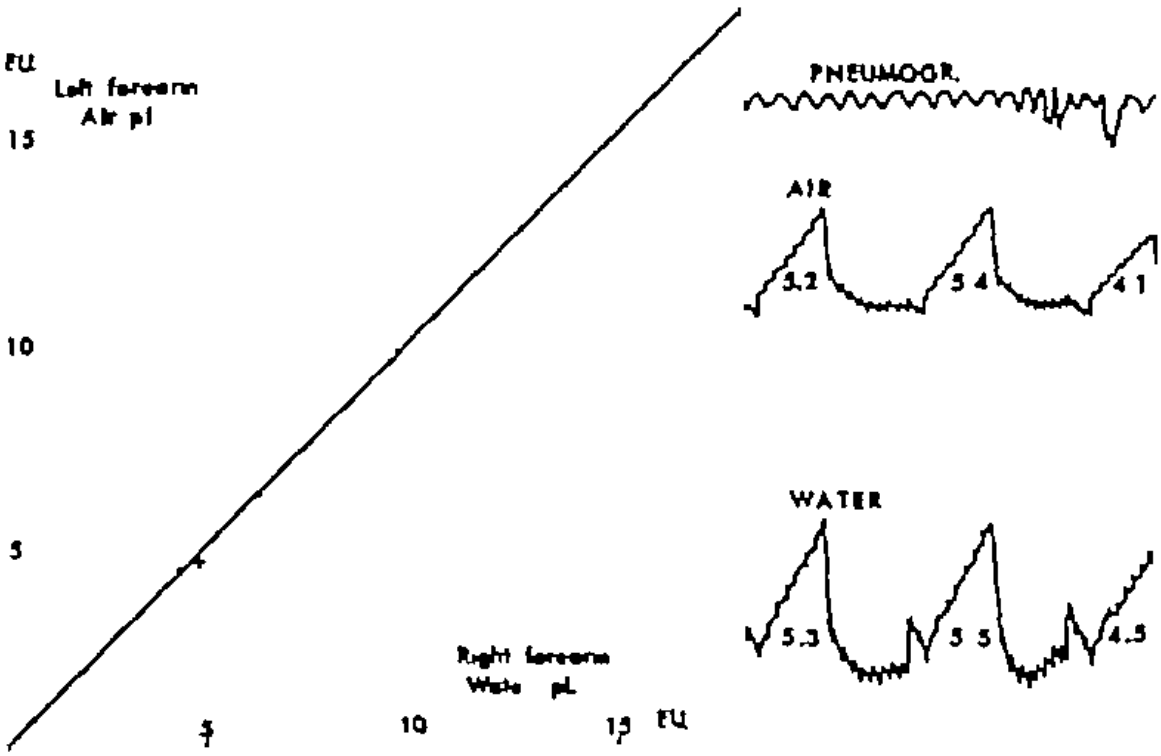


Fig 8 Comparison between air and water plethysmography The cross indicate the average (± SE) of the resting blood flow measured on 31 occasions. The points are paired individual figures. FU = ml blood/100 ml forearm × min.

on a normal subject with the water plethysmograph on the right forearm and the rubber plethysmograph on the left. A set of recordings is shown in Fig 8 The two methods gave essentially equal values at the same skin temperature, also when the mutual location of the two plethysmographs was changed to the opposite forearm. An agreement between two methods found in this way must be more difficult to attain, than by simultaneous measurements on the same arm, so the comparison between right forearm and left is considered fair. The low values (Fig 8) recorded with the Dohn plethysmograph, occurred simultaneously with an incidental temperature of 32 C underneath the Dohn plethysmograph, while the majority were measured at 33 to 34 C, as in the water plethysmograph.

In another normal subject the resting blood flow was recorded 30 times with the Dohn plethysmograph on the right arm, and thereafter 30 times on the left forearm. The mean value ±SE was found to be 3 6±0.04 and 3 7±0 03 FU respectively

Dohn (1916) found the error of a single measurement of the limb segment volume by the air displacement method to be ±7 %, and Graf (1964 b)

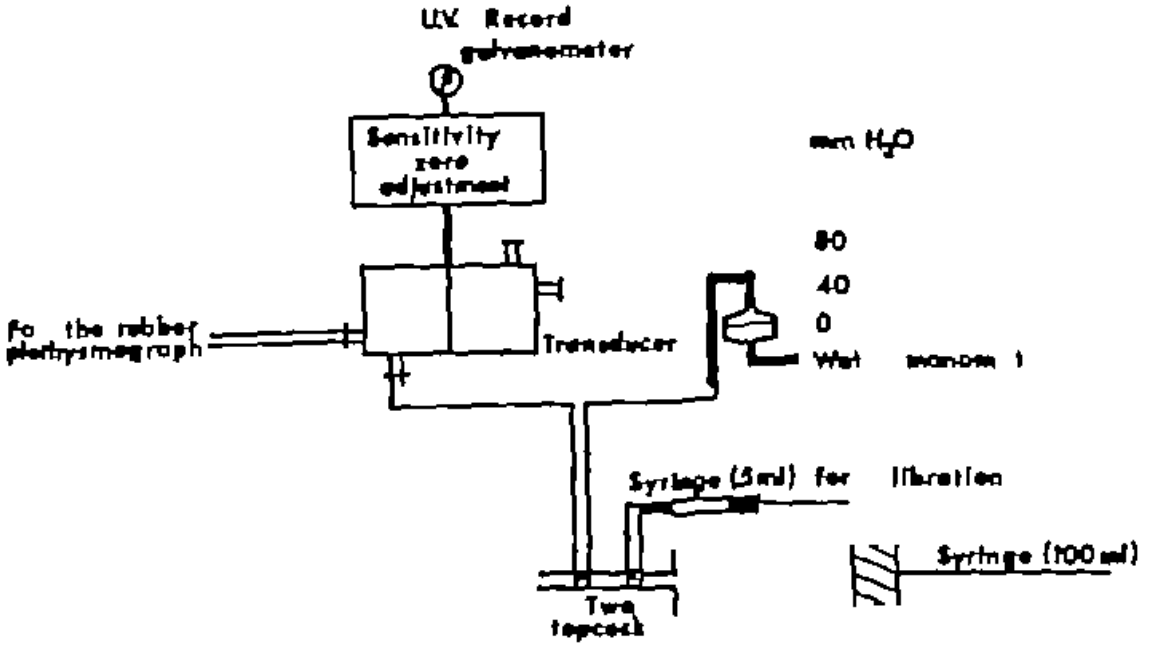


Fig. 9 Simplified design for use with Dohn's rubber plethysmographs. The pistons of syringes are kept carefully lubricated with grease.

and Munc (1967) gave roughly similar values. Since the elastic rubber plethysmograph can be applied to limbs of different thickness, Dohn's measurement of the volume by air displacement is not acceptable, regardless of the apparent accuracy attainable by repeated measurements on a single limb. Results from the air displacement method were not always in accordance with water displacement measurement and with calculation of volume, while the later two methods always agreed well mutually Therefore, these two methods were preferred.

Graf (1964 b) found the error of measurement of the air plethysmography recording device, including the plethysmographs by ± 3.2 % SD (from the pressure level (40 mm H_2O) in the cuff). Values for the apparent rate of arterial inflow of 0.5 to 40 ml/100 ml × min can be measured with sufficient accuracy by venous occlusion plethysmography (Graf 1964 b). A subdiastolic pressure in a proximal cuff produces a negligible decrease of the arterial inflow and thus does not naturally alter the variable to be measured in venous occlusion plethysmography (Graf & Rosell 1964 b).

If a suitable transducer recorder system is available, air plethysmography can be performed with the equipment shown in Fig 9 With simultaneous

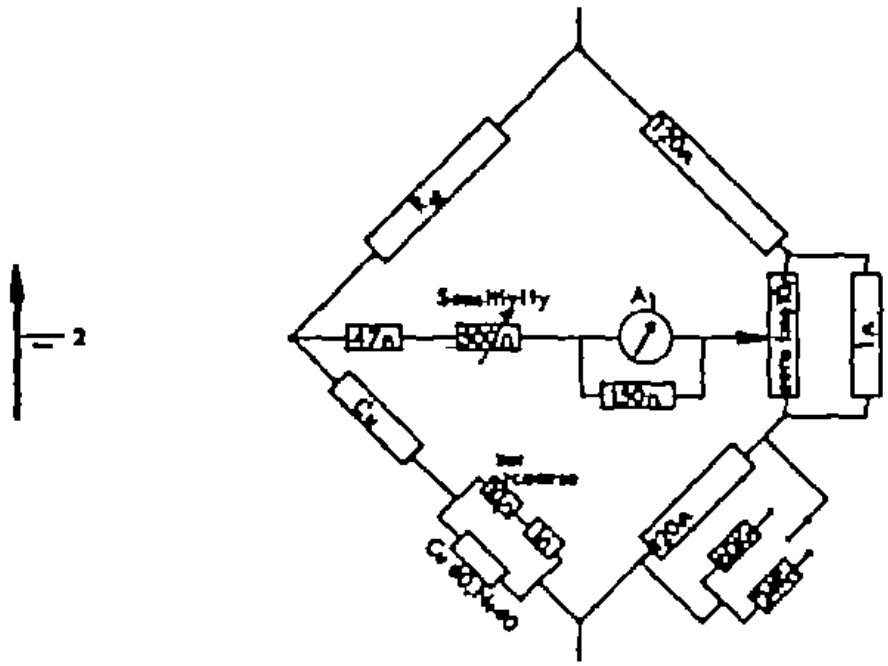


Fig 10 Diagram of the strain gauge supply and Wheatstone bridge circuit. Two separate bridges are used simultaneously The copper wire (Cu) has a resistance of $0.28 \times R_{st}$ and mounted on the strain gauge (R_{st}) The resistance (Cu 60, Ni 40) is—as indicated—of constantan and its size is $0.76 \times R_{st}$ The galvanometer type A_1 (ABEM), is used with a damping resistor of 150 ohms. The 120 ohms resistors are highly stabilized. The 1 % resistors for electrical calibration (60 and 24 Kohms) is of the metal film type, and corresponds to a resistance change of 0.2 and 0.5 ‰, respectively

recording of the temperature underneath the rubber plethysmograph, for comparison with blood flows obtained by others this method may seem convenient.

Strain gauge plethysmography

Whitney's (1953) power supply for the two Wheatstone bridges carried the sensitivity adjustment, common to the two channels at the input side of the circuit, with the consequence that the input voltage to one strain gauge would change, if the other was disconnected during the procedure. In the present study the power supply was highly stabilized with a variable output voltage (0 to 40 V) and a maximal current of 1 amp. The output voltage was fixed at 2 V In order to maintain an accuracy of 1 % on the electrical calibration (Horeman 1958), the 10 ohm zero-fine-potentiometer was shunted with a 1 ohm precision resistor Further modifications are shown in Fig 10

Blood flow was calculated from strain gauge curves in the following way The strain gauges were exact copies of the original gauges used by Whitney (1953), and provided from A. B Charteres, London School of Hygiene. Before the method was adopted for the experiments with breath-holding the strain gauge principle was systematically examined. Whitney mounted the gauge at a tension of 20—30 g We measured the galvanometer deflection by changing the resistance of the Wheatstone bridge circuit (Fig 10) with 0 5 % when the two strands of the gauge were loaded with 10, 20 30, 40 50 and 60 g The galvanometer deflection (*i.e* change in bridge output) was naturally found to be constant, with exactly the same deflection at 20 30, and 40 g An initial tension of 30 g was therefore used throughout.—In venous occlusion plethysmography the variable of interest is the relative change of limb volume (dV/V) per unit of time. Whitney's first assumption is that the entire change in volume of the limb is due to corresponding changes of the transverse sectional area of the limb, with no significant change in length (1953) Whitney also assumed that the transverse section was circular, and that the shape of the section remained unaltered during expansion or contraction. Therefore, if R is the radius of the circular section, it is obvious, if the absolute change in area (A) is small (dA) that the relative change in area (dA/A) will be $2R\ dR/R^2 = 2\,dR/R$, if $(dR)^2$ is small, and the relative change in circumference $2\,dR/2\,R = dR/R$. The relative change in the volume of the limb is therefore equal to twice the relative change in its circumference or girth (dG/G), the change in girth is assumed to be identical with the relative change of the gauge length (dl/l). The resistance of a conductor (r) equals Specific resistance × length/area, so that if the volume of the conductor is constant, dl/l will be half the relative change of the electric resistance, dr/r Hence, dV/V equals dr/r The strain gauge is connected in a Wheatstone bridge (Fig 10). A 0.5 % relative change of the resistance of one of the normal resistors (120 ohms) will cause a galvanometer deflection (d_e) of the same magnitude as a 0.5 / change in resistance of R_{st} (Fig 10). In this way the strain gauge can be calibrated electrically Thus the galvanometer deflection (d_e) corresponded to ½ ml/100 ml limb volume, and $2 \times d_e$ cm ordinate to 1 ml blood per 100 ml. The number of flow units was simply calculated with the equation $FU = S \times \operatorname{tg} \nu/(2 \times d_e)$ where S is the speed of the paper of the recorder in cm/min.

A strain gauge placed on a forearm with a girth of 28 cm showed a galvanometer deflection of 1 1 cm by reduction of the "gap" (Whitney 1953) with ½ mm (*i.e* a relative change in length of 0.178 %). According to this result a similar relative change in volume of 0 5 % should show

a galvanometer deflection of 1 54 cm. However an electrical calibration with a 0 5 % change in resistance gave a deflection of 2.35 cm. When the gauge was detached from the arm and placed horizontally between two fixed points where the length could be altered with an accuracy of ±0.01 mm, a change in length of exactly ½ mm showed a galvanometer deflection of 1 65 cm corresponding to 2.35 cm at a relative change in length of 0.25 % (*i.e.*, a change in resistance of 0.5 %). Thus correct physical calibration is in accordance with the electrical calibration, while the gap reduction on the limb, as suggested by Whitney yields much too low galvanometer deflections. Such a "calibration" should be avoided.

Blood flow was measured on a forearm with a water plethysmograph and with a strain gauge applied in the usual way (Whitney 1953), and close to the proximal border of the plethysmograph. A double loop of mercury in rubber, from one of Whitney's strain gauges, was fixed by rolling a piece of Scotch tape around the gauge at one site (1 cm wide) after which the gauge was placed underneath the sleeve of the water plethysmograph. With these three devices 31 resting blood flows were measured simultaneously and the two strain gauges were calibrated electrically The average value ±SE was 6.2±0 2 FU for the water plethysmograph and 5.2±0 2 FU for the double loop gauge beneath it, while the classical strain gauge close to the water plethysmograph only gave 4.1±0.2 FU although the temperature of the skin underneath it, was almost as high as in the water plethysmograph (33 6 C as compared to 34.1 C). Assuming —at this occasion—the water plethysmograph to measure approximately true blood flows, then the gauge surrounding the entire limb does not measure true values, and the gauge applied as suggested by Whitney with only 2/3 of the circumference in contact with the limb and 1/3 with the mounting plate, gives even lower values. Expansion of the limb covered by the mounting plate may not to its total extent increase the length of the mercury in rubber and hereby lead to fictitiously low results when the blood flow is measured with such a design.

Whitney (1953) found the 'blood flow' measured with strain gauges, to be 20 % larger (Fig. 11) than that indicated by simultaneous measurements with a water plethysmograph, a difference which may be due to his inadequate mechanical calibration and to the fact that his strain gauge was in contact with the limb only over 2/3 of the circumference. It is no consolation that these two pitfalls—under certain conditions—can eliminate each other and produce the same average value as the water plethysmograph. Horeman (1958) and Brakkee & Vendrik (1966) who introduced the electrical calibration, did not report on any comparison between the elec

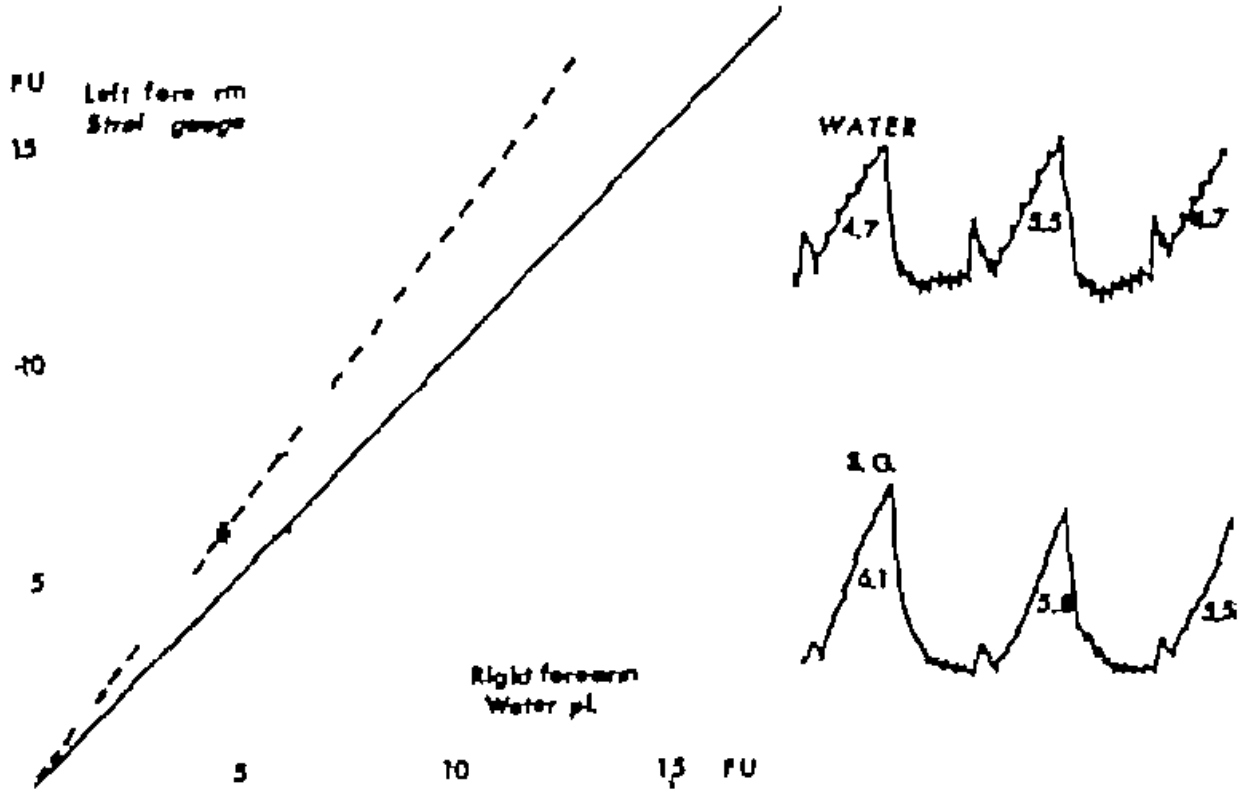


Fig. 11 Comparison between strain gauge and water plethysmography. The above strain gauge results are calculated on the basis of Whitney's original, mechanical "calibration" on the arm. *The cross is the average value (± SE) of 31 determinations.*

trical and the mechanical ('gap reduction) calibration, and seem to have preferred the electrical calibration and a gauge covering the whole circumference for practical reasons. As shown above even these improvements do not permit the strain gauge to measure true blood flows, as judged by the results from the water plethysmograph.

Although serious objections might have been raised against Whitney's method, it is emphasized that blood flow *variations* can be measured with reasonable accuracy with strain gauges, as shown in Table 12.

E. AUXILIARY EQUIPMENT

In classical physiological studies "body temperature" ('core temperature') in man is preferably measured per rectum. Recently Benzinger & Taylor (1963) suggested intracranial temperature (tympanic membrane and nasal cavity) as the measurement of choice. The tympanic membrane—like the anterior ethmoidal region, the anterior wall of the sphenoid sinus, and Rosenmueller's fossa—are supplied with blood from internal branches of

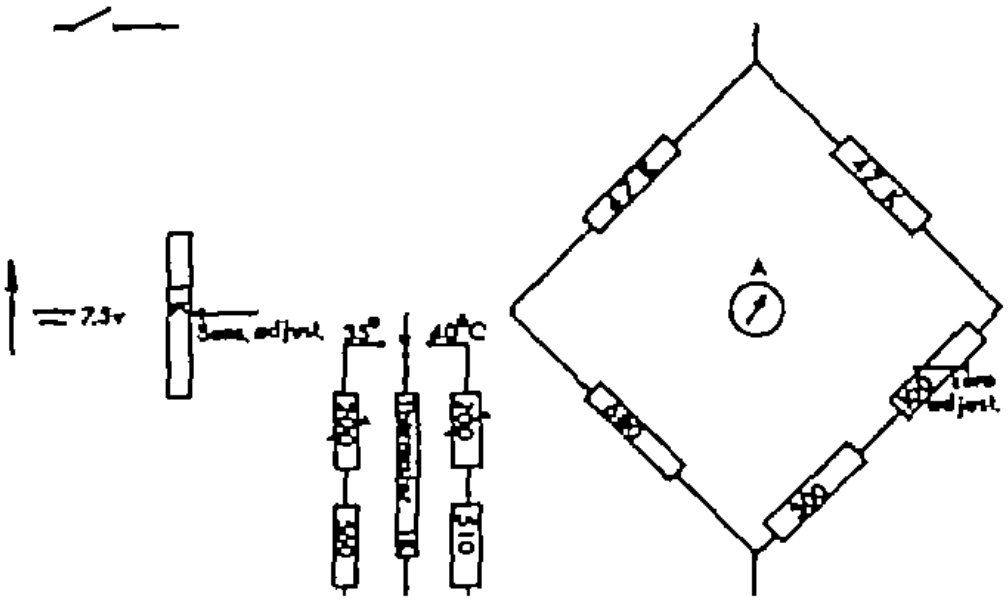


Fig. 12 Thermistor circuit. Philips thermistor type E 214 AE/P 1 K. The thermistor can be calibrated electrically at 35 and 40°C. The maximal sensitivity is 14 mm galvanometer deflection per degree (C). The galvanometer type A (ABEM) has an internal resistance of 110 ohms and is critically damped with 900 ohms. The sensitivity is 10 mm/A at a distance of 30 cm from the mirror. All fixed 1 % resistors are of the metal film type, and the resistance is given in ohms.

the carotid artery. Temperatures measured simultaneously at these sites showed similar relative changes during drastic changes of the external or digestive tract temperature, indicating that hypothalamic temperatures vary in the same way unless the local blood flow and generation of heat varied from one area to another (Benzinger & Taylor 1963). During corresponding drastic environmental changes, the (mutually identical) temperature changes in the cranium were greater and faster than those recorded in the rectum.

Benzinger & Taylor (1963) preferred thermocouples because their response is essentially linear over a wide temperature range (zero to 50 C). In the present study thermistors were used because they could be made equally linear in response over a sufficient large temperature range, and because they could be used directly with the mirror galvanometers available, without amplification. A simple electric calibration was found convenient (Fig. 12). Normal resistors equal to the thermistor resistance at 35 and 40 C were introduced into the bridge circuit instead of the thermistor

(Fig 12). The minithermistor was moulded in araldite and held against the eardrum by a plug of foam rubber The test subjects tried to place the thermistor at the eardrum, always with great care. A light touch of the eardrum elicits a pain sensation of short duration. A minithermistor of the same type was also placed on the skin. The temperatures were read to the nearest 0.1 C. In the course of the earlier experiments, the temperature of other thermistors placed in the rectum or on the skin, were read routinely

By definition a thermometer measures only the temperature of the thermometer. Thus, a so-called "absolute, true temperature" is, at best, a recorded figure with a careful description of the environment in which the bulb is located. The magnitude of some of the tympanic membrane temperatures (Fig 29—31) obtained, indicates that the test subject had not placed the thermistor *on* the eardrum but probably close to it, during these experiments.

Control of intracranial, body surface, and environmental temperature is important when alteration in successive blood flow measurements are to be attributed to other factors. Recording of the respiratory pattern, as a control of normal breathing is equally imperative to exclude flow changes of respiratory origin. In the present experiments the signals from a pneumograph or a pneumotachograph, were therefore always recorded on the U V recorder via a Statham transducer

PART II

PROCEDURES, RESULTS AND DISCUSSION

CHAPTER 5

Breath holding and decompression sickness

As mentioned in the introduction, serious accidents had occurred during training in the submarine-escape tank in Norway. Information obtained at personal interviews with the men and from their case reports (dizziness, eructation, fatigue, pains, pareses, and paralysis, *i.e.*, severe neurological phenomena, immediately relieved by recompression) suggested decompression sickness as the cause of these accidents, *i.e.*, an explanation incompatible with the general consensus of opinion at that time. To check the tenability of such an explanation I dived 60 times to depths of 15—18 meters and occasionally to the bottom of the tank. These dives were performed within a total period of 5 hours. The pattern of each dive ('bottom drop') was as follows: The mean rate of descent was 0.8 meter/second, which implies that the diver took 15 seconds to reach the bottom of the tank, where he remained (rested) prone until the urge to breathe became irresistible. This occurred within one to two minutes, and then he climbed, as quickly as possible, up a rope suspended from above the tank. The rate of ascent was about 1.5 m per second. The interval between consecutive dives varied from a few seconds to one or two minutes.

After three hours' diving symptoms began to appear. Increasing nausea, dizziness and eructation, and after the last dive, mild pain in the left hip. Later the pain became more intense and the right knee also became painful. The right leg and arm felt abnormally weak, and it was difficult to walk and even to stand. After about 2 hours there was severe chest pain that could not be relieved by expiration or by change in posture. The pain was accompanied by inspiratory distress and by a sensation of imminent fainting: paresthesia of the right hand and dullness of vision. After a further hour a colleague found me markedly pale and weak, as if I were about to faint. He examined me and confirmed the visual disturbances and paresis of the right arm. The symptoms and signs progressed and abdominal pain and anesthesia of the ulnar region of the right hand supervened. I was then placed in a recompression chamber. At 6 atm abs the dizziness and nausea disappeared immediately and 5 minutes later the pain and partial paresis regressed and after 25 min at 6 atm abs the paresthesia and anesthesia disappeared, leaving only a feeling of pins and needles in the right hand. I was therefore given treatment according to Table 5 of the U.S. Navy

treatment table (U S Navy Diving Manual 1963), which requires about 20 hours in the recompression chamber. The chest pain, accompanied by an intense feeling of fear of death, reappeared for a few minutes every time the pressure was reduced in the process of stage decompression. The treatment was successful except for persistent weakness of the right hand.

Similar symptoms and signs were experienced by three Norwegian escape-training tank instructors. Cross (1965) has reported a syndrome called taravana among the skin-diving pearl divers of the Tuamotu Archipelago in the South Pacific when severe it comprises loss of consciousness and paralysis of one or more limbs, and it is often fatal. The similarity between the diving patterns of the pearl divers and of subsequent symptoms of taravana and those in the Scandinavian cases described here suggest that the underlying mechanism was the same in both disorders. The response of taravana to recompression has not yet been investigated. The symptoms and signs described are consistent with the diagnosis of decompression sickness. The dramatic response to recompression can probably be explained only on the basis of bubbles in the central nervous system (for further discussion, see ref. *A B*)

Despite convincing evidence of bubbles in the nervous system and other tissues it was still debatable whether they were due entirely to increased tissue nitrogen tensions, as in ordinary decompression sickness, or whether molecules of other dissolved gases, particularly carbon dioxide, might have contributed to the bubble formation.

Since it is extremely difficult to measure mean tissue nitrogen tensions, even in animals the tissue nitrogen tensions were calculated from observed alveolar nitrogen percentages (ref. *B*). The calculation of the tissue nitrogen tensions during repeated breath-hold dives rests on some simple arguments advanced in the famous work on decompression sickness by Boycott, Damant & Haldane (1908) Calculations of nitrogen tissue tensions (here called P_tN_2, in the equations called Y) for hyperbaric exposures are time-consuming. More than 30,000 P_tN_2 calculations were necessary for a systematic examination of all variables. The calculations were made with the use of an electronic computer (GIER, programmed in ALGOL 60). The calculations are based on alveolar nitrogen fractions from samples obtained from divers at the bottom of the submarine-escape training tank. Fig 13 shows the increase in alveolar nitrogen percentage as a function of time (the time of descent plus the time at depth) Assuming that the increase of the F_AN_2 was linear the mean F_AN_2 was 0 83 for one minute breath-hold dives and 0.88 for the two-minute dives. To simplify the calculations it was assumed that the subject was exposed to the mean F_AN_2 values during the entire exposure

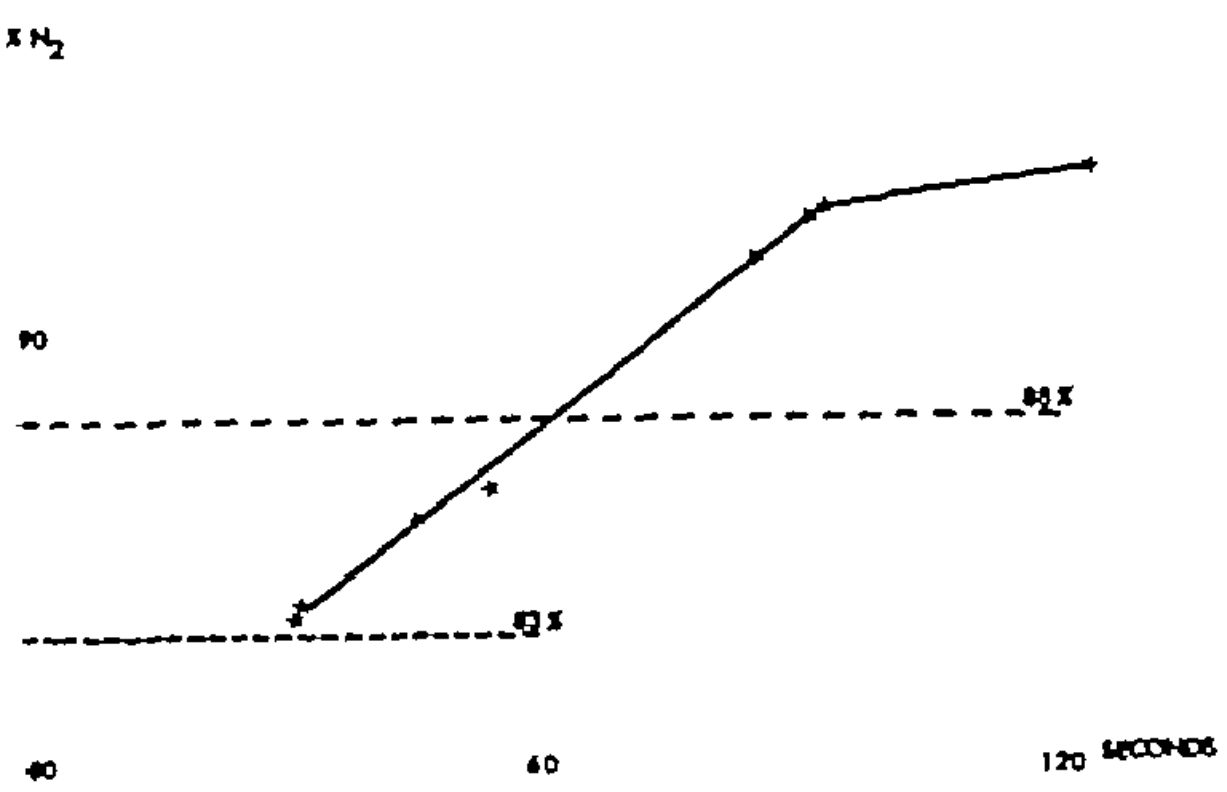


Fig. 13 N_2 percentages from alveolar samples obtained at the bottom of the submarine escape training tank. The abscissa is the period from the start of the dive to the time of sampling

time. The exposure time is defined here as the total time under water, and the surface interval as the time from the end of one dive to the beginning of the next.

The human body may be regarded as a complex of tissues, each 'tissue unit' absorbing or eliminating N_2 exponentially with and individual half time of saturation and desaturation. The half-time is the number of minutes necessary to produce P_tN_2 changes equal to half of the P_{N_2} difference initially existing between the arterial blood and the tissue. The nitrogen tensions before and after each of 48 dives were calculated on the basis of the body model consisting of an array of tissues with 5- 10- 20- 40- 80- and 120-minute half-times, as conventionally assumed in such calculations of N_2-exchange (Yarbrough 1937 Dwyer 1956 Workman 1965) The hypothetical P_tN_2 values obtained for an infinite number of dives—mathematical limit values (lim X_n and $\bar{X}_n$, see ref. *B*)—were also calculated. All P_tN_2 values were expressed in mm Hg and calculated with an accuracy of two decimals. When a P_{N_2} difference occurs between the arterial blood and a tissue, the rate of change in P_tN_2 will at any time be a constant fraction of the prevailing P_{N_2} difference. To describe the rate of the change in P_tN_2, the

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the equilibrium tension may be expressed as a percentage of the largest possible P_{N_2} increment. The largest P_{N_2} increment (mm Hg) possible in the tissues at F_{AN_2} values of 0.83 and 0.88 respectively depends upon the depth (expressed in m of fresh water pressure) of the dive $[[((depth/10 + 1)\ 760) - 47]\ F_{AN_2}] - 567$. This percentage is called the equilibrium saturation percentage of a tissue with a given half-time. In the calculations of the equilibrium tensions for tissues with different half-times, from a representative exposure, the coefficient of variation (SD 100/x) was found to be as small as 2 %. One may therefore talk about a mean equilibrium N_2 tension for the whole organism (P_{tN_2}).

The arithmetic mean (MESP) of the equilibrium saturation percentages of all tissues considered is calculated to express the degree of N_2 saturation obtainable for the organism as a whole. This is done in the following equation

$$MESP = \frac{[\Sigma(\lim Y_n + \lim X_n)/12 - 567]\ 100}{[[((depth/10 + 1)\ 760) - 47]\ F_{AN_2}] - 567}$$

Corresponding to the two P_{tN_2} arrays there are two mathematical limits for each of the six tissues of different half-times and $\Sigma(\lim X_n + \lim X_n)$ is the sum of these twelve limits. For further details see appendix (ref. *B*).

Critical N_2 tensions

On the basis of experiments with air-supplied diving a number of maximum permissible P_{tN_2} (max tens) levels have been specified for each half time tissue (Workman 1965). These are (critical) tensions above which decompression sickness is likely to occur. If the form of analysis described is correct, it should be possible to exceed the max tens, by repeated breath-

Table 4. Number of dives necessary to exceed the maximum allowable tissue N tension (max tens) in different half-time tissues.[1]

Tissue, half-time	5	0	20	40	80	120
Max tens, mm Hg	2,395	2,027	1 658	1 536	1 198	1 175
Depth m						
18 5				41		
50			15	16	24	34
55			7	5	8	17

Surface intervals are of 1 min duration, exposure times of min duration.
Indicated only if max tens is exceeded within the calculated 48 dives.

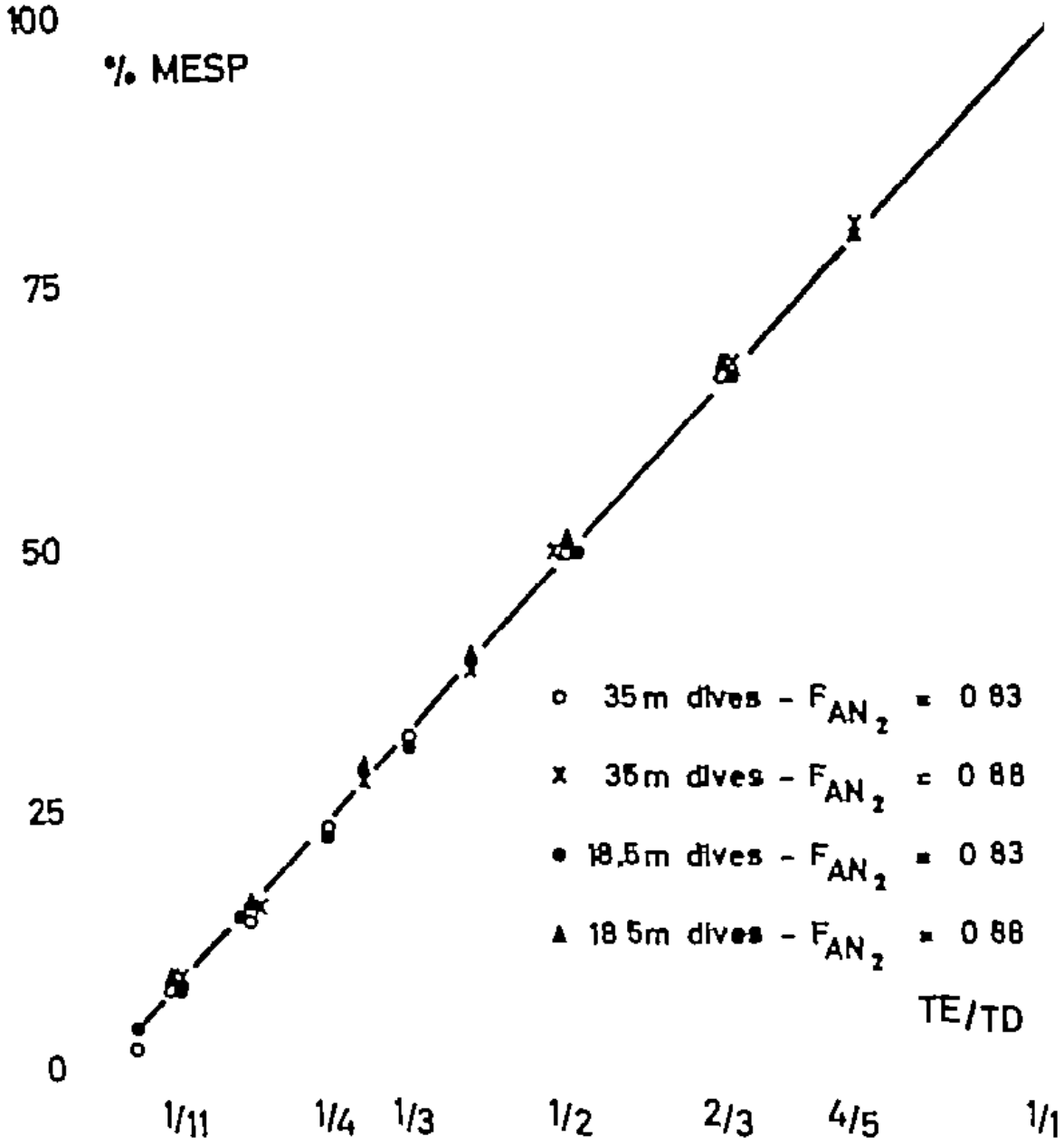


Fig. 15 Mean equilibrium saturation percentage (MESP) at different exposures. The ratio of total exposure time (the sum of all exposure times) to total time of diving series (the sum of all exposure times and all surface intervals) is the abscissa (TE/TD). The calculations are based on surface intervals of ½ 2, 5 10, and 20 minutes duration.

hold diving (Fig 14 and Table 4) and thereby explain the decompression sickness observed (ref. *A*), entirely on the basis of gradually increasing P_{IN_2} values during repetitive diving The observed serious neurological signs disappearing after decompression indicate that the P_{N_2} of the central nervous system is the decisive cause of this condition. This does not, of

course, rule out the possibility of other contributory factors such as the progressive CO_2 retention (ref. *C*) In addition the increased CO_2 tensions in the tissues might favour formation and growth of the bubbles by speeding up the delivery of CO_2 to the bubbles.

N_2 saturation degree

The total exposure time (TE) is defined here as the sum of all exposure times during series of dives. The total duration of a diving series (TD) is defined as the sum of all exposure times and surface intervals during repeated diving Analysis of the MESP equation after substitution of the expressions for $\lim \lambda_n$ and $\lim X_n$ (see appendix, ref *B*) showed that MESP is governed essentially by the ratio between TE and TD As apparent from Fig 15 the relation between MESP and the TE/TD ratio is essentially linear the variation of MESP with the F_{AN_2} and the diving depths is negligible.

When depth and duration of an air-supplied dive do not exceed certain limits, called no-decompression limits (Workman 1965) direct ascent to the surface without stage decompression is considered safe (Workman 1965) But the so-called no-decompression limits" refer to conventional, air-supplied diving and may not be valid for breath-hold diving because of essential differences such as the difference in rate of ascent. The conventional no-decompression limits may therefore not be safe when applied in actual diving A practical procedure for evaluating the risks accompanying a series of breath-hold dives is suggested below At the Mangareva Islands in the South Pacific the native divers use surface intervals of about 10 min. No cases of the decompression sickness-like taravana syndrome are known there, but cases have been known to occur on the neighboring islands (Cross 1965) where the divers use shorter surface intervals. These observations are explained by the low MESP during breath-hold dives with long surface intervals (*i.e.* corresponding to low TE/TD ratios with only a minimal risk of decompression sickness) Thus, surface intervals of 10 min seem to eliminate the risk of decompression sickness during repeated breath-hold diving with exposure times and depths acceptable for humans.

Practical application

The MESP can be determined from Fig 15 and an estimation of the TE/TD ratio in actual diving series. The actual mean equilibrium N_2 tension is calculated by inserting this MESP value, the diving depth and the F_{AN_2} (dependent upon the exposure time) in the MESP equation.

According to Haldane's old rule for air-supplied dives, stage decompression is not necessary as long as the ratio between total pressure at depth and the pressure decrease during ascent does not exceed 2/1 (Boycott et al. 1908). Instead of Haldane's ratio between the total pressure at depth and the pressure decrease during ascent, one may use the ratio between the N_2 partial tension for the whole organism (*i.e.*, P_tN_2, which is dependent upon P_AN_2, here assumed to be 0.85) and the pressure decrease by ascent (*i.e.* for a MESP of 100 % $2 \times 0.85 = 1.7$), yielding a new ratio of 1.7/1. Conventional standard air decompression tables now give a ratio lower than 2/1 in order to increase the safety margin for air-supplied dives. Decreasing the 1.7/1 ratio to a reasonable value for repeated dives, such as a 1.5/1 ratio, of course increases the safety margin still more. If the ratio actually determined between the mean equilibrium P_tN_2 and the pressure decrease (expressed in mm Hg) by decompression is below 1.5 such a small P_tN_2 reduction is not likely to provoke decompression sickness. Under these conditions the planned skin diving series may be considered safe—as far as N_2 is concerned. If the actual ratio is found to be equal to or higher than, 1.5/1 the planned diving series should be cancelled.

Conclusions

Repeated breath-hold diving with insufficient intervals at the surface between consecutive dives can cause decompression sickness, especially if simulated or compressed air-supplied dives have been performed on the same day. Long surface intervals are recommended when repeated breath-hold dives are performed to depths of 10 m or more. The severe neurological signs (paralysis, visual disturbances ect.) reported, emphasize the danger of a long series of repetitive breath hold dives to greath depth. It is therefore preferable in practice to use a calculation of the TE/TD ratio (Fig. 15) and a generous safety margin from Fig. 15 to avoid decompression sickness, instead of using more specific time limits (Lanphier 1965, ref. *A*). It appears that the symptoms and signs observed in breath-hold divers can be explained entirely by the gradual increase of the P_tN_2 during repeated diving, but synergistic mechanisms may still participate in the development of tissue bubbles.

CHAPTER 4

Carbon dioxide retention and hypoxia as a cause of loss of consciousness and drowning

Craig, Jr., (1961 a, b) studied swimmers who hyperventilated before they dived into an ordinary swimming pool, where they swam 50 m in the horizontal plane just underneath the water surface. He ascribed diving accidents under such circumstances to loss of consciousness due to hypoxia. By hyperventilation the underwater swimmer depletes the carbon dioxide (CO_2) stores of his body The partial pressure of CO_2 in the nervous tissue regulating the ventilation seems to be the main stimulus of respiration, the contribution by the low oxygen (O_2) partial pressures being relatively small. During underwater swimming the diver uses up O_2 and produces CO_2, but since the CO_2 is used for repletion of the partly emptied CO_2 stores, the drive for respiration and for surfacing does not become imperative, and the breath-hold dive may end in loss of consciousness and drowning In other words, after hyperventilation prior to underwater swimming, the integrated respiratory drive during underwater swimming, created by the falling O_2 tension and the rising—but still subnormal—CO_2 tension, may be on a level, where it is not an effective stimulus of respiration, at the same time as the O_2 tension of the cerebral cortex is low enough to cause blurring of the mind, rapidly proceeding to loss of consciousness.

These considerations may apply also to drowning after breath-hold diving to great depth, but the evidence available is too little to warrant any conclusions. Schaefer & Carey studied one diver in the submarine escape-training tank at New London (1962). Lanphier & Rahn (1963) and Hesser (1965) described gas exchange during simulated breath hold diving, but these observations have been obtained in pressure chambers without water and the breath-holds were either of a rather short duration or O_2 was breathed prior to the breath-hold, in order to obviate the possibility of unconsciousness. The many studies of Korean and Japanese divers, who mostly perform short dives during which dangerous O_2 and CO_2 tensions (P_{O_2} and P_{CO_2}) are rarely reached, also seem to have had another purpose than studying the risks of extreme breath-hold dives.

To elucidate factors responsible for loss of consciousness and drowning during breath-hold diving to extreme depth and of long duration, the O_2 uptake, CO_2 retention, and alveolar air composition (P_{AO_2} and P_{ACO_2}) were measured during and after a single or series of breath-hold dives in

water and compared with those made during similar breath-holds in air

The dives were performed in the above described submarine-escape training tank (Fig 1) by the so-called 'bottom drop technique, according to which the diver remains vertical and descends feet first. He wore a nose-clip and a pair of pressure compensated goggles (Royal Navy type) Before and between the dives the diver rested submersed up to the neck at the edge of the tank. Before the diving the diver hyperventilated by taking 2 breaths and after a third, a maximal inspiration, pushed himself downward with the arms from the border of the tank, and descended without further movement of the limbs. After the first 2—3 meter loss of buoyancy owing to compression of the lung gas resulted in his descent to the bottom with increasing speed. The mean rate of descent was 0.8 m/sec. After lying a varying time flat on the bottom of the tank, he pulled himself up on a rope fastened above the tank. He ascended at a mean rate of 1 5 m per sec. Five submarine-escape training tank instructors and the author participated in the experiment. The main experiments were undertaken by three of these.

The mean diving time—total time under water— is shown in Table 6. In the single-dive series the diving time reached 124 seconds Attempts to stay as long as possible under water were discouraged. None of the test subjects ever lost consciousness. For the sake of safety surface intervals, (*i.e.*, total time between two successive dives) were between one and two minutes. Subjectively sufficient restitution to permit a new dive was obtained after the first few respirations in all subjects.

Composition of alveolar air

Before diving The effect of repetitive diving is apparent from Table 5 where the mean pre-dive tensions (last) illustrates the degree of pre-dive hyperventilation (here defined as a ventilation producing a P_{ACO_2} below normal level) Most dives were performed after mild hyperventilation (Subject EE and OL). Dives after violent hyperventilation (Subject PP) were unpleasent, mostly because of dizziness during the descent.

At end of dive. Twenty-one samples (7 × 3) were obtained after repeated dives to 18 5 m. As already found by Lanphier & Rahn (1963) in 10 m simulated dives, average P_{ACO_2} values were not far from normal (Table 5). In the very first expiration after each dive the P_{AO_2} values were usually about 60 mm. Two breaths later they were near normal. Some of the P_{AO_2} values of PP were very low (minimum 30 mm), primarily because of the longer diving time. Simultaneously the P_{ACO_2} had again fallen to values below normal. When surfacing the diver was cyanotic and at the

Table 5 Mean alveolar air composition before and after seven repeated dives.

Subject	Sample	PO_2, mm Hg	PCO_2, mm Hg	R
EE PB=746	Last	126 (19—133)	27 (20—32)	1.40
	First	57 (50—65)	43 (37—48)	0.42
	Third	104 (95—115)	33 (27—43)	0.78
OL PB=771	Last	134 (30—135)	25 (23—30)	1 60
	First	64 (48—69)	37 (32—42)	0.27
	Third	118 (116—122)	28 (24—30)	0.80
PP PB=771	Last	137 (135—140)	3 (18—29)	1.90
	First	49 (30—97)	39 (32—45)	0.34
	Third	111 (97—118)	3 (24—40)	0 69

Figures in parentheses are ranges from the seven consecutive dives. Alveolar samples are from the last expiration before a dive and from the first and third expiration after each dive.

border of unconsciousness (Craig 1961 b, Lanphier & Rahn 1963 Otis et al. 1946 b).

In evaluation of the relative contribution of hypoxia and hypercapnia to unconsciousness, it would be desirable to know the gas tensions in the central nervous system at the end of the dive. The partial pressures of the alveolar air at the time of sampling are not ideal measures of these tensions. While alveolar gas tensions fall rapidly during a fast ascent, due to the expansion of the lung gases accompanying the decompression, this de compression does not by itself affect the gas tensions in the tissues. Since the tissue gas tensions do not drop instantaneously to the tensions prevailing in the blood entering the tissue and since the passage of blood from the lungs to the tissues takes time changes in tissue tensions must be expected to lag behind the tensions of the alveolar air at the time of surfacing

Single breath hold dives. Fig 16 shows the composition of the alveolar air during the dive. The samples are obtained while the diver is at the bottom of the tank. Each diver made only one dive (provided only one sample)

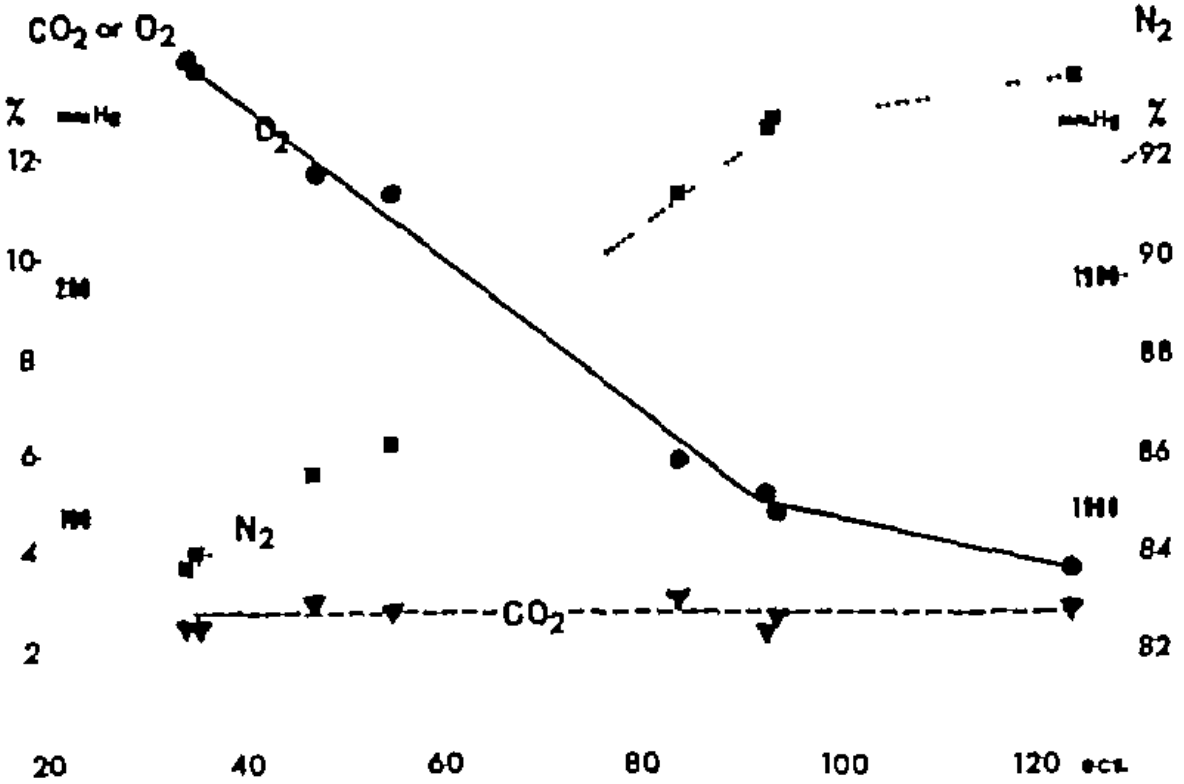


Fig. 16 Relation between diving time (abscissa) and oxygen, carbon dioxide, and nitrogen percentages in alveolar samples taken at 18.5 m of depth during single dives. The alveolar gas tensions at 2.85 atm abs are also shown. Each of 6 divers performed one dive per day and the points represent a total of 8 dives.

per day The carbon dioxide fraction is essentially constant—perhaps with a tendency toward a very small increase—and the alveolar oxygen fraction decreases steadily with diving time. Immediately after the descent to the bottom (2.8 atm abs) the P_ACO_2 is high, probably $2.8 \times 25 = 70$ mm Hg due to the compression of lung gas (P_ACO_2 at the beginning of the dive = 25 mm). Thus P_ACO_2, due to the descent to the bottom of the tank, exceeds the existing $P\bar{v}CO_2$, and CO_2 is transferred from the lungs to the blood. Hereby P_ACO_2 will fall toward the level shown in Fig 16 Initially during a breath-hold at high intrapulmonic pressure the cardiac output will be reduced by 20—40 % (Fig 24) so this CO_2 uptake from the lungs to the body is possibly blood flow limited in some subjects. The arterial CO_2 tension (P_aCO_2) is hereby increased to essentially the same value, and this stimulus probably in association with other stimuli elicited by the small lung volume at the bottom of the tank, makes the diver feel that he is already approaching his breaking point. This subjective sensation may be overcome—although not eliminated—by the will power of trained breath-

bold divers. After 35 seconds an alveolar CO_2 % of 2.7 is measured, which will correspond to a P_ACO_2 ($\simeq P_aCO_2$) of 57 mm at 18.5 m, and standard conditions. At the end of the dive is experienced an irresistible breaking point sensation owing to the decreased lung volume and the high P_aCO_2. This is not always followed by an adequate escape reaction. — It is interesting that when the diver is resting on the bottom of the tank, the P_AO_2 is very high, falling linearly from 380 mm, but still greater after a diving time of 90 seconds (Fig. 16) than when the diver rests at the surface (approximately 105 mm). Thanks to the high P_AO_2, blood passing through the pulmonary capillaries is reoxygenated completely during a dive of 90 seconds duration. However, when the subject rested at the bottom for more than 90 seconds, the P_AO_2 and P_aO_2 values were less than 100 mm. The low P_AO_2 and the Bohr effect (the greater the P_aCO_2 the lower the hemoglobin O_2 saturation) has then in fact caused a significant fall in O_2 saturation. The P_ACO_2 ($\simeq P_aCO_2$) now is still 57 mm and the P_aO_2 is 85 mm after 124 seconds, so that the blood cannot carry as much O_2 from the lungs to the tissues as before. Some divers have been reported to have lost consciousness at the bottom of the tank, possibly owing to the dangerous combination of a low P_AO_2 and a very high P_ACO_2. — During ascent the breaking point sensation disappears because of the increase in lung volume and the fall in P_ACO_2, even though the P_{O_2} will be decreasing considerably as the P_AO_2 falls. Since P_AO_2 may fall below the $P_{\bar{v}}O_2$ there is even a possibility of O_2 being transferred from the blood to the lungs during the last part of the ascent. Also during the ascent the decreasing P_AO_2 causes a fall in S_aO_2, reducing the rate at which O_2 is carried to the tissues from the lungs. Under these conditions part of the O_2 supplied to the tissues must be derived from the blood O_2 stores. However these O_2 stores are only 700 ml or less, which may easily be utilized by the muscles during 10 seconds of powerful ascent. If blood deprived of oxygen arrives at the cerebral cortex, the diver may lose consciousness just as he reaches the surface or before. Unconsciousness during ascent, but below the "buoyancy point" is a potentially fatal condition.

Net change of alveolar gases

The net transfer of gases from the lungs to the body during a "mean dive" (average of seven successive dives performed by one person) was calculated as the difference between the volume of O_2 or CO_2 in the lungs just after the final inspiration before a dive (V_I), and just before the first maximal expiration after the same dive (V_E) ΔVO_2 or $\Delta VCO_2 = (RV\ F_A + V_I\ F_I) - (RV + V_E)\ F'_A$ F_A and F'_A = fractions in end-expiratory

Table 6 Net changes of alveolar gases during a mean dive.

Test Subject	Diving time	ΔVO_2	ΔVCO_2	R
	sec			
EH	42 (40—45)	663	—260	0.39
OL	47 (44—50)	864	—268	0.31
PP	71 (41—80)	673	—164	0.24

Figures in parentheses are ranges. All volumes are ml (STPD) Oxygen is transferred from the lungs to the body carbon dioxide is given off from the body to the lungs (negative values).

sample from final expiration before the dive and in the first expiration after the dive, respectively Dead space volume was assumed to be negligible. The end-expiratory air was assumed to represent alveolar air concentrations in all parts of the lungs There was no loss of air from the lungs (no air bubbles were observed) during the dives because of the tight nose clips (Table 6) Assuming an alveolar exchange ratio (R) identical with a metabolic, respiratory quotient (RQ) of 0 8—1 0, the overall retention of CO_2 in a mean dive was 300—600 ml CO_2 (13 5—27 mM)

Between and after dives

In a separate experiment mixed expiratory air was collected in a Douglas bag during the first three surface intervals in a series of dives (120—119—123 sec) The first expiration after each dive was included. Calculations showed an oxygen uptake per minute surface interval of 590 ml, a CO_2 elimination of 430 ml, and thus an alveolar exchange ratio (R) of 0.73 disregarding a change in the composition of the lung air

The results obtained by fractional sampling of expired air after repeated dives are shown in Fig 17 Two minutes after the 7-dive series R exceeds 1 0 and after 6 minutes decreases toward normal levels. This pattern is less apparent after only four dives The Douglas bag-sampling technique is not adequate for a detailed analysis but, in general, the R alterations reflect both that the emptying of the CO_2 stores of the body occurs with a greater delay than the replacement of the O_2 stores, and that the excess CO_2 is relatively greater than the O_2 deficit. Assuming the metabolic, respiratory quotient (RQ) to be 0.8 during the 16-min collecting period

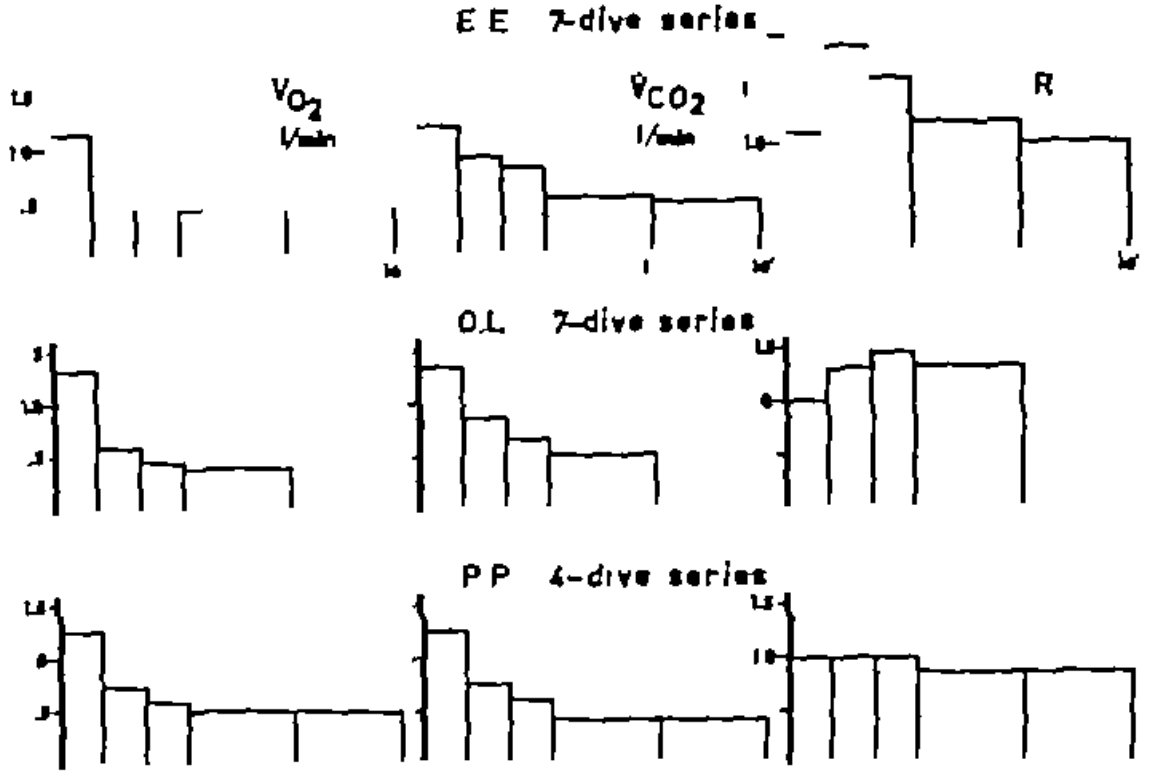


Fig 17 Oxygen uptake, carbon dioxide elimination (liters STPD/min), and alveolar exchange ratio ($R = \dot{V}_{CO_2}/\dot{V}_{O_2}$) after two seven-dive and one four-dive series. The abscissa is the first 16 minutes after the end of the diving series. During this time the subject was resting at the border of the tank in water up to the neck.

and the O_2 uptake for compensating the loss of O_2 from the O_2 stores to be 1 500 ml, subject EE eliminated about 5,000 ml excess CO_2 (223 mM) after 7 dives. Part of this CO_2 retention may help to overcome the respiratory alkalosis due to the pre-dive hyperventilation. However, in the final phase of the longest dives symptoms similar to CO_2 poisoning (headache, dizziness, respiratory distress, fear and a fuzzy state with water swallowing) were present. Although the divers had normal P_{ACO_2} values at the time of surfacing, the corresponding alveolar CO_2 tensions at the bottom of the tank immediately before ascent, must have been abnormally high (Fig 16).

Breath-holding above water

The alveolar air composition before and during prolonged breath-holding while resting above water at normal pressure, is seen in Fig 18 This is a P_{O_2} and P_{CO_2} diagram (Otis et al. 1946 b) Values within the broken lines

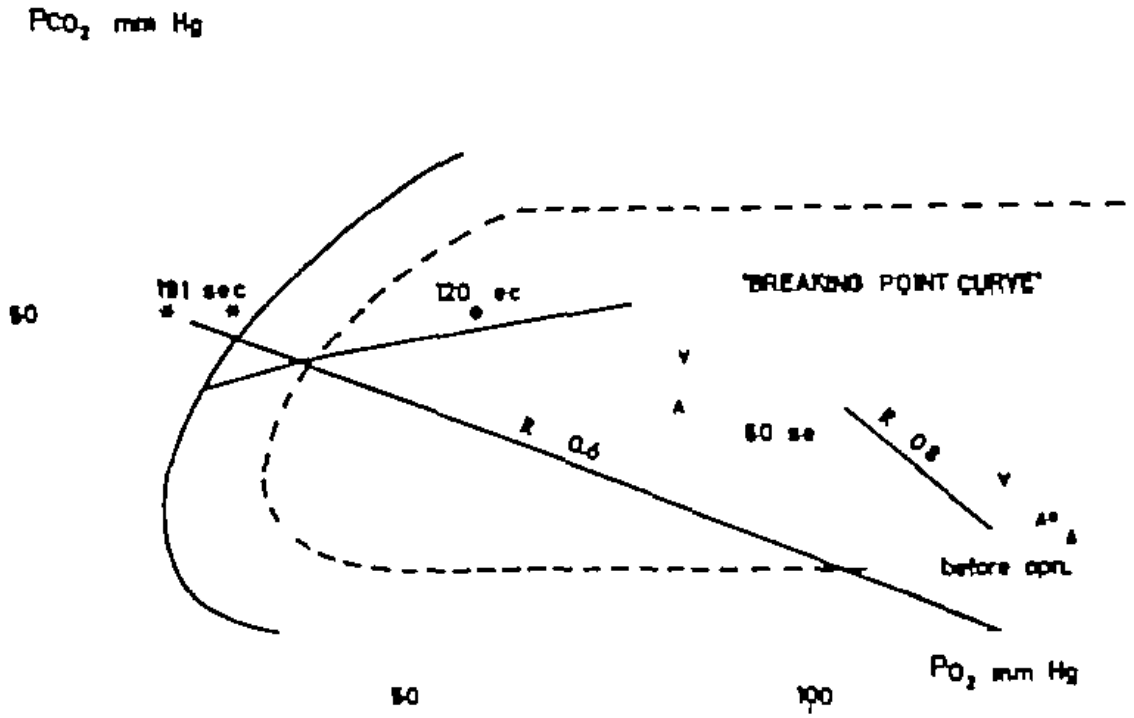


Fig 18 A Penn-Rahn diagram showing the alveolar air partial tensions before and after breath-holds of different duration (60—120—191 seconds). Ten subjects (with individual symbols) *resting supine* in air participated. All tensions are calculated from double determinations of alveolar fractions with a Scholander micro-analyzer

indicate unimpaired consciousness, between the broken and the continuous line impaired consciousness, and outside the continuous line, loss of consciousness (Otis et al. 1946 b). A conventional breaking point curve and two lines corresponding to two alveolar exchange ratios (R) are also shown. Also during breath-holding in air, the CO_2 delivery to the lungs is less than the O_2 uptake from the lungs. All 10 subjects (Fig 18) hyper ventilated with three, deep respirations before the breath-holding One of the subjects lost consciousness on two occasions, both times after 191 seconds breath-holding, lying supine in air. Turning the head and extending the neck in order to commence insufflation resuscitation immediately brought the subject back to consciousness, which commenced with expira tion of the air whereby the alveolar air sampling device was filled. The result of the Scholander analysis is shown at the asterisk to the left (Fig 18). After a resting period the subject insisted on repeating the experiment, and he lost consciousness at exactly the same breath-holding time (see the other asterisk) It seems as if the breaking point curve was past, resulting in a very low P_{AO_2}, while the P_{ACO_2} was not increased much more than

during most breath-holds. Thus, it is possible to lose consciousness by voluntary suppression of ventilation.

Comparison between breath-holding in air and in water

The most interesting difference between the composition of the alveolar air during breath-holding in air at normal atmospheric pressure and below water at a considerable increase in pressure, is that the F_{ACO_2} is low at the bottom of the tank after a 120 sec breath-hold dive (0.027—57 mm Hg), but high after 120 seconds of breath-holding above water level (0 07—49 mm, see Fig 18). During breath-holding the lungs can be compared with an aerotonometer. Immediately after the descent to the bottom of the tank, the P_{ACO_2} will be about 70 mm (see above) causing CO_2 to enter the blood from the lungs. However, after a short period at the bottom of the tank Fig 16 shows that P_{ACO_2} (and F_{ACO_2}) does not change essentially indicating that, in the period considered, hardly any CO_2 is given off to the lungs. This line of reasoning assumed that the decrease of the molar content of gas in the lungs during the dive is negligible. Thus, in the period considered in Fig 16 the difference between the P_{ACO_2} and the $P\bar{v}CO_2$ must be approximately zero, although immediately after descent the P_{ACO_2} will fall from 70 down toward 57 mm due to uptake of CO_2 into the venous blood. The arterial P_{CO_2} must also be essentially equal to that in the alveolar air. At our depth (18 5 m) and with no exercise during the dive, these three tensions are all about 57 mm Hg at standard conditions. The high arterial P_{CO_2} will limit the exit of CO_2 from the tissues, and the P_{CO_2} of the tissues will—although gradually increasing due to CO_2 production—necessarily be lower than 57 mm during a dive. Thus, the exit of CO_2 from the tissues seems tension limited (*i.e.*, inhibited by the high P_aCO_2) and to different degrees during breath-holding in water at increased pressure and above water at normal pressure, where the P_{ACO_2} will rise by cumulation of CO_2 in the lungs. This difference in P_{ACO_2} is not necessarily caused by the hydrostatic factor alone. A blood flow limitation (*i.e.*, a reduced blood flow inhibiting the transport of CO_2 from the cells to the lungs) may of course, also be a factor of importance. During breath-holding at high intrapulmonic pressure we have found the limb (calf and forearm) blood flow decreased by 50 to 100 %, with the subject resting supine in air (Chapter 7) An identical manœuvre performed as a breath-hold dive could hardly lead to an essentially greater fall in limb blood flow (Fig 29) Thus the difference between the overall retention of CO_2 in the tissues during a breath-hold dive, as compared to breath-holding in air may probably not be explainable

as due to the blood flow limitation but a part of the CO_2 retention occurring during both breath-hold diving and breath-holding in air may be caused by a reduction of the blood flow at high intrapulmonic pressure.

When swimmers and divers lose consciousness during breath-holding at sea level and in underwater swimming just beneath the surface, hypoxia may be the main cause (Craig 1961 a) However in repeated deep dives loss of consiousness with the risk of drowning may—in the light of the CO_2 retention discussed above—be caused by CO_2 narcosis (and acidosis) in *combination with the hypoxia.*

CHAPTER 5
Cardiac rhythm

Alterations of the cardiac rhythm during diving and during breath-holding have been demonstrated in many animals (Irving 1964) Among these the pronounced bradycardia of aquatic mammals (e.g duck and seal) has been the subject of intensive investigation (Elsasen 1960, Feigle & Folkow 1963 Murdaugh et al. 1961 Reite et al 1963 Scholander 1962) In aquatic animals important changes occur not only in heart rate, but also in the vascular resistance of organs and limbs, a change which reserves the reduced cardiac output and the limited O_2 stores for those organs most dependent on a continuous supply of oxygenated blood (Elsner & Scholander 1965) These phenomena are known as an "oxygen conserving reflex" or a "diving reflex" but the physiological mechanisms involved have not been fully explained. The problem has been studied systematically in ducks by H. T Andersen (1963) who found an intact trigeminal nerve essential for the development of bradycardia.

Occurrence of bradycardia during breath-hold diving has also been described in man (Craig 1963 Olsen et al. 1962 Hong et al. 1963 Sasamoto 1965 Hong et al 1967) It is assumed that the mechanism by which this bradycardia is brought about, is similar to the diving bradycardia" in animals (Irving 1964, Scholander 1962), though no supportive evidence has been produced. According to Elsner & Scholander (1965) the size of the "drving bradycardia in man is more marked if the breath is held with the face immersed in water than when the breath is held in air an observation suggesting involvment of one and the same type of reflex in the duck and man.

Olsen, Fanestil & Scholander (1962) found rather severe cardiac arrhythmia during breath-hold drving, and recently Wolf (1963) mentioned the possibility of the above suggested mechanism of "diving bradycardia" in man being similar to a mechanism of sudden, unexpected death by cardiac arrest. Lamb et al. (1958) found malignant, cardiac arrhythmia to occur in air pilots during breath-holding and also concluded that malignant arrhythmias might be responsible for cases of sudden, unexpected death.

Despite extensive zoo-physiological work on bradycardia the eliciting mechanism is still obscure (Scholander 1962, Irving 1964) Even in the duck, which has been studied most, the nature of the receptors involved is

unknown (Andersen 1966) It therefore seems unwarranted—though commonly done in the literature—to compare the bradycardia observed in humans during breath hold diving to the diving bradycardia in animals.

Experiments were carried out on 30 subjects to elucidate the nature of the bradycardia reported to occur in human subjects during breath-hold diving Preliminary studies showed that the response of the heart to breath-holding, varies with the intrapulmonic pressure and the ambient temperature. The pressure measured at the mouth was taken as the intrapulmonic pressure during breath-holding, an assumption which obviously require an open glottis to be correct. During these preliminary studies of 25 other subjects, electrocardiograms (paper speed 25—50 mm/sec) were obtained with information about the character of the cardiac arrhythmia found in a group of young normal subjects performing breath-holds in air and water. —Since previous investigators had believed both the "face immersion bradycardia" and the bradycardia described in totally submerged human breath-hold divers to be of similar origin as the diving bradycardia of animals (Elsner et al. 1965 Wolf 1965 Buck 1966 and Corriol et al. 1966), two experimental series were performed. In the first of these the heart rate was measured in the prone position during breath-holding with the face immersed in water at different temperatures. In the second the heart rate was measured during breath-holding, while the subject rested for 30 minutes in water up to the neck.

Procedure

The 30 subjects used were healthy volunteers aged 21 to 52 years. Seven of these were good swimmers (*i.e.*, they swam in a swimming pool or in the sea at least once a week all the year round) All breath-holds were performed after one deep inspiration (see below) and no previous hyperventilation was permitted. The breath-holding time, like the duration of the other manoeuvres, was always 60 seconds. After a single, deep inspiration—without previous overbreathing—they could hold their breath for 60 seconds without involuntary contraction of the diaphragm, as judged by the constant intrapulmonic pressure. Although the subjects claimed that they initiated the breath-hold after a maximal inspiration measurements indicated that 0 to 16 / of VC was still in reserve at the actual inspiratory levels achieved. The subject inspired from the functional residual capacity level (FRC) before each breath-hold. The inspired lung volume in each individual was reproduced with great constancy Thus it is likely that the subjects did not always achieve total lung capacity (TLC) prior to the

breath-hold, but for the sake of simplicity the initial lung volume (mean 90 % of VC, as judged from measurements from the RV level in breath-holds where only volume was measured) in the following is referred to as 'TLC'

The heart rate is here used as the total number of heart beats (all R—R distances) counted in 60 second periods. Even during quiet breathing counting of the number of heart beats for periods of less than 20 seconds showed an unacceptably poor reproducibility. A period of 60 seconds was therefore used. The response of the heart to a specific manoeuvre was expressed as the percentage change occurring during a 60 second manoeuvre, following a 60 second resting control period. Unless otherwise indicated, 30 observations were performed in each group of experiments on the 30 subjects. The mean percentage change ± the standard error (SE) is shown throughout, except in Fig. 20, where the standard deviation (SD) is indicated. In the present text apnoea is defined as a breath-hold without a marked increase of the intrapulmonic pressure. The subjects were instructed to arrest breathing with the respiratory muscles relaxed, but as judged from the intrapulmonic pressures (0 to 5 mm Hg) the subjects were often unable to relax their inspiratory muscles completely (Chapter 1). Breath-holding with a marked increase of the intrapulmonic pressure is called a 'Valsalva experiment or manoeuvre'. As mentioned above, two groups of experiments were performed.

I. Prone position (face immersion and controls)

The subject was placed as shown in Fig. 19 with the head supported by a sling covering the eyes. Before and during the experimental period a noise from a ventilator masked disturbing sounds. The nose was closed by a clip. During breath-holding the subject maintained an intrapulmonic pressure of zero to 5 mm Hg. The subject was studied under the following conditions: 1) apnoea plus face ventilation (see below), 2) apnoea plus face immersion, 3) face ventilation without apnoea, 4) face immersion without apnoea, 5) forearm immersion without apnoea, 6) mental stress.—An assistent started each of the manoeuvres by counting aloud the last 5 seconds down to the time zero.

II. Supine position (whole body immersion and controls)

Experiments were performed with the test subject in a bath with (or without) circulating thermostatically controlled water, in order to study the pulse changes during breath-hold diving. During the control periods —of 2 min duration—the subject rested in water up to the neck (Fig. 20).

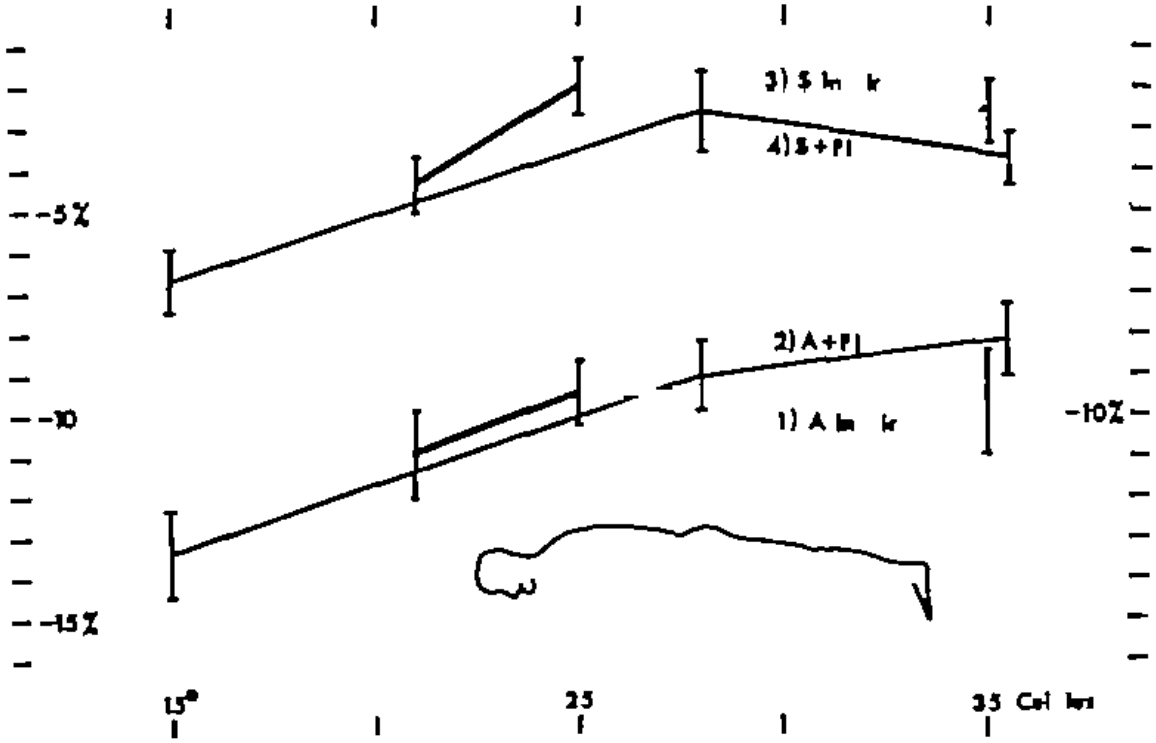


Fig. 19 The abscissa is the water or air temperature.
The ordinate is the percentage decrease of the resting, control heart rate at the different manoeuvres.) Apnoea in air (room air and face ventilation"),) Apnoea plus face immersion in water 3) Snorkel breathing in air 4) 'Snorkel' breathing plus face immersion in water —At each point is shown the mean value of 5 observations (from 30 subjects), except at apnoea in warm air (30 observations only) and in room air (97 observations). The vertical bars indicate the standard error of the mean (SE) The average resting (control) pulse is 75 beats/min.

The apnoea was performed by submersing the head in addition. The subjects spend a total of 30 min in the bath. Two series were made (A. and B) In A. the water temperature was kept constant (35 5 C) at an approximately neutral range" (Craig & Dvorak 1966) and the intrapulmonic pressure was varied from one breath-hold to the next. In B an "approximate neutral (*i.e.* zero to +5 mm Hg) intrapulmonic pressure was maintained during apnoea, and the water temperature was varied. Further details of the procedure are published (ref. *D*)

Results and Discussion

Studies on subjects in the prone position (face immersion)

At an intrapulmonic pressure of +5 mm Hg breath-holding in room air lowered the heart rate by 9 5 % (7 beats per minute, Fig 19). Essentially

the same response of the heart was found during apnoea with face ventilation in warm air (35 C) and during apnoea with face immersion in 28°C and 35°C water (Fig 19) These reactions may be explained as a response to the breath-holding *per se* Apnoea with the face immersed in cold water evidently adds further bradycardia to the apnoic bradycardia and ventilating the face with cold air (21 C) also seems to be additive (Fig 19). For technical reasons it was not possible to obtain the desired low air temperature of 15°C, but Fig 19 like Table 7 inevitably leaves the impression of the response of the heart rate being dependent on the ambient temperature, the temperature of the medium surrounding the face possibly being of particular importance. It thus seems possible to explain the bradycardia occurring as a response to the manoeuvres in the prone position as a combination of an apnoic bradycardia (see below) and a cold bradycardia

The 7 good swimmers showed a mean pulse response of −19 % (−15 beats/min) during apnoea with the face immersed in 15°C water and a −7 % response during apnoea in air. Their apnoic bradycardia then does not seem to deviate much from the average for the whole group (−9.5 %) In contrast with the claim by Corriol et al. (1966) who reported that the bradycardia due to apnoea (intrapulmonic pressure was not measured) has a greater size in trained swimmers the more pronounced cold bradycardia in our swimmers may simply be due to a smaller emotional-intellectual inhibition of the bradycardia, because swimmers are more used to cold water

The effect of local cooling of the face *per se* is apparent from results obtained in experiments where such cooling was produced by water or air in subjects continuing to breathe (Fig 19) in those cases (face immersion in water or 'face ventilation in air) the subjects were breathing through a respiration valve (von Döbeln type) with two rubber tubes of 30 cm in length (a two way snorkel without dead space) —Immersion of the forearm in 35°C water elicited a heart rate decrease of 2.4 % from the resting control pulse (average 75 beats/min ±2.2) while forearm immersion in 15°C water produced a 3 % decrease, when the usual counting down procedure was used. Identical figures were found when the immersion was performed without counting down aloud.—The subjects faced with an arithmetical problem all responded with considerable *tachycardia* to this—emotional—stress (+17 2±1.0 %)

During the above six manoeuvres the resting, control heart rate of the 30 subjects averages, as mentioned, 75 beats/min, with the subject at room temperature, dressed and prone. The 7 accomplished swimmers also had a mean resting heart rate of 75 but with a SE of ±5 9 beats/min. Thus

with a few exceptions the control pulse was below 80 beats/min. In each subject the control heart rate remained essentially the same during the 30 min procedure.

STUDIES ON SUBJECTS PLACED SUPINE IN A BATH (WHOLE BODY IMMERSION)

A. The recordings in the 6 subjects who kept their breath at different intrapulmonic pressures in a bath with (35 5 C water) or without water are shown in Fig 20 It is seen that the pulse response of the heart to apnoea is dependent upon the intrapulmonic pressure. Between −5 and +5 mm Hg, there is slight bradycardia and above +10 mm Hg the response becomes one of tachycardia, increasing almost linearly with the increase in intrapulmonic pressure. The relationship is present, whether the subject is in air or in water, though the slope of the curve was smaller for breath-holding in air than those for breath-holding in water The difference between the results obtained with water up to the neck and those obtained with total immersion of the head and body is very small and not significant.

B In the 100 procedures on 5 subjects resting supine in 35 C water up to the neck, apnoea suppressed the heart rate by 6 % (4 beats/min), compared with 14 % (10 beats/min) when the subjects were in 22°C water (Table 7). This difference is statistically highly significant ($p<0.001$) The difference is not correlated to changes in the respiratory pattern of the control periods or to the duration of the stay in water (Table 8) Thus also during whole body immersion the apnoic bradycardia can be augmented by exposure of the skin to cold water

Strangely enough breath-holding with only the face immersed produces a response of the pulse (14 %=10 beats/min) equal to that of apnoea with the subject up to the neck in cold water This might be explainable by the

Table 7 Heart rate response (percentage decrease of resting heart rate) upon apnoea lying supine in water

Water temperature	22.0°C	35 5°C
Water to the neck:	−14±1 5 %	−8±1.4 %
Total immersion:	−14± .0 %	−4±1 7 %
Sum:	−14±0.9 %	−6±1.0 %

All breath-holds were performed with an intrapulmonic pressure of zero to +5 mm Hg The results are mean values ±SE from five representative subjects. Five identical observations were performed on each subjects, yielding total number of 25 observations in each of the four groups (*i.e.* 5 observations at each temperature) The resting (control) heart rate is 71±0.7 beats/min in the warm and 75±0.8 beats/min in the cold water

Table 1 Respiration and heart rate response during apnoea.

	First	Last
Respiratory rate (breaths/min):	19.6±0.7	17.2±0.6
Respiratory depth (cm amplitude):	2.5±0.2	3.9±0.4
Heart rate response (% change):	−15.2±1.4	−14.3±1.1

The values are means of 25 observations in each group, and the SE is indicated. The first and the last half part of the immersion period (whole body immersion in 22°C water) is compared. The average resting (control) heart rate is 73 beats/min.

fact that in the case of the face the cold stimulus (15 C) was suddenly applied simultaneously with the beginning of apnoea, while in the body immersion experiments (in water of 22 C) the skin of the body had been exposed to cooling for a considerable time before the apnoea, *i.e.*, the skin (body) had had time to adapt itself to the lower temperature in the later case, but not in the former.

Previous reports often fail to include the information about the intrapulmonic pressure during breath holding and the water temperature. A detailed discussion of such reports is not considered pertinent, as these two variables, demonstrated in the present study to affect the heart rate during breath-holding, were either not measured or were not varied with the aim of studying their effect on the heart rate. A similar argument has eliminated an extensive number of reports from the discussion in Chapters 6 & 7

Cause of "apnoic bradycardia" and "diving bradycardia"

The bradycardia during apnoea at intrapulmonic pressures around zero and the relative tachycardia during breath-holding at high pressures (Fig 20) can be explained by the following mechanism. At intrapulmonic pressures of zero, the intrathoracic pressure (*i.e.*, the pressure in the pleural space minus the atmospheric pressure) is approximately −10 mm Hg at 90 % of VC ("TLC") (Otis et al. 1946a, Knowles et al. 1959, Mead & Millic Emili 1964) This intrathoracic pressure is somewhat lower than the pressure with the lungs in the mid-position, such as in the control period, and *by itself* adds a stimulus to the baroreceptors of the aortic arch. Furthermore, by facilitating the diastolic filling and the venous return to the right half of the heart it should, according to Starling's law (Starling 1918)

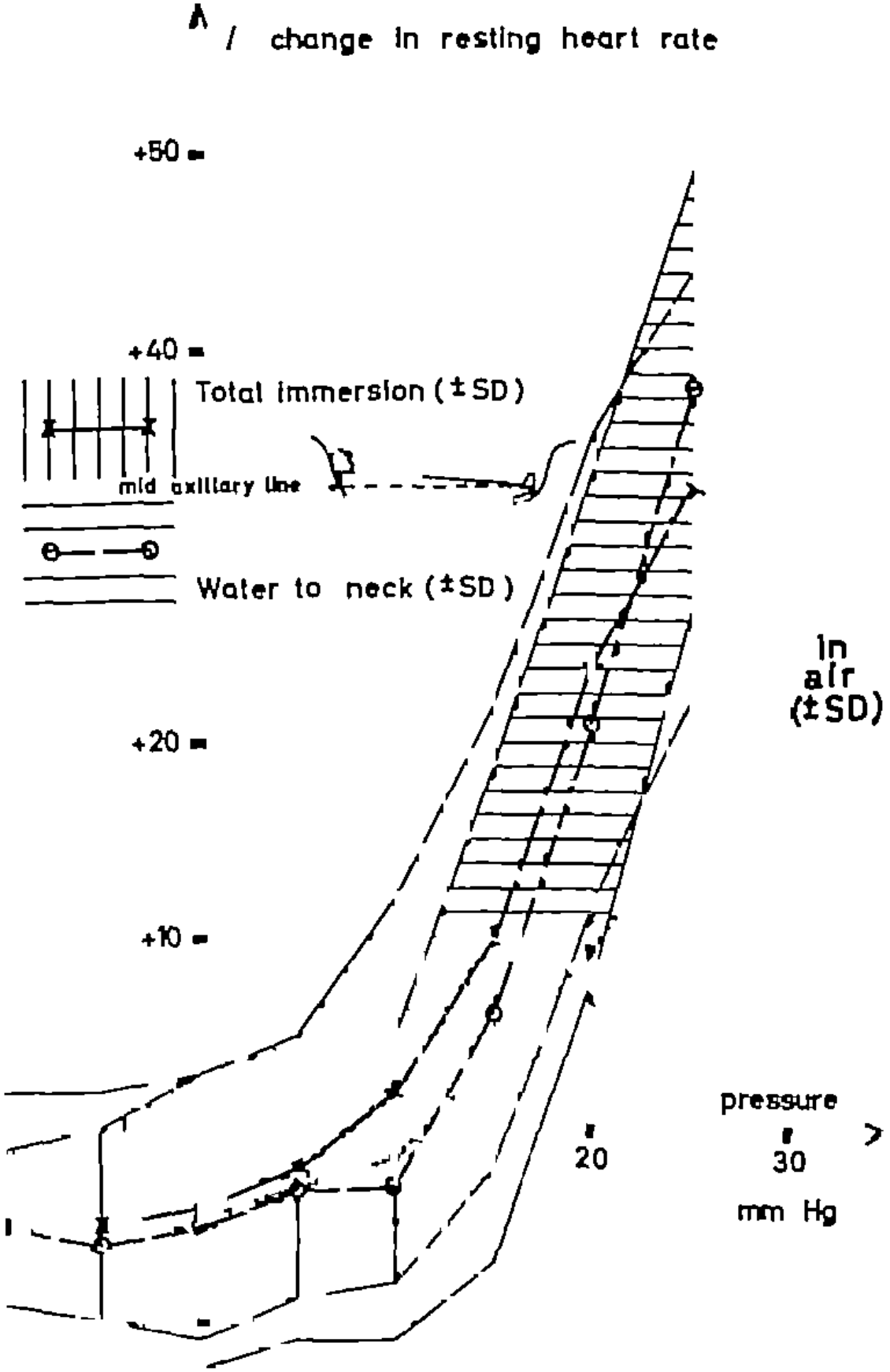


Fig 10 The mean heart rate response (±SD) to breath-holding varying with the intra-

increase the stroke volume, leading—with a short latency—to a rise in systemic arterial pressure. This would add a further stimulus to the baroreceptors (aortic and carotid), which might in turn explain the bradycardia actually observed during such breath-holds (Fig 20) At an intrapulmonic pressure of +8 to +12 mm Hg, no pulse response is to be expected, since the intrathoracic pressure is of the same—slightly negative value—as in the normal midposition in the control periods. It is in keeping with this argument that no alteration of the heart frequency was found in any of the 3 experimental series at breath-holds at an intrapulmonic pressure of +8 to +12 mm Hg (Fig 20) On the other hand, when a subject holds his breath at 'TLC' and with an intrapulmonic pressure of +20 to +30 mm Hg, the intrathoracic pressure will be positive (+10 to +20 mm). Thus, under such conditions the situation will be the reverse of that considered first, *i.e.*, there will be an immediate lowering of the stimulus to the aortic receptors followed by a lowering of the stimulus to the baroreceptors because of a fall in systemic arterial pressure due to impaired venous return to the heart and a fall in stroke volume. In fact, the tachycardia expected occurred under such conditions (Fig 20).—Measurements of blood pressure during breath-holding (14 subjects) in this laboratory showed mean pressures of +1 to +5 mm Hg in intrathoracic veins, and an increase of the mean arterial blood pressure (direct) of 30 % from the resting control values, when breath-holding was performed at 'TLC' with an intrapulmonic pressure of zero Breath holding at 'TLC' and an intrapulmonic pressure of +20 to +30 mm was accompanyed by an initial fall of 35 to 47 % of both the pulse pressure and the resting mean arterial blood pressure. There is adequate stimulation of the high pressure baroreceptors to explain the changes observed in heart rate (Chapter 7).

Judging from the present results, the bradycardia described in the literature as occurring in humans during breath-hold diving can be interpreted as consisting of two independent components an apnoic bradycardia varying in severity with the intrapulmonic pressure (Fig 20) the other, a cold bradycardia varying in severity with the temperature of the water. The cold bradycardia demonstrated in man in the present investigation is different from the temperature-*independent* (Andersen 1963)

pulmonic pressure. The water temperature was 35 5 to 36°C, and the room temperature was 25°C. The curves are the mean values of twelve observations in six subjects. The mean resting, control heart rate is 7 beats per min.

"diving bradycardia" described in ducks In the light of these considerations Irving's generalisation (1939)—a *diving bradycardia reflex* common to animals and man—appears unfounded. Raper et al. (1967) found no evidence of oxygen conserving reflexes in man during breath-holding in air at an initial lung volume of FRC, and at an airway pressure which they assumed to be zero Already Mutdaugh et al. (1961) suggested that the bradycardia reported in some vertebrates may reflect nonspecific effects, rather than a "true diving adaptation"

Cardiac arrhythmia and other ECG abnormalities

Five persons out of 25 studied electrocardiographically (Elema Schomander mingographs) in a previous project, showed definite rhythm disturbances during breath-holding (atrial premature contraction, partial sino-auricular block without symptoms) Seven subjects out of 25 showed no sign of cardiac arrhythmia, and the remaining 18 showed occasional sinus arrhythmia during the deep respirations before and after the breath-holds. The sinus arrhythmia occurred in the subjects both in the air and in water and was seen both before and after the breath-holds. *During* the breath-holds several ECG wave alterations were seen Low voltage, isoelectric P_{III}—Q_{III}—T_I Q_I and Q_{II} of 5 mm, prolonged QRS and RS-T depression. *After* the breath-holds the ECG assumed its original appearance, apart from some deep (3—5 mm) Q_I and Q_{II} in a few subjects.

Sudden death

Olsen et al. (1962) found premature ventricular beats to be more common than premature atrial beats in pearl divers. Premature beats of ventricular origin are often more malignant (*i.e.* often associated with a severe decrease in the functional capacity of the heart) than those of atrial origin (Cecil & Loeb 1959) Lamb et al. (1958) also reported serious arrhythmia following breath-holding These findings suggest that breath hold diving carries substantial risks. Lamb et al (1958) and Wolf (1965) felt that cardiac arrhythmia during breath holding, especially the "diving bradycardia" might become severe enough to arrest cardiac activity and cause sudden death. But no severe arrhythmia could be demonstrated in more than 2,000 dives performed by the present 25 young subjects The sinus arrhythmia in 18 of the 25 subjects, the occasional premature beats of atrial origin in 3 and the ventricular beat observed in one, is of no importance (Cecil & Loeb 1959). The ECG wave alterations described reflect only the respiratory effort of the manoeuvres. The increased intrathoracic ("intratruncal")

pressure during the Valsalva manœuvre (*i.e.*, during breath-hold diving micturition, defecation, strenous lifting etc.) is normally not dangerous in man, but the effect of the stress on the circulation may be quite different in patients with severe myocardial damage (Wolf 1965) Judging from the observations made in the present young normal subjects accidents during breath holding and skin diving are probably due to loss of consciousness caused by hypoxia and hypercapnia (Chapter 4) rather than to cardiac arrhythmia.

CHAPTER 6
Cardiac output

The changes in blood pressure and heart rate during and after a Valsalva manoeuvre have been studied by among others, Hamilton et al. (1936) Dornhorst et al. (1952) Candel & Ehrlich (1953), and Sharpey-Schafer (1955) but the effect of such manoeuvres on the cardiac output ($\dot{Q}$) in normal man has received only little attention. Otis et al. (1946 c) found a decrease in stroke volume during a Valsalva manoeuvre in 2 subjects examined with a ballistocardiograph.

The changes in heart rate during breath-holding (Chapter 5) made it reasonable to measure the cardiac output and the blood pressure, in order to study further the mechanism of the observed variations.

The method is described in Chapter 2 and the procedure has been published (ref. *E*). The experiments were performed in association with Dr. H. Wetterqvist in Lund 1965

Results

Out of the 8 subjects examined, 4 (group A) made Valsalva manoeuvres after three deep inspirations ("TLC") with intrapulmonic pressures of about 20—30 mm Hg, thereby increasing the mean central venous pressure to 12—18 mm Hg (Fig 21 and 23) During the first few seconds of the manoeuvre a moderate rise in the arterial pressure was observed (Fig 21), but it was quickly followed by a fall of the mean arterial blood pressure to some 75 % of the control level. The pressure in the brachial artery is assumed to be representative of the arterial systemic pressure.—The other 4 subjects (group B) held their breath after three deep inspirations (also "TLC") with intrapulmonic pressures about 0 to 10 mm Hg Such breath-holds did not influence the mean central venous pressure (control values 1 to 5 mm Hg). The manoeuvre started with a 15 % fall of the arterial mean pressure (Fig 22) All mean pressures were recorded with an electronic integrator. A detailed description of the pressure curves has been published (ref. *E*).

In the subjects in group A who performed Valsalva manoeuvres, a fall of cardiac output was observed, while in those of group B this variable was found to be higher during breath-holding (Table 11) than the control

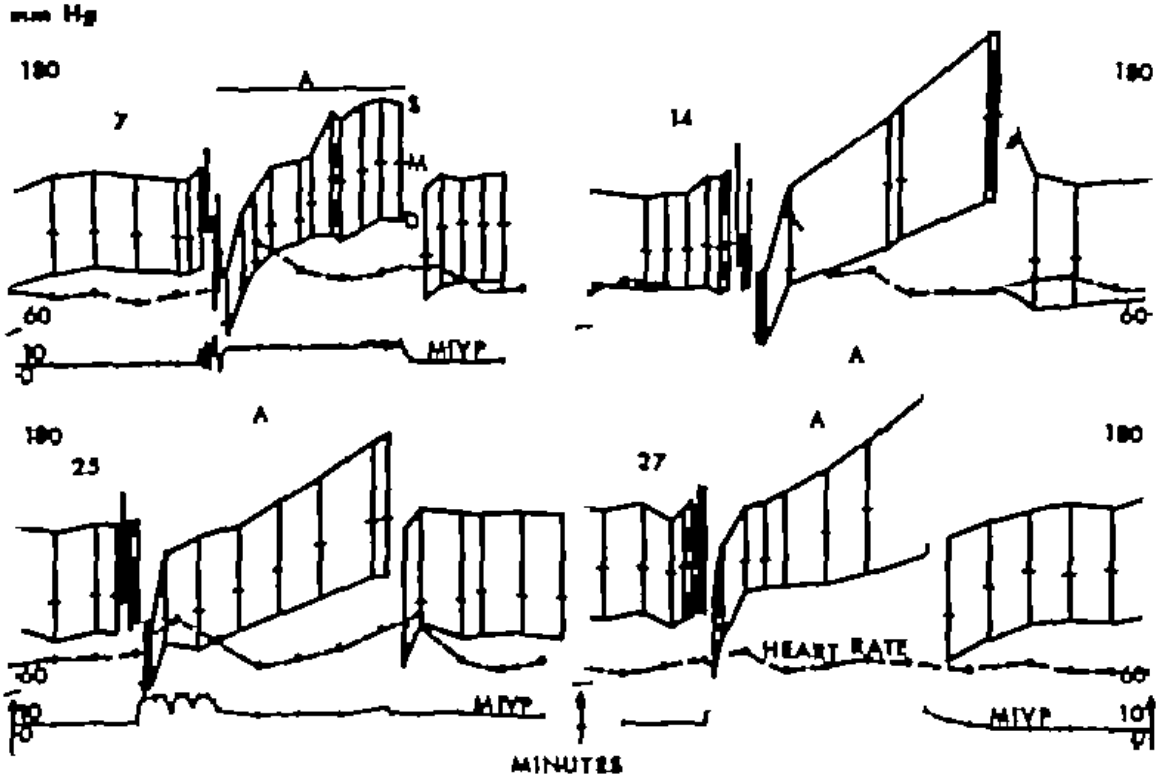


Fig. 21 Systolic (S), diastolic (D) and mean (M) brachial artery pressure, heart rate (broken curves), and mean intrathoracic (central) venous pressure (MIVP) of the group A subjects performing Valsalva manœuvres (A). Ordinate: mm Hg and beats/min. Pressure oscillations from the three inspirations before the manœuvre and from involuntary contractions of the diaphragm in the last part of the manœuvre, are interposed on the venous curve of subject 7. Central venous mean pressure of subject 14 was not recorded in this experiment, but it was usually about +2 mm during rest and rose to about +12 mm Hg.

level (Table 11). A decrease of $\dot{Q}$ at the commencement of the Valsalva experiment corresponded to a decrease in stroke volume in group A (Fig. 24, Table 11). Apnoea (group B) produced only a slight alteration of the stroke volume (Table 11). The stroke volumes of Table 11 are—for the sake of simplicity—converted to percentage changes from the control values (Fig. 24). Group A consists of subjects with relatively high resting cardiac indices (liter blood/min $\times$ m^2 body surface area), while group B consists of subjects with somewhat lower indices (Table 9).

Analysis of the arterial blood gas tensions, pH and base excess showed the oxygen partial tension and the pH decreased, while the carbon dioxide partial tension increased, the change varying with the duration of breath-holding (Table 10). The base excess showed no consistent changes during the breath-holds.

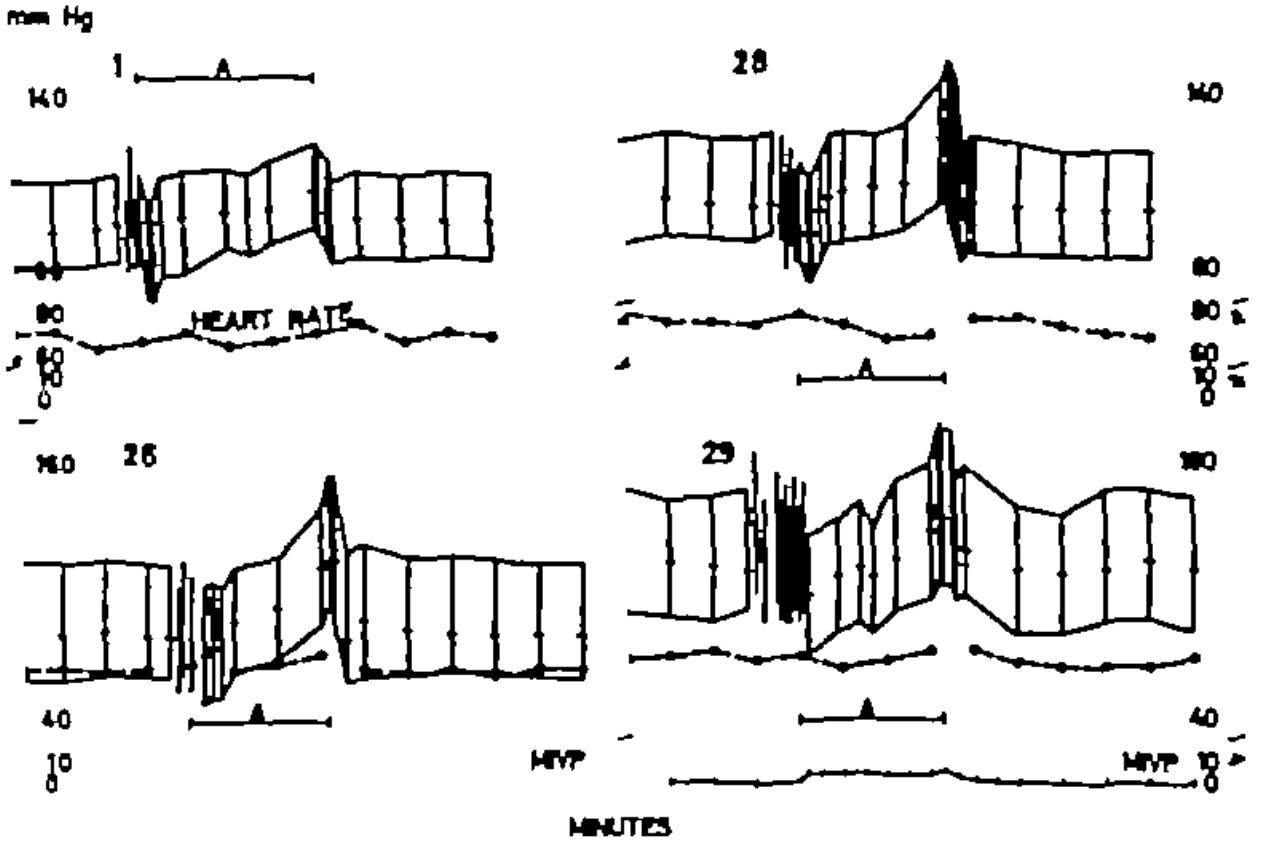


Fig 22. Results from apnoea (group B) with the same variables as in Fig. 2 The venous pressure of subject 1 did not vary with the alterations in intrathoracic pressure with deep inspirations, and was assumed to be peripheral venous pressure (catheter advanced 5 cm).

Table 9 Characteristics of the test subjects.

Subject	Height/weight cm/kg	Body surface area m^2	Resting heart rate beats/min	Resting cardiac index $l/min \times m^2$
7 (A)	87/81 7	2.07	68	3 8
4 (A)	177/76.4	1 93	69	5.9
5 (A)	187/91.0	8	63	3.6
7 (A)	176/78.0	2.00	56	4.5
(B)	64/57.9	.63	63	3.2
26 (B)	79/78.0	.97	66	3 5
28 (B)	170/60.	.69	66	3.4
29 (B)	180/67 5	1.86	67	6

Calculated from the monogram of DuBois and DuBois (Arch Intern. Med. 17 863 196).

Discussion

The variables measured in this and the following Chapter should preferably be measured simultaneously The $\dot{Q}$ ought to be measured during the first 30 seconds of the breath-hold in an attempt to get the greatest variation per unit of time, and preferably at different levels of intrapulmonic pressure and lung volume during breath-holding A beat to beat registration of stroke volume would be even more useful, instead of the present indicator dilution technique. If the present method is used in a period, where $\dot{Q}$ is steady and where recirculation is avoided, it can—at best—measure $\dot{Q}$ rather inaccurately When $\dot{Q}$ is thought determined repeatedly during a Valsalva manoeuvre it is reasonable to assume that none of these provisions are fulfilled. However, the indicator dilution technique was available in Lund as a routine method, and it was assumed acceptable for the purpose, which was a rough estimate of relative changes in $\dot{Q}$ As it will be shown in Chapter 7 a simultaneous measurement of blood pressure and limb blood flow has later been performed.

During breath-holding at high intrapulmonic pressures (+ 20 mm Hg or more) the vessels running to the right atrium will be considerably compressed, with diminuition of the venous return, and according to

Table 10 Blood-gas and acid-base values.

Subject	Breath-hold sec[1]	P_aO_2 mm Hg	P_aCO_2 mm Hg	pH	Base excess meq/l blood
7 (A)	130	84 to 60	41 to 46	7 39 to 7 34	−0.5 to −1 5
	130	86 to 60	39 to 45	7.40 to 7 35	−0.5 to −2.0
4 (A)	170	87 to 56	44 to 54	7.40 to 7 3	0 to −0.5
25 (A)	150	92 to 55	4 to 54	7 59 to 7 55	−1.0 to −1 5
27 (A)	75	93 to 74	55 to 45	7 57 to 7 53	−2.0 to −2.0
1 (B)	105	92 to 66	40 to 47	7 59 to 7 35	−1.0 to −1 5
6 (B)	60	95 to 70	42 to 49	7 56 to 7 3	−1.0 to −2.5
8 (B)	60	100 to 72	38 to 43	7 58 to 7 36	−3.0 to −1 5
29 (B)	65	99 to 75	39 to 48	7 39 to 7 35	− 5 to −1.0

[1] Duration of the breath-hold at the time of sampling In each group the first figure refers to the control value, while the second (after 'to') refers to the value obtained during the breath-hold. Subject 7 was studied twice.

Table 11 Heart frequency (p) in beats/min, stroke volume (sv) in ml, and cardiac output ($\dot{Q}$) in liters/min measured before (Rest), during and after a breath-hold.

Subject		Rest			During breath-holding early			During breath-holding late		After breath-holding	
	p	72	64	65	72	72	67	80	88	65	69
7 (A)	sv	115	111	109	68	74	84	81	99	129	119
	$\dot{Q}$	8.3	7.1	7.1	4.9	5.5	5.6	6.5	8.7	8.4	8.2
	p	69	68			78		70		74	70
14 (A)	sv	109	110			78		99		135	117
	$\dot{Q}$	7.5	7.5			6.1		6.9		10.0	8.2
	p	67	66	63		60		58		61	
23 (A)	sv	119	108	124		105		124		148	
	$\dot{Q}$	8.0	7.1	7.8		6.3		7.2		9.0	
	p	54	56	52		52		55		58	58
27 (A)	sv	163	159	164		146		167		207	176
	$\dot{Q}$	8.9	8.9	8.5		7.6		9.2		12.0	10.2
	p	64	63	61	71	68				65	
1 (B)	sv	86	81	82	82	78				9	
	$\dot{Q}$	5.5	5.1	5.0	5.8	5.5				6.0	
	p	66	67	65	72	69				65	
26 (B)	sv	104	102	97	100	100				112	
	$\dot{Q}$	6.9	6.8	6.3	7.2	6.9				7.5	
	p	67	64	68	69	68	71			73	
28 (B)	sv	88	89	81	91	87	8			92	
	$\dot{Q}$	5.9	5.7	5.5	6.5	5.9	5.8			6.7	
	p	73	69	71	100	102				71	69
29 (B)	sv	67	74	65	65	74				80	97
	$\dot{Q}$	4.9	5.1	4.6	6.5	7.5				5.7	6.7

During the breath-hold, $\dot{Q}$ was measured approximately 40—70 seconds (early)—and in group A also 120—15 seconds (late)—after the commencement. Each subject was studied during —5 breath-holds.

Starling's law it is reasonable to expect a decrease of the stroke volume of the right half of the heart (Table 11 see also Chapter 5) Bolt et al. (1955) believed that the normal flow equality in output from the left ($\dot{Q}_L$) and the right ($\dot{Q}_R$) half of the heart, is preserved during a Valsalva manoeuvre. However, Cudkowicz and O'Neill (1965) have measured $\dot{Q}$ in patients, with a single injection of Coomassie Blue into the superior vena cava, and

simultaneous withdrawal from the pulmonary artery representing $\dot{Q}_R$ and the femoral artery representing $\dot{Q}_L$, during short voluntary apnoea with open glottis, and lung volumes about TLC, FRC and RV they found a rather great difference between $\dot{Q}_L$ and $\dot{Q}_R$ and postulate a temporary disequilibrium during breath holding at TLC. At the RV level both $\dot{Q}_R$ and $\dot{Q}_L$ decreased ($\dot{Q}_L$ most). A corresponding decrease was recently found in a study of the $\dot{Q}$ measured with the pressure-gradient technique during short Valsalva manoeuvres at an intrapulmonic pressure of +40 mm Hg (Greenfield et al. 1967) no information of the lung volume was reported. A 50 % decrease of the $\dot{Q}$ was found at the end of the manoeuvre. The pressure-gradient technique measures simply the difference in lateral pressure above the aortic valve and 4 cm from this point via a double-lumen catheter introduced percutaneously into the femoral artery and advanced to the aortic location. By a modified Poisseuille equation the aortic blood flow is calculated from the pressure difference per unit of length and an angiographic estimate of aortic radius. During periods where the mean arterial pressure and the aortic radius does not vary appreciably this method may well provide acceptable results for stroke volume of the left ventricle. However during a Valsalva manoeuvre both these values decreases considerably so even relative variations must be considered with reservation, also as far as this method is concerned. Nevertheless, all of the above mentioned methods—including the present (Fig 24, group A)—indicated a decrease in stroke volume during a Valsalva manoeuvre, as already predicted in the preceding Chapter, so the results seem acceptable in principle. —The first few seconds of a Valsalva manoeuvre are accompanied by a rise of the arterial pressure owing to the increase in the intrathoracic pressure (Sharpey-Schafer 1965). A few beats later this increase begins to disappear. Both the mean arterial blood pressure and the pulse pressure are reduced (Fig 21). The considerable fall in these pressures must be accompanied by a fall in impulse frequency from the baroreceptors in the carotid sinuses and from those in the aortic arch, where the increase in external pressure further decreases the transmural arterial pressure. Thus, the "negative stimulus" of a Valsalva manoeuvre to the sino-aortic baroreceptors must be extraordinary strong The carotid sinuses are generally regarded as being very sensitive in vagotomized cats a maximal "amplification factor" of 15 mm systemic pressure change per mm pressure change in the carotid sinuses, has been described (Scher & Young 1963). The medullary vasomotor and cardiac control centers respond with an arteriolar and possibly a venous constriction (Wallis et al. 1963) which will be considered in the next Chapter.

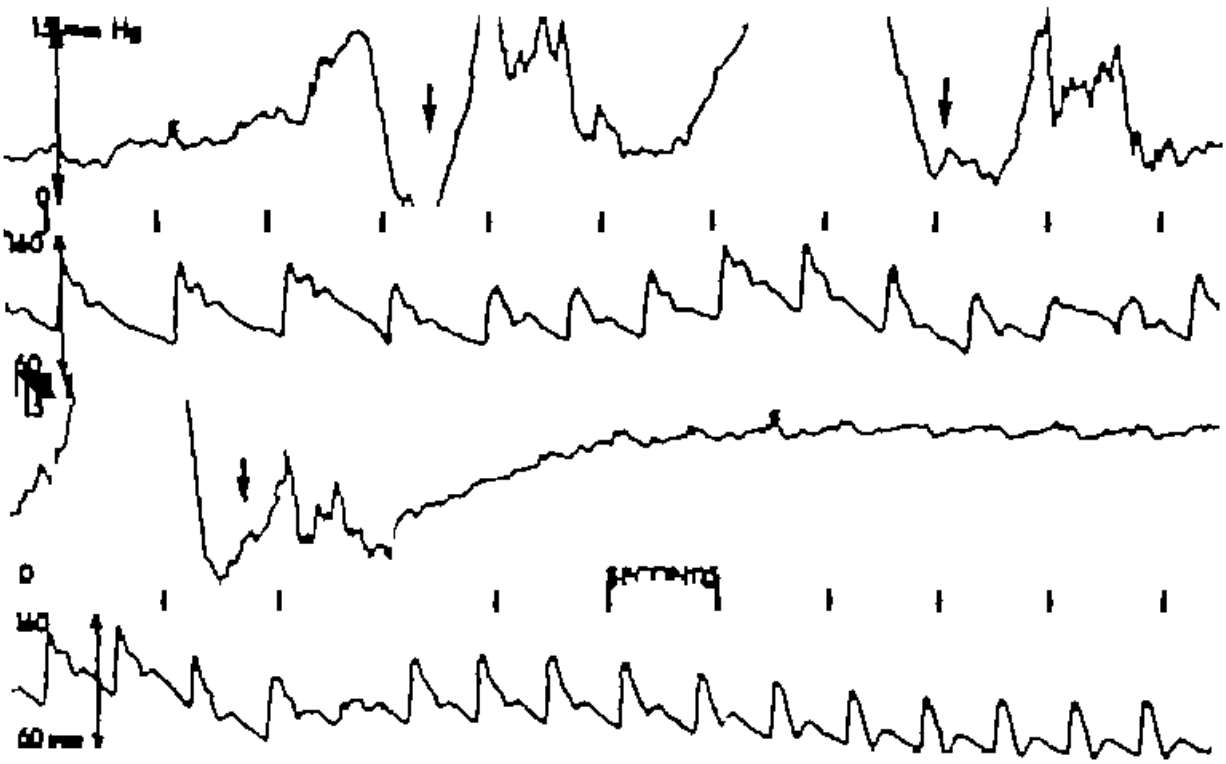


Fig 23 Blood pressure records from an intrathoracic vein and the brachial artery of subject 7 Arrows indicate the three, deep inspirations before the Valsalva manœuvre. The venous pressure follows the alterations of the intrathoracic pressure, which shows that the tip of the catheter is in the thorax. The ordinate is mm Hg, the abscissa is time in seconds, and the vertical line indicate the onset of the Valsalva manœuvre.

The subjects of group B performed the breath-hold with intrapulmonic pressures (*i.e.* apnoea) corresponding to an intrathoracic pressure at 'TLC' of −10 to −2 mm Hg and a mean central venous pressure of +1 to +5 mm (Fig 22). Apnoea was only accompanied by minor changes in arterial blood pressure (Fig 22), and cardiac stroke volume (Fig 24, group B) The small fall in the arterial blood pressure may be due to the preceding CO_2 elimination by the three deep respirations.

The venous return to the right heart is dependent upon a reasonable difference between the peripheral and central venous pressure (or rather right ventricular diastolic pressure). Because of the distensibility of the veins, a high intrapulmonic pressure maintained during prolonged breath-holding, would be able to deprive the heart for blood, regardless of the arterial response. However, as it will be shown later, a venoconstriction, possibly generalized, will protect the normal organism during such a condition.

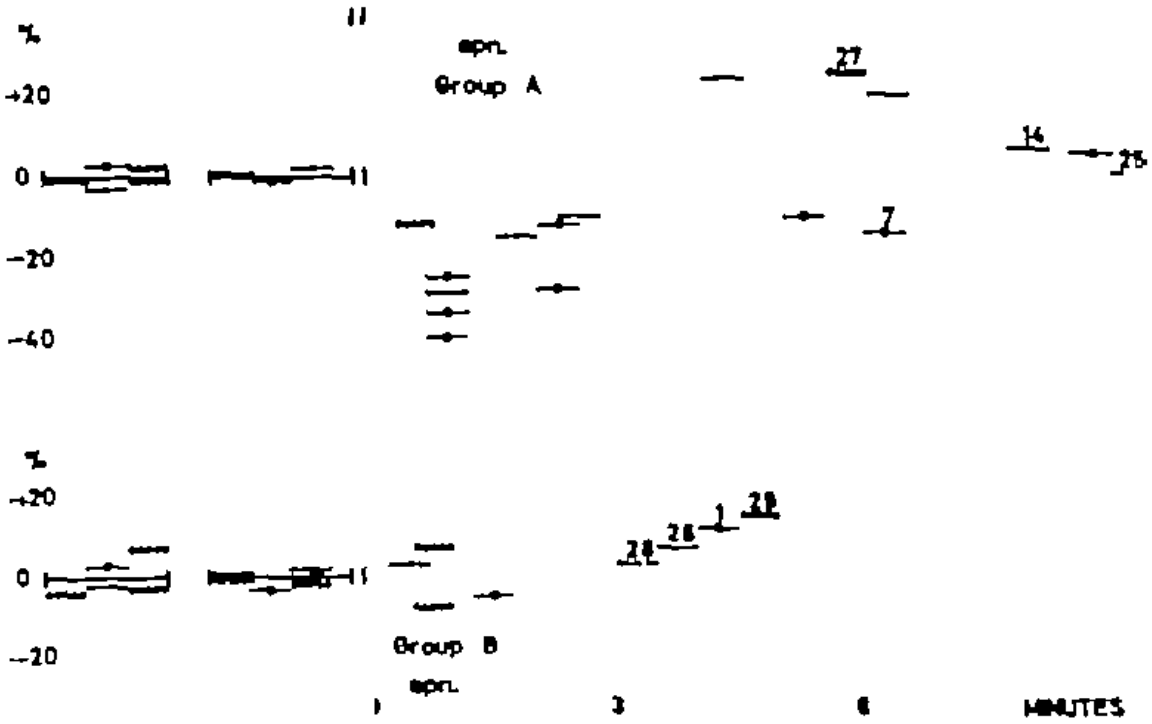


Fig 24. The percentage change in control stroke volume by breath-holding (ordinate). The time is the bachea. Before the breath-holds, the variation around the individual mean value in control stroke volumes is shown. The horizontal bars cover the periods in which the heart rate and Q̇ were determined. The figures to the right indicate the different subjects. Positive indicates higher negative lower values, than the control stroke volume.

The high P_aCO_2 (and possibly the low P_aO_2) found at the end of the prolonged breath-holds (Table 10) stimulated peripheral (and possibly central) chemoreceptors, and may be the cause of the arterial hypertension (Fig 21) —with a relative blood flow increase (Fig 25 27)– late in the breath-hold. In addition the adreno-medullary secretion may be involved possible via the sino-aortic receptors (Heymans & Neil 1958)

In conclusion the presented Q̇ determinations –although absolute values must be considered with reservation—confirmed the predictions of a small stroke volume during Valsalva manœuvres and an almost unaltered (or slightly increased) stroke volume during apnoea

CHAPTER 7
Blood pressure and limb blood flow

Although the *cardiac* function during breath-holding may seem explained in principle through Chapters 5 & 6 our knowledge of the circulatory changes occurring during apnoea and the Valsalva manoeuvre is still incomplete.

Sharpey Schafer (1955 1965) measured limb blood flow in association with the Valsalva manoeuvre, but the measurements were confined to the periods before and after rather short periods of a Valsalva experiment. Elsner & Scholander (1965) have performed some plethysmographic measurements (strain gauge) during immersion of the face in water, but the character of the breath-hold and the size of the blood flow is not clearly stated. Also Brick (1966) studied the forearm blood flow in relation to face immersion, but neither the intrapulmonic nor the intrathoracic pressure was measured during the breath holds.

It appears that no systematic investigation has been made of the human limb blood flow during breath-holding with Valsalva experiment or during breath-holding without a marked increase of the intrapulmonic pressure.

Material and procedures

A PRELIMINARY EXPERIMENTS

The first series of experiments were performed in Lund, Sweden, in collaboration with Dr I. Dahn. The subjects (three volunteers) lay supine with either both calves, or with one calf and one forearm or hand in the plethysmograph filled with water (34°C). The venous occlusion plethysmographs designed by Dahn were used (Dahn 1964, 1965). Two types of experiments were carried out

PROLONGED BREATH HOLDING. The procedure was started with a 4 minute period with intermittent registration of the control blood flow. The nose was closed with a foam rubber nose clip. The prolonged breath-holdings were performed after 3 deep inspirations. After the third, during which the subject filled his lungs, he held his breath at increased intrapulmonic pressure (Valsalva manoeuvre). With a little practice the test subjects

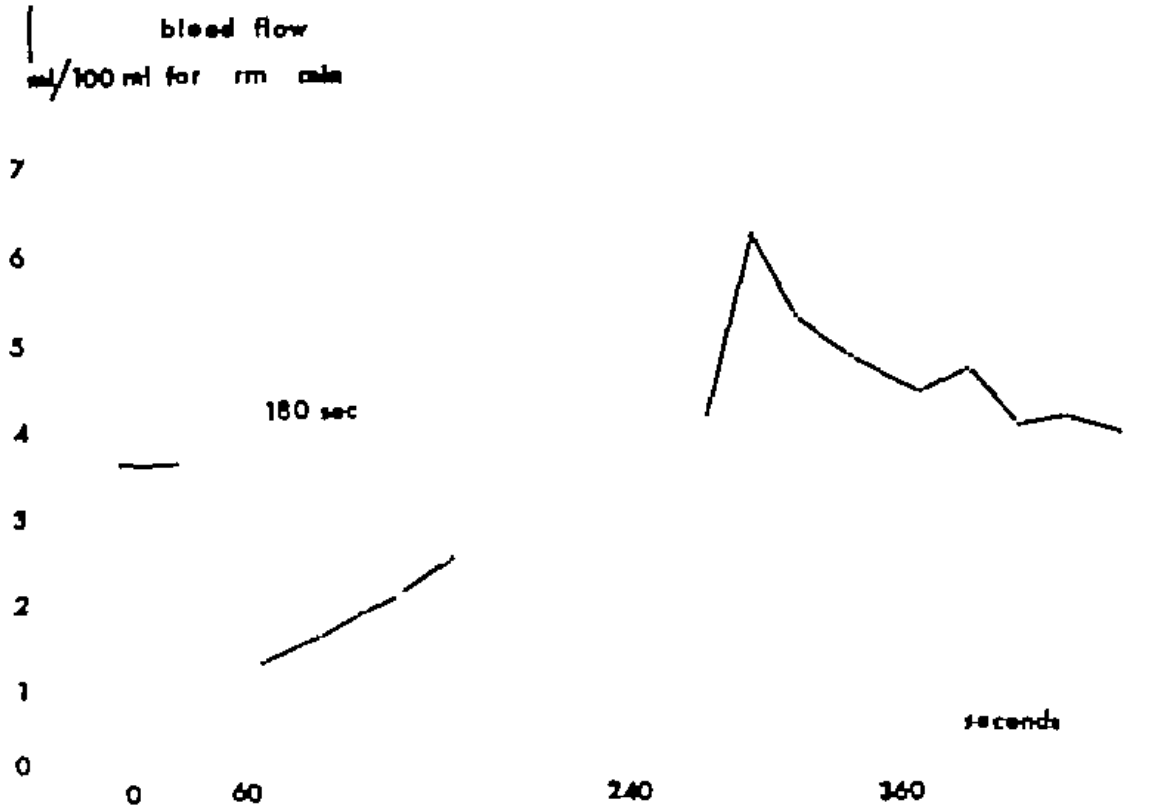


Fig 25 The blood flow in the right forearm measured before, during and after a breath-hold (Valsalva experiment at "TLC") of three minutes duration. The ordinate is ml blood/100 ml forearm × min and the abscissa is the time. Each point indicates the middle of a plethysmographic occlusion period.

increased their breath-holding time to 215 seconds. The flow of blood through the limb was measured both before, during and after each breath-holding (Fig 25 26 and 27)

PROLONGED BREATH HOLDING DURING SYMPATHETIC BLOCK. The same procedure was used here, but left stellate ganglionic block was performed beforehand, by insertion of a needle between the common carotic artery and the thyroid cartilage, and deposition of 10 ml Lidocaine hydrochloride (1 %) in the retropharyngeal space. A few minutes after the injection the solution reached the lower cervical and the upper thoracic ganglia, and a typical Horner syndrome developed. In the period from 10 to 45 minutes after the injection, plethysmographic recordings were made of the left hand or forearm (and both calves) during Valsalva manoeuvres.

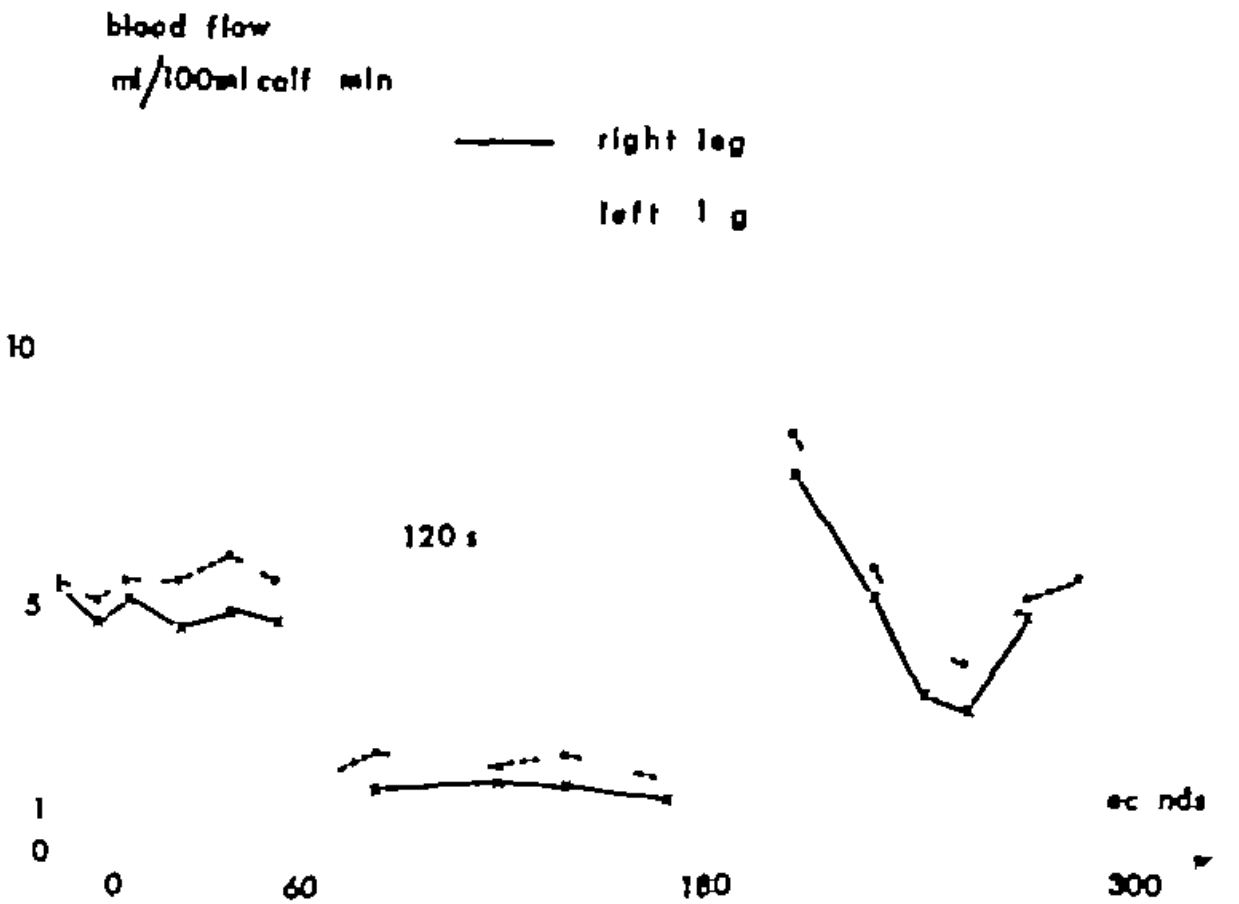


Fig. 16 Calf blood flow measured before, during and after 120 sec Valsalva experiment ('TLC')

B SUPPLEMENTARY EXPERIMENTS

The investigations performed in Lund (in 1965) were followed up by studies carried out in Copenhagen on 27 volunteers, well trained in the procedure. The observations were made in association with the development of the methods set forth in Chapter 2. Some of the results of these personal studies bearing on the limb blood flow and blood pressure during breath-holding, are given and commented below. All the subjects rested supine during the experiment (Chapter 2).

1 CONTROL EXPERIMENTS. In three subjects the venous pressure was measured via a catheter (Bardic Deseret Intracat model 1614), the tip of which was placed in a vein in the middle of the forearm segment enclosed in a Dohn (air) or a water plethysmograph. The other forearm was placed in a water or air plethysmograph, and its blood flow was measured three times each min as a control. In an attempt to avoid the usual increase in peripheral venous pressure during a Valsalva manoeuvre (Fig. 29) the veins

of the forearm were drained for blood in 30—60 seconds prior to the venous occlusion period by passive elevation of the forearm (30 cm above the horizontal level) of course, no pressure was applied to the cuffs of this arm during the elevation. Four seconds after the commencement of the Valsalva manoeuvre the arm was lowered into the horizontal position, and on the 5 sec, 60 mm Hg pressure was applied to the distal cuffs of both arms. The proximal cuffs were automatically inflated 2 seconds after the distal ones. Each person performed 9 Valsalva experiments of 1 minute duration and with 3 minute intervals. During each Valsalva manoeuvre the blood flow was measured only once in the previously elevated arm, either between the 7—15., the 27—35., or the 47—55 second. The subjects performed the same kind of Valsalva manoeuvre each time after a deep inspiration ("TLC") through a pneumotachograph (which was then closed by an assistant), the subject created an intrapulmonic pressure of 30 mm Hg —In the second phase of the control experiment a narrow cuff (6 cm) was placed around the right arm at the axilla. The cuff was inflated to 30 mm Hg for 60 seconds in order to simulate the increase in peripheral venous pressure of the limb caused by a Valsalva experiment (Fig 29) Of course, the subject relaxed and breathed normally during this simulated "Valsalva manoeuvre"

2. Standard experiments with water plethysmographic measurement of the blood flow through a forearm were carried out in 27 volunteers Each test subject held his breath at different intrapulmonic pressures, either after a deep inspiration ("TLC") or after a normal expiration (FRC) The breath-holdings lasted 60 ("TLC") or 35 (FRC) seconds, respectively During breath-holding the subject kept the intrapulmonic pressure constant at one of the following values 0, 10 20 30, or 40 mm Hg

3 Comparison The flow of blood was measured before, during and after Valsalva manoeuvres ("TLC" at 30 mm Hg). The left calf was placed in a Dohn plethysmograph and the right forearm in a water plethysmograph (34 C) The left forearm was surrounded by a double loop of mercury in-rubber, fixed—at one point—with 1 cm wide Scotch tape. One of Whitney's strain gauges was used for the loop but his mounting plate and calibration method was excluded for reasons given in Chapter 2. The experiment was performed several times with intervals in which control blood flow was measured (Table 12)

4. Blood flow and pressure. In 14 of the subjects the flow of blood through a forearm was measured with a water or a Dohn plethysmograph, while the arterial blood pressure, as well as the central and peripheral

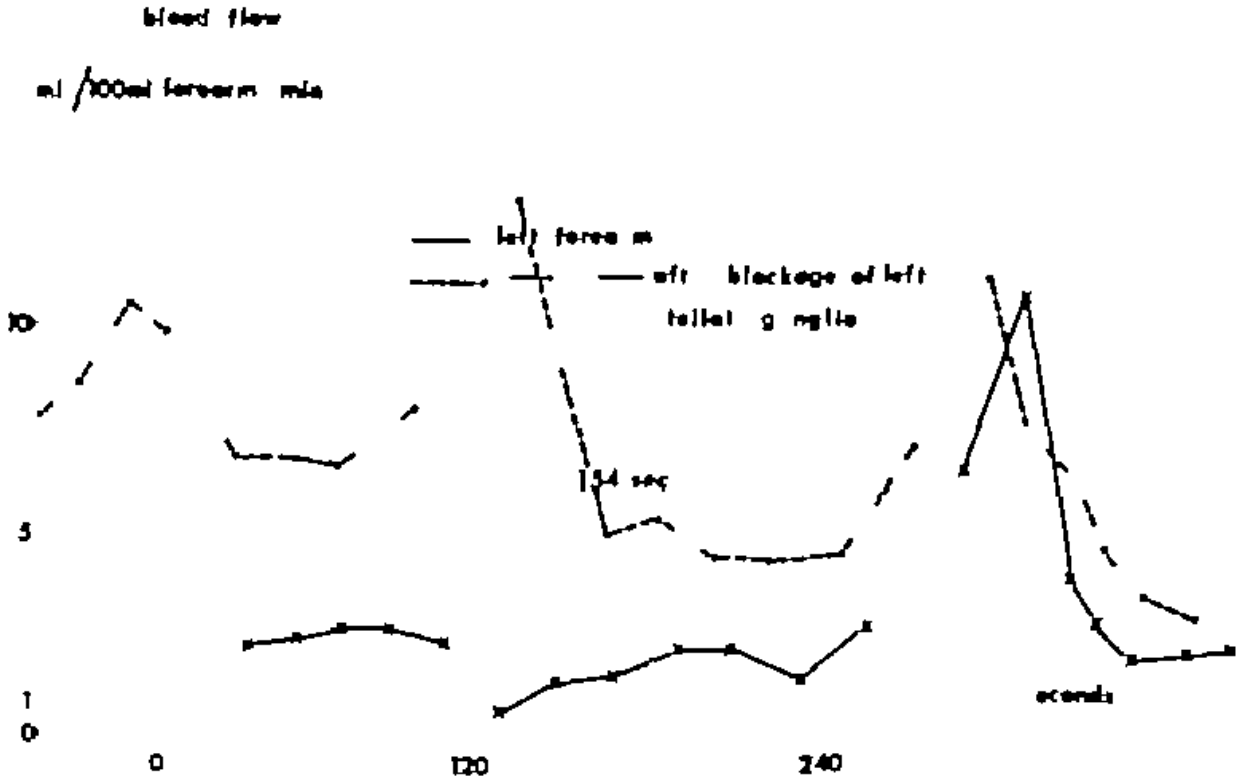


Fig. 27 Forearm blood flow altered by a Valsalva manoeuvre ('TLC') performed both before and after sympathetic blockage.

venous pressures, were measured via catheters introduced percutaneously in the vessels of the other arm. Separated by intervals of three minutes of *normal* breathing, the subject held his breath at *different* intrapulmonic pressures and lung volumes, as described above.

5 Muscle and skin vessels. Hand and finger blood flow has not been the topic of interest, for reasons that will become obvious for anybody who tries to measure this variable. However, the right hand blood flow of one subject was measured. The distal exit of the chamber of the water plethysmograph was closed. The subject was wearing a glove of thin rubber fixed with latex glue to one of the stiff rubber plates (Fig 6) which was used to close the proximal hole of the chamber. The plethysmograph chamber was kept at a temperature of 37°C. The flow of blood through the hand was measured for three minutes as a control (9 determinations). The subject then performed a Valsalva experiment of 60 seconds duration ('TLC' at an intrapulmonic pressure of 30 mm Hg) during which the blood flow was measured. Then the temperature of the plethysmograph chamber was lowered in steps of 1—3 C, and at each step the above experiment was repeated the lowest temperature tested was 10 C. The control blood flows decreased from 12.3 at the 37°C to 2 2 FU at 10°C.

6 Venoconstriction In three subjects the right forearm was placed in a water plethysmograph, and the venous pressure was measured in a vein in the middle of the segment. The right forearm segment volume and the venous pressure was recorded. A blood pressure cuff (12 cm wide) was placed around the upper right arm and inflated to 190 mm Hg for 3 minutes. After one min of this period had elapsed, the subject held his breath ('TLC' 30 'TLC' 0 FRC 30 FRC 0) Simultaneously as a check the flow of blood through the left forearm was measured intermittently with a Dohn plethysmograph.

Results and comments

The modified water plethysmograph was found to be more reliable than the air and the strain gauge plethysmographs, probably due to the fixed, local temperature and to the tailor made sleeves. The Dohn (air displacement) plethysmograph was hardly acceptable, since the volume of the limb segment was difficult to obtain with a reasonable accuracy The strain gauge method, even in the modified form covering practically the whole circumference and with correct calibration, was only applied for measurements expressed in percentage change.

1 *Does* the plethysmographic method fail to measure essentially true blood flows during a Valsalva manoeuvre? Since the peripheral venous pressure increases to a magnitude of 20—25 mm Hg, it would be reasonable to assume that the distended veins would create a back-pressure interfering with the arterial inflow (ignoring—for a moment—the distensibility of the capillary bed) In the first control experiment described above, the Valsalva manoeuvre—as usual—produced a fall in the flow of blood through both forearms approaching zero. In a forearm which has not been drained of blood prior to the flow measurement the Valsalva manoeuvre and the proximal venous occlusion elicit an increase in peripheral venous pressure of approximately 20 mm Hg However, in the previously elevated (drained) forearm the venous pressure, assumed to be representative of that of the veins of the segment in general, only increases to approximately 10 mm Hg Consequently the blood flow reduction is not a function of the increase in venous pressure. On the other hand, no drastic blood flow reduction was observed in the experiments where the peripheral venous pressure was raised with a cuff at the axilla during normal respiration. Thus, the plethysmographic method seems to yield the same results, regardless of the size of the venous back pressure (10—20 mm Hg). It seems reasonable to assume that the flow of blood measured during a Valsalva experiment approximates the 'true value.

2. *The results of the preliminary experiments made in Sweden showed* that the Valsalva manœuvre was accompanied by a certain reduction of the flow of blood through the forearm (Fig 25) and calf (Fig 26) The results of the standard experiments *indicated* that the marked drop in the limb blood flow was only seen in the initial phase of a breath-hold at *increased* intrapulmonic pressure. Breath-holding after a deep inspiration ('TLC') and at an intrapulmonic pressure of zero to 10 mm Hg reduced the limb blood flow only slightly from the control level, while at 20 mm Hg a considerable drop in blood flow was seen, and at 30 to 40 mm Hg the reduction of the flow of blood through the limb was so drastic that the flow even approached zero. Breath-holding after a normal expiration (FRC) hardly reduced the limb blood flow at intrapulmonic pressures of 0 to 10 mm Hg, but at 20 to 30 mm the flow of blood was reduced to almost zero in 6 cases. Curiously enough—at that time—21 of the 27 subjects did not show this dramatic blood flow reduction at FRC with an intrapulmonic pressure of 20 to 30 mm Hg

Simultaneously measured intrapulmonic and central venous pressures showed a linear relationship during Valsalva manœuvres with the glottis open. Owing to the risk that some subjects, contrary to the instructions, closed the glottis and produced the intended intrapulmonic pressure with the upper part of their airways the central venous pressure was measured during standard experiments. Such false Valsalva manœuvres were not accompanied by a marked fall in limb blood flow and the central venous pressure did not rise much, although the mouth pressure was 30 to 40 mm Hg Consequently the details of the many standard experiments are without much value, except for the overall picture given above, and conclusions will be based on other experiments (4.)

3 *The results* of the experiment where the blood flow changes were measured simultaneously with 3 different plethysmographs are given in Table 12. The Valsalva experiment evoked a marked reduction of limb blood flow and the relative reductions were of a similar magnitude in the forearm and the calf (Table 12)

4. *Simultaneous* recording of arterial and venous blood pressure and limb blood flow gave the following results. Breath-holding at 'TLC' and intrapulmonic pressures of 20 to 40 mm Hg produced a marked fall in arterial blood pressure and limb blood flow along with an increase in the venous pressures (Fig 29). Breath-holding at 'TLC' and intrapulmonic pressures of 0 to 10 mm Hg only changed the blood flow and pressures to a small extent, and since the main point of interest was the marked blood flow reduction these experiments are only considered as controls.—The short

Table 12 Percentage reduction of the blood flow by breath-holding after a deep inspiration ("TLC") and with an intrapulmonic pressure of +30 mm Hg.

	FU	% change
Water pleth., right forearm	8.1/1.9	77
Strain gauge left forearm:	(5.6/1.5)	73
Dohn pleth., left calf:	3.8/1.0	74

FU denotes flow units (*i.e.* ml blood/100 ml limb × min) The figure above the oblique line indicate the control blood flow and below the line is shown the blood flow measured during the initial part of the Valsalva experiment. The blood flow is the average of 10 flow observations in one subject.

FRC breath-holds at high intrapulmonic pressure produced a more or less pronounced decrease in the arterial pulse pressure. However, in most cases the systolic pressure increased (Fig 30), and these subjects showed no marked reduction in the flow of blood through the limbs (Fig 30) On the other hand, in three cases both the systolic arterial pressure and the pulse pressure dropped during breath-holding at FRC and high intra pulmonic pressure, and in all of these a marked fall in limb blood flow followed (Fig 31) In Fig 30 the increase in peripheral venous pressure is delayed as compared to the rise in the cases with marked flow reduction (Fig 31)

Catheterisation of all together 64 blood vessels (brachial artery (14), superior vena cava (22) peripheral vein (28)) was never attended by any complications

5 *In the hand* surrounded with water at different temperatures the Valsalva manoeuvre, at each temperature level produced a definite reduction of the blood flow down to about 0.5 ml/100 ml hand × min. Of course, a plethysmograph used on intact limbs can never reveal with certainty whether skin or muscle blood vessels are involved in the blood flow reduction. However, the hand when cooled down to low temperatures showed a blood flow reduction during Valsalva manoeuvres, to the same absolute value as at higher temperatures. This suggested involvement of the muscle blood vessels, since the cutaneous vessels should be essentially closed beforehand at such low temperatures.

6 *In order to* demonstrate an increase in venous tone it is necessary to show an appreciable pressure rise in veins unable to change their volume. The segment volume of the arm (560 ml) whose circulation was arrested

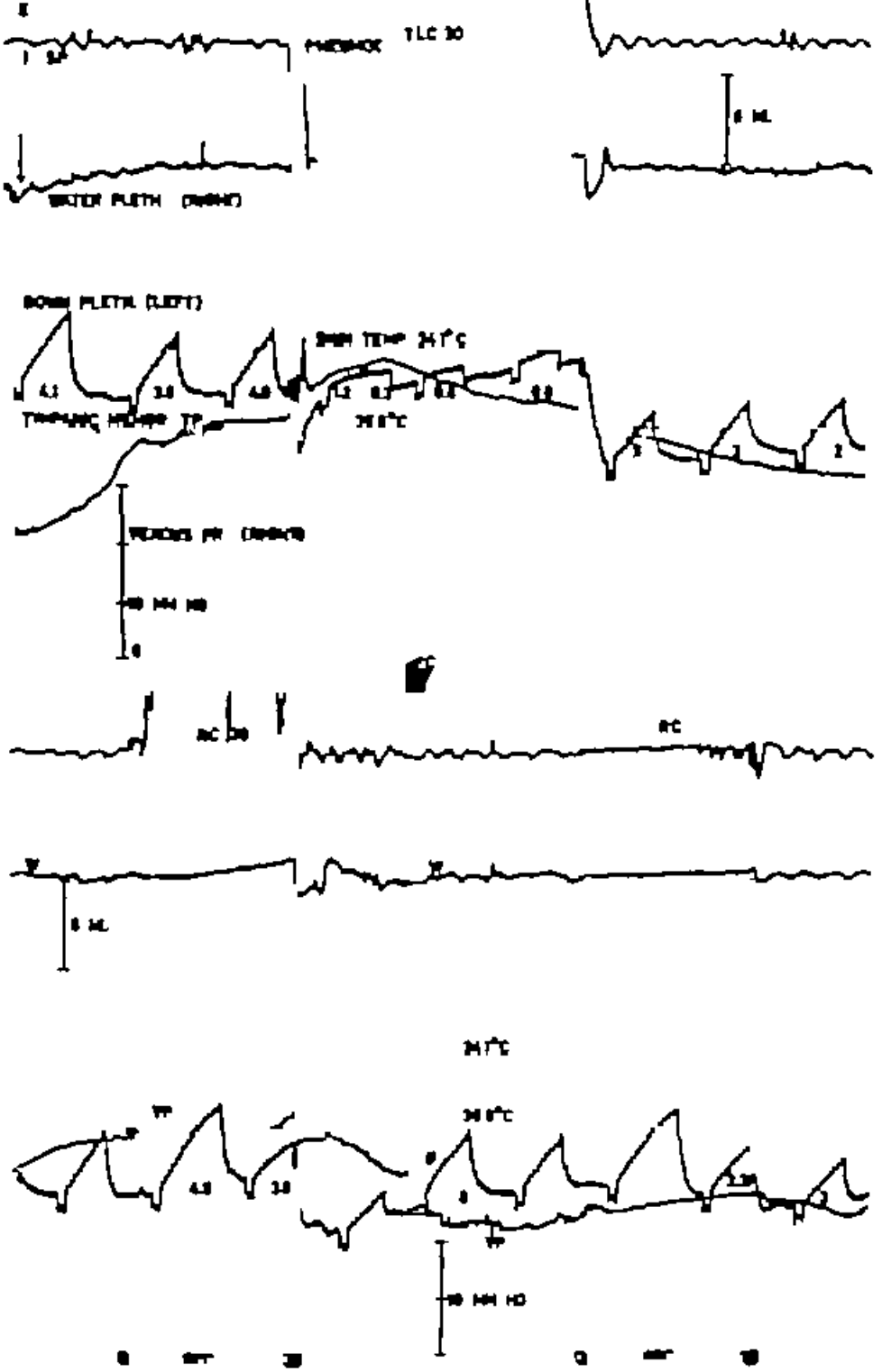


Fig. 18 Left forearm blood flow (Dohn plethysm. D) in FU V rietions in the right forearm segment olume measured with water plethysmograph (W), and in the venous pressure (VP) of the segmental veins, were recorded during arterial occlusion (90 mm Hg) of the right arm. Several secoods after the application of the arterial occlusion (A), the VP reached certain level whereafter the subject performed a breath-hold ("TLC" o, "TLC" 30, FRC o FRC 30 mm Hg). In the control phase VP was 1 mm Hg, to which must be added water column equal to 5 mm Hg The VP level reached before the breath-hold, varied between 20 and 40 mm Hg, dependent upon the content of blood of the veins when the arterial occlusion was applied (A). Pneumotachogram (P) and temperatures are recorded as control.

did not vary essentially with time (Fig 28) so input or output of blood through the vessels in the humerus or any other source is excluded. Only breath-holds ('TLC' 30) at high intrapulmonic pressure elicited a marked rise in venous pressure (11 mm Hg) without essential change of the volume of the segment. Immediately after circulatory arrest the blood volume of the limb will be distributed to the different categories of vessels according to their elastic and active tension. After this equilibration the passive tension of the vein wall will probably be constant, and a marked increase in venous pressure presumably due to an increase in active tension of the venous wall, *i.e.*, venoconstriction.

Although it was not proved with simultaneous volume an pressure measurements, previous experiments (Page et al. 1955—and later other authors) on isolated venous segments *in situ* made it likely that the Valsalva manœuvre provoked venoconstriction in human limbs. The above increase in peripheral venous pressure of 10 mm Hg is difficult to explain on any other basis than venoconstriction.

Cause of the limb blood flow reduction

From a theoretical point of view the fall in the flow of blood through the limbs during the Valsalva manœuvre must be due to a fall in the arteriovenous pressure difference (*i.e.*, perfusion pressure) a passive or active increase in the resistance of the small vessels of the limbs, or a combination of these.

Reduced perfusion pressure According to the Poiseuille equation, the flow is identical with perfusion pressure divided by resistance. The mean pressure of the brachial artery drops from about 100 mm Hg in the control period to approximately 75 mm during the initial part of a Valsalva manœuvre (Fig 29) while the peripheral venous pressure rises from 5 to 20 mm Hg Thus the arteriovenous pressure difference has fallen less than 50 % while the simultaneous percentage drop observed in limb blood flow was always greater and often approached 100. Therefore, the conclusion must be drawn that only part of the observed fall in blood flow can be accounted for by the reduction in perfusion pressure *alone*

Passive increase in resistance However, the fall in arterial pressure and rise in venous pressure leading to a reduction in perfusion pressure may have produced a change in the dimensions of the resistance vessels of the limb and thus reduced the flow of blood. In rigid straight tubes, as used by Poiseuille, the resistance is constant and—if the coefficient of viscosity and the length of the tube do not vary—inversely proportional to the fourth

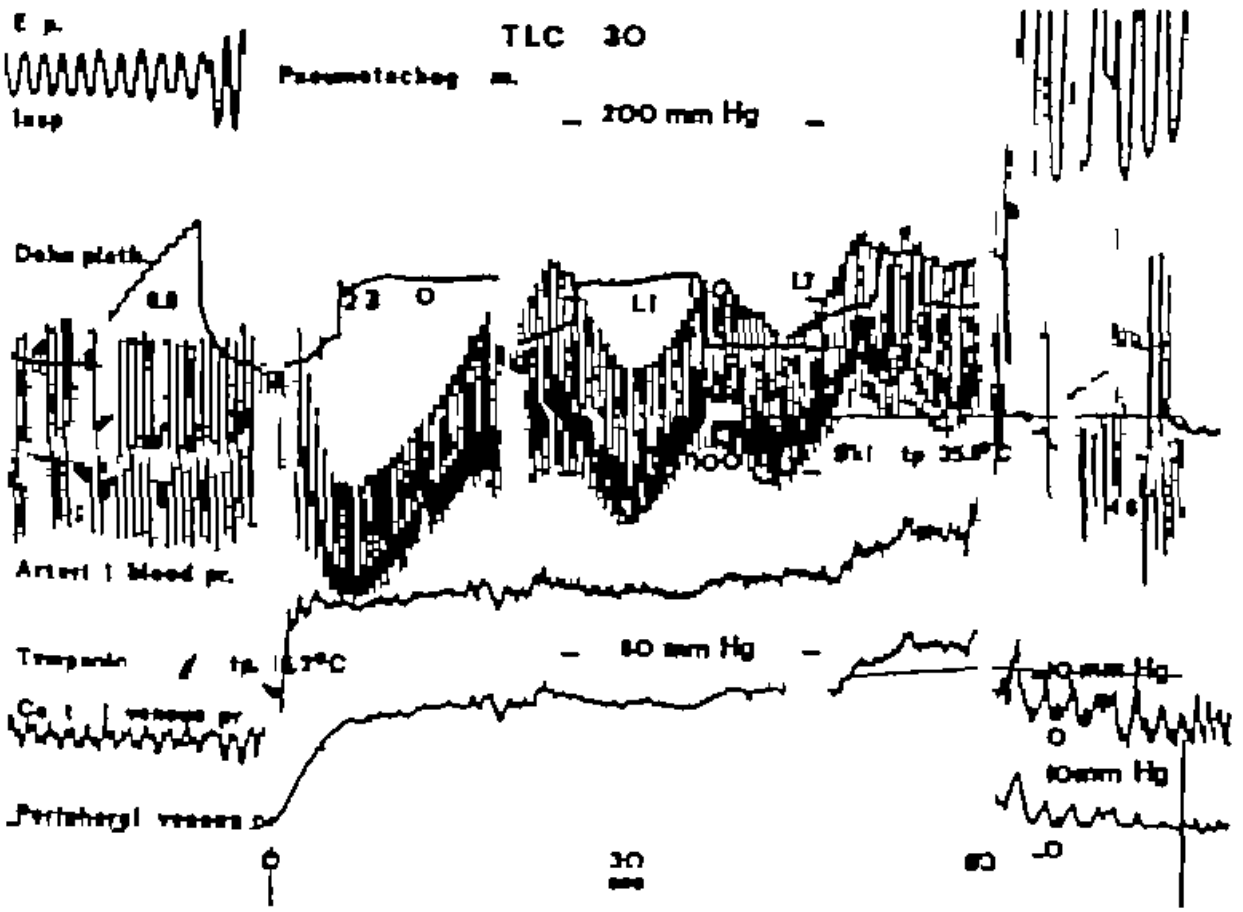


Fig. 29 Simultaneous recording of blood pressure and blood flow The breath-hold was performed after a deep inspiration and at an intrapulmonic pressure of 50 mm Hg On the ordinate 0 to 10 mm Hg are venous pressures, while 5 100, 150, and 200 mm Hg are arterial pressures. The blood flow values are given along the plethysmogram. The temperatures are practically constant. Eighty similar experiments confirmed the covariation between blood flow and arterial pressure shown above.

power of the radius. Since blood vessels are distensible, their radius depends upon their transmural pressure (*i.e.*, the difference between the intravascular pressure and the tissue pressure) The resistance vessels are here defined as those producing the major part of the resistance it is to be expected that the intravascular pressure of these vessels should, on an average, be close to the mean of the arterial and the venous pressures. It is also likely that the tissue pressure of relaxed limbs is about zero and remains so during a Valsalva manœuvre. Thus—in the control period as well as during the Valsalva manœuvre—the transmural pressure of the resistance vessels should, on an average, be close to half the sum of the arterial and venous pressures. In the control period the transmural pressure will be approximat ely (100 + 5)/2 mm Hg, while during the Valsalva experiment, at the time where the flow approaches zero, it will be (75 + 20)/2 mm Hg (Fig 29)

Thus, according to this reasoning, the Valsalva manœuvre should only lead to a reduction in the transmural pressure of the resistance vessels of a few mm Hg Even considering the fourth power relation between resistance and radius mentioned above, it can hardly be expected that such a moderate reduction in transmural pressure could lead to a decrease in the radius of the resistance vessels responsible for a major increase (toward infinity) in their resistance.

Active increase in resistance Since even the combined effect of the 2 factors just mentioned, cannot alone explain the observed fall in flow to nearly zero one seems compelled to assume that contraction of smooth muscle in the wall of the resistance vessels is also an important contribution to these falls in blood flow Such a contraction, or vasoconstriction, is probably brought about reflexly since vasoconstriction was not found during Valsalva manœuvres in limbs with sympathetic block (Fig 27) Part of the limb blood flow reduction can therefore most likely be explained by a reflex vasoconstriction with a sympathetic efferent path to the resistance vessels

The initial marked fall in the flow of blood during a Valsalva manœuvre was followed by a slow rise (Fig 25) This may in part be accounted for by a gradual increase in arterial blood pressure (Fig 21) but some relaxation of the resistance vessels possibly evolved by local hypoxia may also be involved, especially during some of the prolonged breath-holds. However in most cases the blood flow was reduced also in the later part of the manœuvres (Fig 26 29) suggesting a *marked* vasoconstriction, especially since the arterial blood pressure had reached normal levels (Fig 29).

The afferent path of the reflex arc

An essential part of the limb blood flow reduction during a Valsalva experiment, is due to a reflex vasoconstriction of the resistance vessels with a sympathetic efferent path, so it seems worth while to search for receptors related to the afferent side of the reflex arc.

Since the venoconstriction shown above is seen only during the same types of breath-holds as is the marked constriction of the resistance vessels, the trigger mechanism is assumed to be the same for both phenomena.

First the above results will be compared with results obtained by others, in an attempt to solve the receptor problem. Sharpey Schafer (1965) recorded limb blood flow immediately *after* short breath-holds with unknown lung volume and sometimes unknown intrapulmonic pressure (in certain cases the intra-œsophageal pressure was approximately 40 mm

Hg), and found a blood flow reduction, which—because of the short duration of the breath-hold—may have certain characteristics in common with that observed *during* a Valsalva experiment here. Sharpey-Schafer (1965) assumed the existence of a reflex constriction of both arteries and veins reducing the blood flow and concentrated his interest upon the "paradoxical role of vasoconstriction" in the period *after* the Valsalva manoeuvre. The marked degree of peripheral vasoconstriction with a limb blood flow approaching zero during the initial phase of the Valsalva manoeuvre, was not recognized. The important effect of a fall in perfusion pressure, and of a fall in transmural pressure of the resistance vessels, was not considered. However, Sharpey Schafer's (1965) interpretation is in accordance with the essential part of the explanation given above (*i.e.*, active increase in resistance).

Recently Brick (1966) reported a mean decrease in the flow of blood through the human forearm of 25 % on immersion of the face in water with the subject prone. The intrapulmonic pressure was not measured.—We repeated Brick's experiment and found the forearm blood flow to decrease by 68 % (mean) during 60 sec breath-holding with the subject prone ('TLC' and an intrapulmonic pressure of 20 mm Hg). The same blood flow decrease was found during similar breath-holds with the face immersed in water at 15 and 28° C. Thus, the blood flow reduction, which Brick attributed to face immersion, seems to be elicited simply by breath-holding at increased intrapulmonic pressure, and his suggestion is not a fair solution to the receptor problem.

Other possibilities must be considered.—*Receptors* might be situated in the thoracic wall, pulmonary tissue (stretch receptors) or in other compartments of the respiratory system. Assuming an adequate stimulus of such receptors to be an increase in receptor length per unit of time, it is clear that pulmonary stretch receptors can be excluded as a possible explanation, since the marked blood flow reduction is also seen during breath-holds at FRC (Fig. 31), namely where both pulse pressure and the arterial systolic pressure fell. The basis for these exceptions may be a relatively insufficient blood volume (Nordenfelt 1964). The limb blood flow drops with the arterial blood pressure, regardless of the lung volume during the Valsalva experiment (Fig. 30, 31).—*Receptors* might be located in the venous system, in the right half of the heart, or in the pulmonary circulation. Especially the 'low pressure baroreceptor reflex to skeletal muscle vessels of man' (Barcroft 1963), is a highly attractive explanation of the vasoconstriction. The evidence for the low pressure receptors is the increase in forearm blood flow by raising the legs of recumbent subjects or by negative pressure

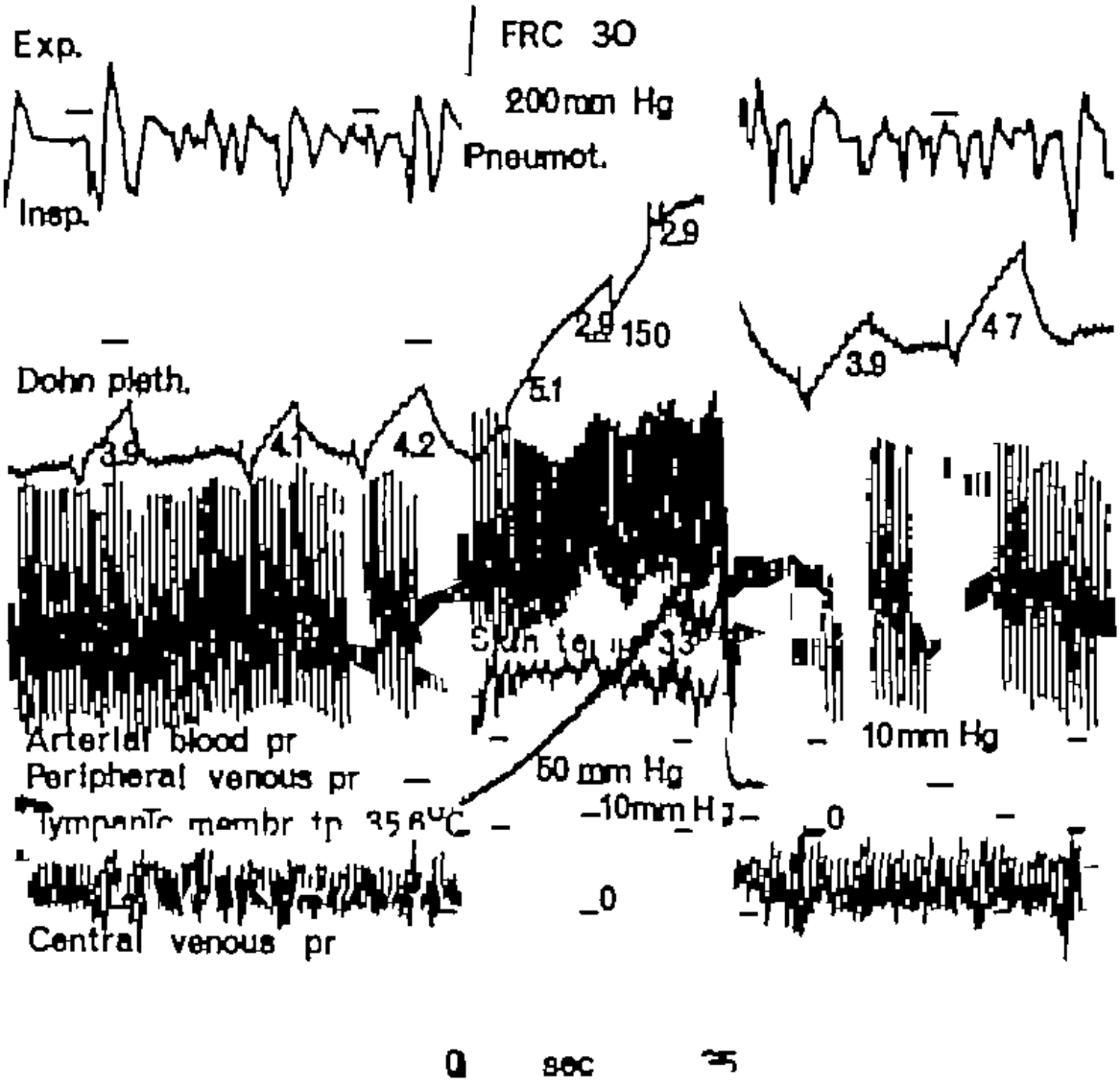


Fig 30 Record of a 55 sec breath-hold after normal expiration (FRC) and at an intrapulmonic pressure of 30 mm Hg. Variables as in Fig 29. Twelve similar records (FRC, pressure of 20 to 30 mm Hg) and eight control experiments (FRC, pressure of zero to 1 mm Hg) are available.

breathing. The effect of these experiments on the arterial blood pressure is not considered of importance in such argumentation. Other reflexes are considered in the monograph by Heymans & Neil (1958). Unfortunately most of the evidence of their existence and function is indirect, as shown in the above example.—*Receptors* might also be located in the left half of the heart and the systemic arteries. Receptors other than sino-aortic have been postulated (Heymans & Neil 1958) but most of the evidence

put forward is indirect and the functional role of such receptors is neither clear nor proved. The baroreceptors believed to exist in the myocardium of the left ventricle may be an exception. In contrast, the receptors found in the carotid sinuses and in the wall of the aortic arch have a proved functional importance (Heymans & Neil 1958) but even their function is not yet properly understood. Adequate stimulus of these receptors is not only the mean transmural pressure of the vessels, but also the size of the pulse pressure, and the rate at which it changes. The dramatic fall in arterial blood pressure due to a Valsalva manoeuvre, is a strong "negative" stimulus to the sino-aortic baroreceptors (possibly the receptors cease to fire) The reaction to such a condition is in healthy persons—after a certain delay—a vasoconstriction of both arterioles and veins. The profound vasoconstriction was demonstrated above and the problem seems explained by well-known physiological concepts, without assuming the existence" of "receptors of unknown origin" or other hardly informative hypotheses as is commonly done in the literature.

In some cases breath-holding at FRC and high intrapulmonic pressure did not lead to a marked fall in limb blood flow These cases differed from those represented in Fig 31 because the systolic and the mean arterial pressure *rose* although the pulse pressure fell (Fig 30), in contrast to the cases represented in Fig 31 In these cases the negative stimulus to the baroreceptors may not have been present, indicating sufficient venous return, probably mainly from the abdominal blood deposits (Nordenfelt 1964) Moreover the high arterial mean pressure and relatively low peripheral venous pressure counteracts the fall in perfusion pressure and in transmural pressure of the resistance vessels.

Although a major part of the reduction of the flow of blood through the limbs seems explainable by the classical high pressure baroreceptor reflex, this does not necessarily exclude influence of other parts of the nervous system. Afferent impulses from many sources including the central nervous system itself, may influence the activity of the circulatory control centers. Besides the output from these centers to the heart, the arterial, and the venous systems may not always follow the same relative pattern from one situation to the next.

Since the flow of blood through the limbs approaches zero during breath-holding at high intrapulmonic pressure, a greater blood flow reduction could not be expected during the same manoeuvre performed in water (regardless of its temperature) During breath-hold dives to depths of some meters, the pressure difference across the lungs and chest wall will most likely be small and not lead to marked changes in limb blood

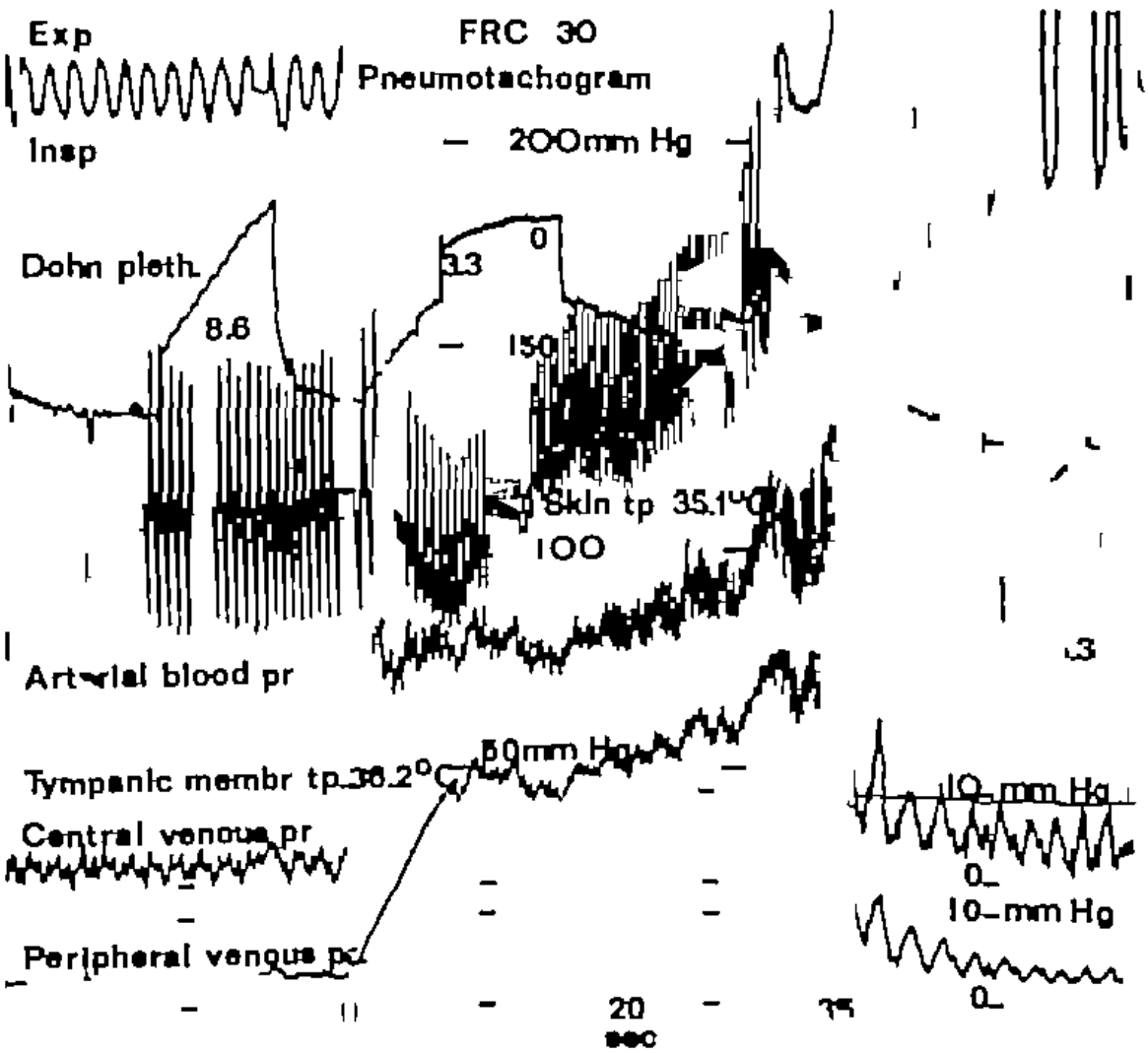


Fig. 31. As Fig. 30, but with faster speed of the recorder. A 35 sec breath-hold is performed after normal expiration and at an intrapulmonic pressure of 3 mm Hg. Three similar records (FRC, pressures of 20 to 3 mm Hg) and two control experiments (FRC, pressure of zero and mm Hg are available).

flow except where the subject performs a Valsalva manoeuvre. During deep dives toward the limit of human performance, the intrapulmonic pressure will probably be negative, and the blood content of the thoracic vessels will increase. This rather special condition is possibly accompanied by a small relative increase in limb blood flow. In general the cardiovascular changes are not likely to potentiate essentially the serious consequences of gas accumulation during breath-hold diving in otherwise healthy subjects. —The Valsalva experiment and unconsciousness have been analyzed by Nordenfelt (1964).

In the above argumentation it was mentioned that the function of the high pressure baroreceptors is not completely understood at present and that even more information is needed as far as other receptors are concerned. For persons interested in circulation and neurophysiology these problems should be highly attractive, especially when sufficient knowledge from animal experiments is provided to justify a study in man.

Summary and conclusions

Serious accidents in a submarine escape training tank prompted an investigation of the basis of the:
loss of consciousness and drowning
changes in cardiac rhythm and
alterations in limb blood flow cardiac output and blood pressure occurring in association with breath-holding (in air and in water).

Experiments were carried out on 129 persons some of whom participated in 2 or more projects.

In these experiments determinations were made of the
composition of alveolar and expiratory air with a modified Scholander micro-analyzer and electrodes
air volumes with gas meters, pneumotachographs and spirometers
intrapulmonic pressures with water manometers
ECG with conventional device and with equipment modified for use under water
direct blood pressures with percutaneous technique and strain gauge transducers
cardiac output (mean stroke volume) with the indicator dilution technique
tympanic membrane and skin temperatures with thermistors, and
limb blood flow with water air and strain gauge-plethysmographs.

All variables were registered simultaneously with selected mirror galvanometers and recorded on a UV recorder. Only ECG registration made it necessary to use an amplifier between the detector and the recorder.

The following observations of interest were made:
In 4 divers who got a severe neurological disease after repeated breath-hold diving, treatment with recompression produced immediate and total relief
after repetitive dives to depths of 20—30 m of 1—2 min duration and with surface intervals less than 10 minutes, the predicted N_2 tissue tensions exceeded the maximum permissible tensions of conventional air supplied diving

on two occasions a test subject lost consciousness by suppressing the ventilation voluntarily for 190 seconds each time

carbon dioxide retention occurred during repeated breath-hold diving

the alteration in heart rate observed in humans during breath holding varied with the intrapulmonic pressure ('apnoic bradycardia and tachy cardia during Valsalva manoeuvres) and the environmental temperature ('cold bradycardia')

breath-holding with an intrapulmonic pressure of 20—40 mm Hg (Valsalva manoeuvre) produced a drastic reduction of limb blood flow (approaching zero) if the arterial blood pressure fell essentially this reduction could be partially prevented by sympathetic block.

These findings appear to warrant the following conclusions

Avoidance of decompression sickness requires surface intervals of at least 10 minutes between consecutive, deep breath-hold dives

decompression sickness calls for immediate recompression treatment, especially because of serious neurological signs

psychological factors (will power feeling of competition) may be of decisive importance in breath-hold diving accidents

loss of consciousness with the risk of drowning may be caused by CO_2 narcosis in combination with the well-known hypoxia during ascent from deep breath-hold dives

apnoic bradycardia is elicited from the sino-aortic baro-receptors and the cold bradycardia is caused by cold stimulation of the skin

the reduction in limb blood flow by breath-holding at increased intra pulmonic pressure is caused by the fall in perfusion pressure and by a sino-aortic baroreceptor reflex with a sympathetic efferent path to the arterioles

Despite the complexity of the circulatory regulation in general, it seems as if no hypothetical mechanisms are necessary in the interpretation of the cardiovascular changes observed during breath holding since these changes are presumably explainable alone on the basis of classical physiological mechanisms, whose existence is generally accepted.

Resume og konklusioner

Alvorlige ulykker i en tank til optræning af U-bådsmandskab i fri udslusning startede en undersøgelse af grundlaget for

Bevidstløshed og drukning

forandringer af hjerterytmen, og

ændringer af perfusionshastigheden i extremiteterne, hjertets minut volumen og blodtrykket, optrædende i forbindelse med vejrholdning (i luft og i vand)

Der udførtes forsøg med ialt 129 personer af hvilke nogle deltog i 2 eller flere projekter.

I disse forsøg undersøgtes

Sammensætningen af alveole og exspirations-luft med en modificeret udgave af Scholanders mikro-analysator og med elektroder,

luftvolumina med gas-ure, spirometre og pneumotachografer,

intrapulmonalt tryk med vandmanometre,

EKG med konventionelt udstyr samt med udstyr modificeret til under vandsbrug,

direkte blodtryk med perkutan teknik og 'strain gauge" transducere,

hjertets minutvolumen (middel-slagvolumen) med indikatorfortyndingsteknikken,

trommehinde- og hud-temperaturer med termistorer, og

perfusionshastigheden i extremiteterne med vand luft og "strain gauge" plethysmografer

Alle variable blev registreret simultant med udvalgte spejlgalvanometre og opskrevet med en UV-skriver (ultraviolet følsomt papir) Kun ved EKG registrering var en forstærker nødvendig imellem detektor og skriver

Følgende observationer synes af interesse

Fire dykkere med en alvorlig neurologisk sygdom efter gentagne dyk under vejrholdning, blev behandlet med rekompression som medførte øjeblikkelig og total helbredelse,

efter en serie dyk til dybder på 20—30 m af 1—2 minutters varighed og med mindre end 10 minutters overflade-intervaller overskred beregnede vævstensioner af kvælstof de maximalt tilladelige tensioner ved konventionel trykluftdykning,

ved 2 lejligheder holdt samme forsøgsperson vejret i 190 sekunder indtil han besvimede,

efter gentagne dyk under vejrholdning fandtes en betydelig kuldioxyd-ophobning

hjertefrekvens under vejrholdning varierede med det intrapulmonale tryk ('apnoe-bradycardi' og tachycardi under Valsalva manovrer) og den omgivende temperatur ('kulde bradycardi')

vejrholdning med et intrapulmonalt tryk på 20—40 mm Hg (Valsalva manovre) fremkaldte en drastisk reduktion af perfusionshastig heden i extremiterne (nærmende sig total ophør), dersom det arterielle blodtryk faldt væsentligt denne reduktion kunne delvis forhindres ved blokade af de sympatiske efferente tråde.

Disse fund synes at berettige til følgende konklusioner

For at undgå dekompressionssygdom må man benytte overflade intervaller på mindst 10 minutter mellem dybe dyk under vejrholdning,

de alvorlige neurologiske fund understreger betydningen af at denne form for dekompressionssygdom straks behandles med rekompression,

psykologiske faktorer (viljestyrke, konkurrenceforemmelse) kan være afgørende ved drukneulykker

beruselsestilstand med risiko for drukning kan forårsages af kuldioxyd-narkose kombineret med den velkendte iltmangel under opstigning fra dybe dyk,

'apnoe-bradycardi' udløses fra de sino-aortiske baro-receptorer og 'kulde bradycardien' af kuldestimulation af huden,

faldet i perfusionshastigheden i extremiteterne ved Valsalva's manovre forårsages af faldet i drivende tryk (perfusionstryk) og af en reflektorisk vasokonstriktion udløst fra de sinoaortiske baroreceptorer med en sympatisk efferent bane til arteriolerne.

Kredsløbsregulationens kompleksitet må erkendes i almindelighed, men hypotetiske mekanismer synes ikke nødvendige i fortolkningen af de cardiovaskulære ændringer der er iagttaget under vejrholdning Disse ændringer lader sig tilfredsstillende forklare alene på grundlag af klassiske fysiologiske mekanismer hvis eksistens der er almindelig enighed om.

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Abbreviations in accordance with World Medical Periodicals. The numbers in brackets indicate the page(s) on which the reference concerned has been quoted.

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Subject index

Numbers refer to pages of the present review and letters (A—E) to previously published papers, in which the subject is discussed.

	Page — Letter code
Acidosis	62 — C
Adreno-medullary secretion	82 — E
Air plethysmography	23 35
Alveolar air	10 — B C
Alveolar exchange quotient (R)	58 — C
Apnoea	
definition	65
bradycardia	67 69 — D
Arterial	
baroreceptors	71 95 — E
occlusion	3
systemic pressure	71 75 80 89 — E
Arteriolar constriction	94, 97 — E
Auxiliary equipment	39
Ballistocardiography	75 — E
Baroreceptors	7 95 — E
Blood pressure	
measurement	19 — E
changes	7 7 75 80, 89 — E
Body-temperature	39
Boyle Mariotte law	50
Brachial arterial pressure	71 75 80 89 — E
Bradycardia	7 63 — D
Breaking point	60 — C
Buoyancy point	57 — B, C
Calculation of blood flow	53
Calibration	
of plethysmographs	32, 38
of transducers	19
Capillary permeability	5
Carbon dioxide	
narcosis	6 — C
retention	53 — C
tension	11 12, 57 6 82 — C

	Page — Letter code
Cardiac	
arrhythmia	7 63 72 — D
oscillation	29
output	7 27 75 — E
rhythm	63 — D
Central venous pressure	75 — E
Chemoreceptors	82 — E
Core temperature	39
Dead space	
apparatus	10 — C
subjects	11 — C
Decompression sickness	7 45 — A, B
Direct blood pressure	75 86 — D E
Diving bradycardia	7 63 — D
Diving reflex	63 — D
Dizziness	7 45 — A, C
Dohn plethysmographs	24
Douglas bags	12 13 — C
Drowning	7 53 — C
Dye dilution	21 — E
Ear drum temperature	39
Electrocardiography	17 19 64 — D
Electrodes	1 17 22 — C, D
Eructation	7 45 — A, C
Expirograph	12
Exposure time	47 — B
Face immersion	66 — D
Fatigue	7 45 — A, C
Free escape	7 — A
Functional residual capacity	64, 80 86
Gas meter	— C
Gas volumes	— C
Hand blood flow	87
Heart	
arrhythmias	7 63 72
rate	63 76, 79 — D
Hypercapnia	75
Hyperventilation	54 — C
Hypoxia	55 62, 75 — C
Intra-cranial temperature	39
Intra-oesophageal pressure	15 94 — D
Intra-oral pressure	14
Intra-pulmonic pressure	14, 87 — D E

	Page — Letter code
Intra-thoracic pressure	69 81 — D F
Intra-thoracic venous pressure	81 — E
Limb blood flow	7 83 — E
Loss of consciousness	53 62 — C
Manometers	
water	14 — D E
mercury	19
Medical gas analyzer	12
Metabolic exchange quotient (RQ)	58 — C
Micro-electrode chain	22 — C, E
Modulus of volume elasticity	21
Nausea	7 45 — A C
Nitrogen	
percentages	47 — B
saturation degree	51 — B
tissue tension	47 48 49 — B
Oxygen	
conserving reflex	63 — D
stores	57 59 — C
tension	12, 56, 82 — C, E
Pains	7 45 — A, C
Paralysis	7 45 — A, C
Peripheral venous pressures	85 9 — E
Physiological gas analyzer	
Physiological reactance	1
Plastic tubes	15 9
Plethysmography	22 88
Pneumographs	41
Pneumotachographs	4
Polyethylene	
bags	14
catheters	1 — E
Pulse wave velocity	5
$\dot{Q}$ —Cardiac output	2 75 — E
Receptors	63 71 80, 82, 94 —
Recompression	45 — A, B
Rectal temperature	39
Residual volume	2, 37 65 80 — C
Respiratory valves	12, 67 — C, D
Ruben valve	1

	Page	—	Letter code
Scholander's microanalyzer	11 60	—	C
Seldinger technique	20, 21	—	E
Severinghaus PCO_2-electrode	11 22		
Sino-aortic receptors	71 80 97	—	E
Skin temperature	36 41		
Spirometer	12	—	C
Square wave generator	20	—	D
Starlings law	69 79	—	D E
Statham transducers	4, 19 20, 29 31		
Strain gauges	25 36 88		
Stroke volume	71 79 8	—	D E
Submarine escape training	7 45	—	A, B
Sudden death	7 72		
Surface interval	47 52	—	B
Sympathetic blockade	84		
Tachycardia	71	—	D E
Tetanus	46	—	A, B
Temperature dependency			
of blood flow	24, 36		
of heart rate	68 71	—	D
Thermistors	40		
Thermocouples	40		
Thermodynamics	50		
Total lung capacity (TLC)	64, 75 86	—	D E
Transducers	14, 19 20 9, 31		
Transmission loss	6		
Tympanic membrane temperature	39		
Ultraviolet recorder	1, 4, 17 19 31 33 36 40	—	D
Unconsciousness	7 33 73	—	C
Valsalva manoeuvre	65 73 73 83	—	D E
Vascular resistance	9 93	—	E
Venous			
compliance	25		
constriction	88 92 94	—	E
occlusion	22		
pressure	88 9	—	E
tone	25 92		
Vital capacity		—	C, D
Volume displacement	19 1		
Voluntary apnoea	33 43 53 63 75 8	—	A, D E
Water			
immersion	7 63	—	D
plethysmography	6, 85 88		

Symbols

The symbols used here conform essentially to those published in Federation Proceedings 1950: 9 602–605

V	Air volume
$\dot{V}_A$	Alveolar ventilation per min (the expired alveolar ventilation)
V_D	Dead space
P_B	Barometric pressure
F	Fractional content of gas in the dry phase
f	Respiratory frequency per min
p	Pulse frequency per min
R	The alveolar exchange quotient, *i.e.*, VCO_2/VO_2 (the exchange between the atmosphere and the air in the respiratory system, or the exchange across the total alveolar membrane).
RQ	The metabolic exchange quotient (ratio) across the total surface of the cellular membranes.
I	Inspired air
E	Expired air
A	Alveolar air
	Arterial blood
v	Mixed venous blood
	Capillary blood
t	Tissue
P_{IO_2}	The fractional content of O_2 in the dry phase of the inspired air
$\dot{Q}$	Flow or volume rate of blood
$\dot{Q}_c$	Volume rate in the capillaries
S_vO_2	Saturation of hemoglobin with O_2 in mixed venous blood
P_{CO_2}	The partial tension of CO_2 in arterial blood
P_tN_2	The mean partial tension of N_2 in a (homogeneous) tissue compartment
RV	The lung volume after maximal expiration, *i.e.*, the residual volume
TV	Tidal volume, *i.e.*, the volume of air expired per breath
IRV	Inspiratory reserve volume
ERV	Expiratory reserve volume
VC	Vital capacity *i.e.*, the maximal volume expirable after maximal inspiration
FRC	The lung volume after normal expiration, *i.e.* the functional residual capacity =RV+ERV
TLC	The lung volume after maximal inspiration (total lung capacity = RV+ ERV+TV+IRV)
STPD	Standard Temperature and pressure, dry (0°C, 760 mm Hg)
BTPS	Body temperature and pressure, saturated with water vapour (37°C, P_B)
ATPS	Ambient Temperature and pressure, saturated with water vapour ($t°$ P_B)
t°C	The ambient (room) temperature in degrees Celsius

$\dot{V}O_2$	Alveolar O_2 exchange per min
VCO_2	Alveolar CO_2 exchange per min
atm abs	Atmospheres absolute pressure
min	Minute
sec	Second
mM	mill Molar
PE	Polyethylene terephtalate
PVC	Polyvinyl chloride
FU	Flow units (*i.e.*, ml blood/100 ml limb segment × min)

As indicated above a bar over a symbol denotes a mean value while a dot over a symbol indicates a time derivation (in general per min).

Capitals in suffixes symbolize a gas in an air phase, while small letters in suffixes indicate a gas dissolved in a liquid phase.

Generally accepted abbreviations in physics and chemistry are used (mm, μl, O_2, N_2, CO_2, v for Voltage, etc)

ACTA PHYSIOLOGICA SCANDINAVICA
SUPPLEMENT 325

ELIMINATION OF IODINE-LABELLED FIBRINOGEN IN INTRAVASCULAR COAGULATION AND IN THE NORMAL STATE

EXPERIMENTAL AND CLINICAL STUDIES

BY

ERIC ZETTERQVIST

STOCKHOLM 1969

ACTA PHYSIOLOGICA SCANDINAVICA

SUPPLEMENT 325

Department of Blood Coagulation Research, Karolinska Institutet, Thoracic Surgery Research Laboratory and King Gustav V Research Laboratory Karolinska Sjukhuset, Stockholm, Sweden.

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Stockholm 1969
Kungl. Boktryckeriet P. A. Norstedt & Söner

The present dissertation comprises a summary of the following papers

I A. Blombäck, B., L. A. Carlson, S Fransén and E. Zetterqvist, Turnover of ^{131}I-labelled fibrinogen in man. Studies in normal subjects, in congenital coagulation factor deficiency states, in liver cirrhosis, in polycythemia vera and in epidermolysis bullosa. Acta med. scand. 1966, 179 557—574

I B. Zetterqvist E. and I von Francken, Coagulation disturbances with manifest bleeding in extrahepatic portal hypertension and in liver cirrhosis. Preliminary results of heparin treatment. Acta med. scand. 1963 173 753—760

II Nordström, S and E. Zetterqvist, Effects of thrombin infusions upon ^{131}I labelled fibrinogen in dogs. Acta physiol. scand. 1968 72. 85—99

III Nordström, S and E. Zetterqvist, Effects of epsilon aminocaproic acid (EACA) and Trasylol on thromboplastin-induced intravascular coagulation studied in dogs with iodine labelled fibrinogen. Acta physiol. scand. In press.

IV Nordström, S P Olsson, A.-C. Teger Nilsson and E. Zetterqvist, A procoagulant from human postpartum serum. Effects on coagulation and hemodynamics in dogs. Acta physiol. scand. In press.

V Zetterqvist, E., Fibrinogen degradation products in experimental intravascular coagulation Gel filtration and immunological studies. Acta physiol. scand. In press.

VI. Blombäck, B. and E. Zetterqvist, Incorporation of ^{131}I into dog fibrinopeptides. Acta chem. scand. In press.

Reference to these papers will be made in the text by numerals I—VI

CONTENTS

INTRODUCTION 7

TURNOVER OF FIBRINOGEN IN MAN 9
Review on other investigations 9
Present investigation 10
Labelling of human fibrinogen 10
Half life times in normals and in different disorders 11
Summary 13

EFFECTS UPON THE TURNOVER OF FIBRINOGEN BY INTRAVENOUSLY ADMINISTERED PROCOAGULANTS IN DOGS 14
Review on general effects 14
Present investigation 16
Labelling of dog fibrinogen 16
Experimental conditions 16
Results in control dogs 17
Effects of thrombin, thromboplastin and procoagulant from postpartum serum (PPS) 18
Effects of thrombin and PPS in heparinized dogs 20
Summary 20

INFLUENCE UPON THE TURNOVER OF FIBRINOGEN BY FIBRINOLYSIS INHIBITORS TOGETHER WITH PROCOAGULANTS IN DOGS 22
Review on other investigations 22
Present investigation 24
Experimental conditions 24
Effects of epsilon aminocaproic acid (EACA) and Trasylol 24
Summary 26

SEPARATION OF FIBRINOGEN AND FIBRINOGEN DEGRADATION PRODUCTS IN INTRAVASCULAR COAGULATION IN DOGS 27
Previous investigations 27
Present investigation 27
Experimental conditions 28
Gel filtration and immunological experiments 28
Summary 30

INCORPORATION OF ^{131}I INTO DOG FIBRINOPEPTIDES 32
Other investigations 32
Present investigation 32

Isolation and location of fibrinopeptides 32
Discussion 33
Summary 35

GENERAL SUMMARY 36

ACKNOWLEDGEMENTS 37

REFERENCES 38

INTRODUCTION

The final step in the formation of blood clots in vitro as well as of thrombi in the circulation consists of the thrombin mediated conversion of fibrinogen to fibrin. The dissolution of clots or thrombi occurs through the action of fibrinolytic enzymes, giving rise to fibrin degradation products. Therefore estimation of the elimination rate of tagged fibrinogen seems to be valuable in the study of clinical disorders or experimental states, in which abnormal metabolism of fibrinogen can be expected. Such disturbances may involve the conversion to fibrin the fibrinolytic breakdown or the synthesis of fibrinogen. Obviously several mechanisms can occur simultaneously

Part of the present investigation has been devoted to clinical studies on the fibrinogen turnover in man. Normal subjects as well as patients with different coagulation disorders have been studied.

The greater part of the work has been experimental studies in dogs, in which the coagulation system has been activated by intravenous infusion of different procoagulants—thrombin, thromboplastin and a procoagulant isolated from human postpartum serum. Special interest has been devoted to the secondary changes, following the initial stage of intravascular coagulation A fibrinolytic "response to the coagulation trauma has been found and analysed with respect to quantity and quality of the appearing split products and the influence thereupon of fibrinolysis inhibitors.

In parallel with the experimental studies the incorporation of ^{131}I isotope into dog fibrinopeptides has been studied.

Review of the results of other investigations

In the last decades several studies have been made on the turnover of fibrinogen in human subjects. Such investigations have comprised *normal subjects* (Christensen 1958 Hammond and Verel 1959 Adelson et al 1961 Blombäck et al 1961 McFarlane et al. 1964 Amris and Amris 1964 Hart 1964 Takeda 1966) investigated by means of ^{131}I or ^{125}I labelled fibrinogen. Some earlier studies were made with the use of transfusion of plasma fibrinogen, labelled in vivo by means of ^{35}S-amino acids, from yeast or yeast hydrolysate (Madden and Gould 1952 Volwiler et al 1955 Gerdes and Maurer 1957) The authors using iodine-labelled fibrinogen have reported a half-life time for the protein of around 4 days (2.3—5 1) whereas in the studies made with radioactive amino acids a longer half life time (6.7 days) was found. Reutilization of radioactive amino acids from the catabolism of labelled body proteins seems to introduce an appreciable error in studies using fibrinogen labelled by the latter method.

Concerning states of *congenital coagulation defects* several different disorders have been studied. Gitlin and Borges (1953) followed the elimination of administered fibrinogen in ? cases of congenital afibrinogenemia. They determined the plasma fibrinogen concentration by an immunochemical method and found the half life time to be around 4 days. Rausen et al. (1961) using ^{131}I fibrinogen found in one case of congenital afibrinogenemia a half-life 3 0 days, and in 6 cases of hemophilia A, it varied between 2.8 and 3 6 days. Later Hart (1964) also with ^{131}I fibrinogen, investigated 10 patients with different types of hemorrhagic diathesis and found the half life times for those to be 3 6—4.5 days. These times were all within the normal range found by this author Among the patients of Hart were one case of each of von Willebrand's disease, prothrombin deficiency hemophilia A, hemophilia B and factor X-deficiency Takeda and Chen (1967) with the use of ^{125}I labelled fibrinogen, and also measuring the radioactivity with a whole body counter found the half-life for 10 patients with hemophilia A to be 2.34 ± 0.17 days, which is shorter than the time obtained by this author in normal subjects (mean 3 4)

Also patients with acquired or induced forms of coagulation disturbances have been investigated. Thus Hart, in the studies mentioned, followed the elimination of I fibrinogen in cases of platelet deficiency and Glanzmann's disease in which states the half life time also was found to be within normal limits The same author also studied the influence of anticoagulants as coumarin and heparin, which were found not to evoke any significant effects upon the fibrinogen survival. The influence of fibrinolysis inhibitor (epsilon aminocaproic acid, EACA) has been studied by Hart (1964) who found a prolongation of the fibrinogen survival in a normal subject and the same in a patient with factor X-deficiency Amris and Amris (1964) in one case of liver cirrhosis under treatment with EACA also found a prolongation

of the initial survival time to about the double. After induced fibrinolysis by means of streptokinase Hart found a distinct fall in labelled and unlabelled fibrinogen.

A further group although very heterogeneous, is represented by the *so-called "hypercoagulable states"* To these have been conferred several conditions, in which clinical experience has shown an increased incidence of thrombosis and where also hypercoagulability as judged by the findings of conventional coagulation investigations, has been more or less clearly demonstrated. In patients with polycythemia vera Christensen (1958) Blombäck et al. (1964) as well as Hart (1964) have found cases with a distinct shortening of the half life for fibrinogen. In patients with cancer Christensen (1958) and Hart (1964) also found a diminished half-life, especially in metastatic carcinoma With reference to the hypothesis of Duguid on the thrombogenic pathogenesis of atherosclerosis, studies in patients with coronary heart disease and/or hyperlipemia have been performed. Christensen (1958) in 3 patients, like later Blombäck and Carlson (1963) in a larger series, found no evidence of a changed half-life time Hart, on the contrary demonstrated shortened times in patients with arterial vascular disease In cases of liver cirrhosis, Christensen in one of two patients noticed a slight shortening. Blombäck et al (1964) Amris and Amris (1964) as well as Tytgat et al. (1968) found that an accelerated fibrinogen elimination can occur in this disorder In patients with burns, Davies et al. (1966) found half life times decreased to 1.3—2.1 days as against 4.5 days in their normals. In 2 of 6 patients with cerebral malaria Devakul et al (1966) observed a very rapid elimination of fibrinogen radioactivity

In disorders with increased plasma fibrinogen values as rheumatoid arthritis and nephrosis the half life times of ^{131}I labelled fibrinogen were within normal limits (Takeda 1967 Takeda and Chen 1967) In both states, besides the high plasma fibrinogen level, they found increased amounts of interstitial fibrinogen, transcapillary transfer rate and catabolic rate of the protein which changes were secondary to an increased synthesis of fibrinogen.

Present investigation

Labelling of fibrinogen

Human fibrinogen, prepared according to Blombäck and Blombäck (1956) was labelled with the jet iodination technique essentially as described by McFarlane (1956) The coagulability of the fibrinogen after labelling was around 92 per cent. Less than 0 4 per cent of the radioactivity was found in the supernatant after precipitation of the protein with trichloroacetic acid (TCA) The specific radioactivity (μC/mg) of fibrin, obtained by clotting the labelled fibrinogen with thrombin, was on the average 93 per cent of that in the labelled protein before clotting The yield of ^{131}I-isotope in the labelled protein was on the average 15 per cent of the isotope added. The mean label was 2.0 atoms of iodine per mole of fibrinogen.

McFarlane (1963) studying the turnover of ^{131}I labelled rabbit fibrinogen concluded that in this protein not more than 0.5 atoms of iodine can be incorporated into the fibrinogen molecule if denaturation is to be avoided. He defined denaturation as a rapid initial catabolism of injected labelled protein. In the present study on human fibrinogen the label was between 0.8 and 2.7 atoms per mole of protein. As no significant difference with regard to the extrapolated values for the plasma pool of fibrinogen was found it seems as if no denaturation had occurred. Also the fact that the specific radioactivity of fibrin was only a few per cent lower than that of fibrinogen suggests that only an insignificant degree of denaturation had taken place during iodination. Discussing our results with respect to those obtained by McFarlane we suggested that in rabbit fibrinogen the fibrinopeptide part, containing a tyrosine-O-sulphate residue, might be labelled (I A) In that case the iodination might have given rise to a molecular species liable to rapid excretion. Later Regoeczi and Walton (1967) however found no evidence that the fibrinopeptide part of rabbit fibrinogen is labelled with ^{125}I Therefore, other explanations must be sought.

Plasma half-life of I fibrinogen in normals and in different disorders

Normal subjects In our study the mean values of T for normal men and women were 4 7 and 4 4 days, respectively No significant difference in half life was found between the sexes, nor could any influence of age be observed. The values are in good correspondence with the majority of those obtained in other investigations. In some studies performed with the use of fibrinogen labelled with the monochloride method of McFarlane (1958) the latter author (1964) and Takeda (1966) obtained half life times in normal subjects of 3 1 and 3 4 days, respectively However Amris and Amris (1964) also with this method obtained a mean value of 4 3 days. According to Takeda (1966) long half lives as obtained by Hammond and Verel (1959) of 5 1 days, suggests the presence of labelled globulins. It has been found for rabbit fibrinogen (Atencio et al. 1965) that altered ^{131}I fibrinogen does not show a changed half life. Recently Tytgat et al. (1968) also using ^{131}I fibrinogen obtained with the monochloride method, found a half-life in normals of 5 0 days.

The average extrapolation value for the plasma pools in our study was 66 per cent, which is lower than those obtained by Amris and Amris (75—90) and by Takeda (84) This might indicate overiodination of our batches. As mentioned above, however there was in this respect no significant difference between batches labelled with 0 8 to 2.7 atoms of iodine per mole of fibrinogen.

Coagulation factor deficiency states. In most studies on the fibrinogen turnover in cases of congenital coagulation defects a normal half-life has been found (Gitlin and Borges 1953 Adelson et al. 1961 Rausen et al. 1961 Hart 1964) Also this study showed no significant differences from the normal values. This suggests that the rapid turnover rate of fibrinogen, as compared to other plasma proteins, can only be accounted for to a small degree by consumption through intravascular fibrin formation. This finding seems to speak against an often proposed theory of a

continuous coagulation and fibrinolysis taking place within the blood stream. On this background the results obtained by Takeda and Chen (1967) studying the metabolism of iodinated fibrinogen in patients with hemophilia A, seem difficult to explain. They found a half life time for ^{125}I fibrinogen of 2 3 days as against 3 4 days in their normals. The catabolic and synthetic rates of fibrinogen were found to be markedly increased whereas the plasma fibrinogen concentration, intravascular and interstitial fibrinogen, and the transcapillary transfer rate of fibrinogen were not significantly different. Takeda and Chen suggested for the hemophilia A patients an increased rate of breakdown of fibrinogen with or without previous fibrin formation. However it should be remarked that the fibrinogen used for labelling by these authors was obtained by precipitation of plasma with ammonium sulphate This might imply that the protein was not sufficiently pure for use in metabolic studies, although the clottability was reported to be 95 per cent or more. Furthermore the radioactivity of plasma but not that of the fibrinogen fraction was assayed. For the reasons mentioned the validity of the results obtained by Takeda and Chen must be put into question.

Polycythemia vera In 3 out of 6 patients with this disorder significantly increased turnover rates of fibrinogen were demonstrated. Although in all patients studied the marrow findings indicated an intense platelet production, only those with normal platelet counts in the peripheral blood showed short half lives. This may be explained by an increased peripheral destruction of platelets, leading to thromboplastic activity which can initiate a hypercoagulable state The latter again could explain a rapid consumption of fibrinogen. Christensen (1958) also found a shortened half life in one case. Increased fibrinolysis has been reported to occur in polycythemia vera (Björkman et al 1956) but could not be found in the present study Even if with the use of sensitive methods an increased fibrinolytic activity can be demonstrated this is not contradictory to an occurrence of intravascular coagulation. Increasing evidence has been obtained for a close connection also in clinical disorders between these two expressions of abnormal coagulation (Merskey et al. 1964 and others)

Liver disorders In 4 patients with liver cirrhosis (and in one with fatty liver) an increased turnover rate of fibrinogen was found. This could not easily be ascribed to increased fibrinolytic or fibrinogenolytic activity as no consistent increase in plasma fibrinolytic activity could be demonstrated From the results obtained on the turnover of other proteins in liver cirrhosis (Volwiler et al. 1955 Wilkinson and Mendenhall 1963) our findings can not either be explained as depending on a generalized increase in protein catabolism. A hypothesis of increased consumption of coagulation factors by intravascular coagulation has been strengthened by clinical and laboratory observations in liver cirrhosis (Bergström et al. 1960 Zetterqvist and von Francken 1963—I B Johansson 1964) Recently Tytgat et al. (1968) have demonstrated the occurrence of intravascular coagulation in liver cirrhosis. These authors in 25 patients with liver cirrhosis of varying severity found an average half

life time for ^{131}I fibrinogen of 3 1 days as against 5 0 days in their normals. Furthermore, they found that in nearly all the patients, the half-life time was fully normalized during heparin treatment. Simultaneously there was a continuous increase in the plasma fibrinogen concentration. As a possible mechanism for intravascular coagulation in this disorder they suggested that a functional insufficiency of the hepatocytes and disturbances in the portal circulation could lead to a decreased neutralization of activated coagulation factors or of thromboplastic activity in the reticuloendothelial system of the liver This is in line with their finding of a shortened fibrinogen half life in two patients with essential portal hypertension splenomegaly and extensive collateral circulation but with normal function and structure of the liver This, in turn, fits well with the good clinical effect of heparin upon bleeding in a corresponding case (Zetterqvist and von Francken 1963 I B) In this connection, Fletcher et al. (1964) have suggested that their finding of an abnormal fibrinolytic response after nicotinic acid can be due to impaired clearance of plasminogen activator by the liver Thus, it might be that both coagulation active components and fibrinolytic activators are not sufficiently cleared in liver parenchymal injury An important contribution in this field was the observation by Spaet et al. (1961) that in dogs blockade of the reticuloendothelial system resulted in failure to clear thromboplastin and thrombin injected into mesenterial veins.

Epidermolysis bullosa. Two patients were studied because of previous findings of a shortening of the whole blood clotting time and of a resistance to intravenously administered heparin in this disorder (Bauman and Krause 1954 Fuchs 1952) No shortening of the fibrinogen half life was, however observed.

Summary

The half life time of ^{131}I fibrinogen in humans was studied with a labelled protein of high clottability There were no indications of denaturation of the fibrinogen by the labelling procedure.

In normal male and female subjects the half-life time was found to be 4 7 and 4 4 days, respectively which is in accordance with the results obtained in earlier investigations. Patients with different forms of coagulation factor deficiency states showed half life times within the normal range. This is in line with previous findings. However in recent studies Takeda has found a shortening of the half-life in patients with haemophilia A. In polycythemia vera the turnover rate in some cases was abnormally rapid. As this was in patients with relatively normal platelet counts in the peripheral blood, it was supposed that here a consumption of platelets occurred. This as well as the faster elimination of fibrinogen might indicate a state of intravascular coagulation. In some patients with liver cirrhosis a shortening of the half life time was found. As no consistent fibrinolysis could be demonstrated, it was supposed that also in this disorder a mechanism of intravascular coagulation could occur Strong support for this has been obtained through later studies by Tytgat. Finally in 2 patients with epidermolysis bullosa normal half life times for the labelled fibrinogen were found.

Review of general effects of intravenously administered procoagulants

Since the experiments of Naunyn (1868) using infusions of hemolyzed red cells and Wooldridge (1886) a very large number of investigations have been made on the effects of procoagulants upon blood coagulation in vivo Wooldridge injected intravenously ein Gemisch oder vielleicht eine Verbindung von Eiweiss und Lecithin" and obtained the whole circulation system filled with thrombi if doses large enough of the substance were injected. H noticed that the thrombotic effect was proportional to the amount of substance injected and also found that blood obtained from animals given smaller doses did not clot spontaneously In this blood furthermore he found only traces of "Fibrinferment"

Concerning the *primary effects* of procoagulants a very large number of observations have been published and reviewed in detailed monographs (McKay 1965 Hardaway 1966, Selye 1966) The main symptoms of clinical intravascular coagulation are hypotension or shock, bleeding, and diffuse fibrin deposition in one or several organs. Extensive clotting in vivo like in vitro produces thrombocytopenia and reduced levels of fibrinogen, factor V and factor VIII As to prothrombin and the so called "serum factors —factors VII IX and X—their behaviour in such states has been somewhat disputed However later investigations have confirmed that also these are consumed (Rapaport et al. 1966) Anyhow in clinical states of intravascular coagulation as well as during experimental conditions the level of platelets, fibrinogen, factors V and VIII seem to be the most useful in the diagnosis of consumption coagulopathy

Evidence has been obtained, that *pathological fibrinolysis* can appear in connection with or as a consequence of intravascular coagulation, which in some way triggers plasminogen activation as a compensatory response (Fletcher et al. 1962, Mersky et al. 1964 Kowalski et al. 1965 Izak 1965 Girolami et al. 1966, Leandoer and Nilehn 1969) As expressed by Penick et al. (1965) it may be that both mechanisms are simultaneously or sequentially operative. The appearance of an *anticoagulant* has also been observed after intravenous infusion of thromboplastin (Ratnoff and Conley 1951 Lewis and Sreto 1962) and thrombin into dogs (Kowalski et al. 1965)

Concerning the *fate of fibrin and/or fibrinogen fibrin degradation products* formed as a result of intravascular coagulation special interest has been focused on the role of the reticuloendothelial system (RES) A basic observation was that of Monkhouse and Milojevic (1960) that in low-grade intravascular coagulation, although insufficient to provoke intravascular thrombosis, still conversion of fibrinogen to fibrin goes on. Spaet et al. (1961) in studies on rats showed that blood thromboplastin is selectively taken up by reticuloendothelial cells following intravenous injection and also demonstrated that blockade of the RES resulted in

enhancement of defibrination produced by blood thromboplastin In 1962 investigations by Lee led him to the assumption that the bulk of fibrin aggregates found in the circulating blood are rapidly removed by the RES In further studies, where immunohistochemical methods (Lee and McCluskey 1962) as well as electron microscopy (Prose et al 1965) were applied it was clearly demonstrated that the RES can operate as an efficient fibrin clearing mechanism in states of diffuse, low grade intravascular coagulation, where fibrin is formed in an incompletely polymerized state and consists of minute shreds, capable of being phagocytized (Lee et al. 1965) The phagocyte action was found to be exerted by the Kupffer cells (Prose et al. 1965) Later Gans and Lowman (1967) in studies on the uptake of fibrin and fibrin-degradation products by the isolated perfused rat liver using ^{75}Se labelled rat fibrin, found that this fibrin in the perfusate was eliminated at a rate comparable to that of aggregated ^{131}I BSA (bovine serum albumin) and colloidal gold in the same system. The loss of activity from the blood closely paralleled the increased activity recorded in the liver Thus, there is evidence that the RES plays a significant role in the clearance of fibrin, and possibly also of other coagulation products (Gans 1966)

With respect to potential protection mechanisms against the effects of intravascular coagulation an important contribution has been given by Lipinski et al. (1967) Using isotope-labelled fibrinogen these authors showed that fibrinogen, fibrinogen-fibrin intermediates and fibrinogen degradation products (FDP) in vitro may form soluble complexes, which are unclottable by thrombin. They presumed that such complexes may explain the occurrence of cryofibrinogen in various pathological states and postulated them to be an important factor involved in the development of the hemostatic defect. The authors thought that such complexes might be the expression of a defence mechanism against intravascular fibrin formation by rendering unclottable the intermediates of the fibrinogen fibrin conversion.

Of great general interest to clinical medicine have been also investigations on the effects of procoagulants with respect to *tissue damage* in different organs. Thus, a number of studies have been made on pulmonary vascular lesions following repeated intravascular administration of thromboplastin (Gore et al. 1962, 1963) or thrombin (Studer and Lorez 1965) into rabbits. Gore et al. found that repeated intravenous injections caused thrombi in the pulmonary arteries predominantly localized to medium and small arteries. By organization of the thrombi intimal fibrosis developed. As in the test tube thromboplastin was found to form an insoluble complex with arterial acid mucopolysaccharides, Gore et al. suggested that this reaction also in vivo was responsible for the intimal injury Cholesterol was found to accumulate in the changed intima. Studer and Lorez in studies by means of intravenous thrombin infusion, found that this gave rise to embolization in pulmonary arteries with mural incorporation and finally intimal fibrosis. In their experiments inhibition of fibrinolysis by epsilon aminocaproic acid (EACA) in doses corresponding to those used in the clinic, did not influence upon the results. They therefore suggested that fibrinolysis is of minor importance for the fate of pulmonary emboli

in rabbits Of great interest are the studies by Vassalli et al. (1963) who found that slow intravascular coagulation produced by thromboplastin infusion in rabbits, caused ultrastructural lesions in the kidneys resembling those in toxemia of pregnancy The authors suggested that entrance of placental thromboplastin in the maternal circulation could be responsible for the glomerular changes in toxemia, which was further supported by the demonstration with immunofluorescence of large amounts of fibrinogen derivatives in toxemic glomeruli (Vassalli et al. 1964)

Finally a recent observation on the influence of procoagulants upon the *platelets* is the one by Farburzewski et al. (1968) that thrombin, administered intravenously to rabbits, releases large amounts of platelet factor 4 (PF_4) into the circulating blood. It has been suggested that PF_4 may be a potent and specific agent triggering platelet aggregation and blood clotting in vivo. A significant elevation of this factor was also found in patients with fresh coronary thrombosis, thrombophlebitis and thromboembolic complication Ultrastructural platelet changes after thrombin injection in rabbits have also been demonstrated (Vassalli et al. 1964)

Present investigation

Labelling of fibrinogen

The preparations used in 3 different series (II—IV) were labelled with ^{131}I using the jet iodination technique essentially as described by McFarlane (1956) or with ^{125}I according to the monochloride method of the same author (McFarlane 1958) The coagulability of the fibrinogen preparations after labelling was 90—95 per cent. The radioactivity in fibrin in per cent of that in fibrinogen was 80—85 The yield of isotope in the preparations was for ^{131}I fibrinogen around 15 per cent and for ^{125}I fibrinogen 50—60 per cent of the isotope added. Between 0.2 and 2.6 atoms of iodine were introduced per mole of fibrinogen.

In comparison to ^{131}I-labelled human fibrinogen (I A) the radioactivity in fibrin in per cent of that in fibrinogen is for ^{131}I and ^{125}I-labelled dog fibrinogen around 10 per cent less than in the labelled human protein. This is explained by the finding that on iodination of dog fibrinogen about 15 per cent of the isotope is bound to material released by thrombin during the fibrinogen fibrin transition. Of this material, in turn, about 50 per cent, i.e. around 9 per cent of the total label in dog fibrinogen, seems to be associated with dog fibrinopeptide B, The other half of the radioactive unclottable material most likely is bound to denatured fibrinogen or to other proteins, present as contaminants in the fibrinogen preparations (VI)

Experimental conditions

Dog with a weight of 7—21 kg were injected with iodine-labelled fibrinogen in doses giving an initial radioactivity of 40—60 $\mu C \times 10^{-3}$ per ml plasma. For blocking

of the thyroid the dogs were given potassium iodide tablets from 2—3 days before the injection. Blood samples were drawn from the 1st to the 5th or 7th day to permit determination of the turnover of the labelled fibrinogen. On the 5th—7th day after the injection, experiments with procoagulants were made. After i.v anesthesia, bovine thrombin was given as i.v infusion during 1 hr whereas dog brain thromboplastin and a procoagulant from human postpartum serum (PPS) were injected i.v during 10—20 sec. The dogs given thromboplastin and PPS were ventilated artificially from 15 to 45 min after the injection. Blood samples were taken before anesthesia, then at 15 30 60 min and 2, 4 and 6 hrs after the start of thrombin infusion or injection of the other procoagulants, respectively usually also on the following day Hematocrit, platelet count, fibrinogen concentration, fibrinolytic activity radioactivity in plasma and in the coagulated fibrinogen were determined. In some of the dogs given thrombin infusions and thrombin together with epsilon aminocaproic acid immunological studies with respect to the appearance of fibrinogen-fibrin degradation products as well as measurements of radioactivity in serum were made on the samples obtained on the day of the experiment (V)

As *controls* 9 dogs were used In 4 of these the radioactivity decay in plasma and in fibrinogen was followed up to 10 days for determination of the basic half life time. For studies on the influence of anesthesia and indwelling catheter 2 dogs were used On the 6th day after injection of ^{131}I fibrinogen, one of these dogs was only anesthetized in the other also a catheter was inserted, through which a saline infusion was given during 1 hr Three dogs were given iodinated fibrinogen without isotope and followed during 10 days.

Results obtained in control dogs (II)

The mean half life times as measured in plasma and in coagulated fibrinogen were 2.0 and 1.9 days (range 1.2—3.0 and 1.2—2.8) respectively The values are in good agreement with those obtained by other investigators (II)

Anesthesia, saline infusion or catheters inserted were not found to influence upon the half life time to such a degree that the results obtained in the experiments with procoagulants could be markedly affected. However it must be pointed out that the information obtained in another investigation indicates that indwelling polyethylene catheters for 8 hrs may double the elimination rate of iodine labelled fibrinogen (Zetterqvist, Carlson, Liljedahl 1968) Such an effect would not, however influence upon the evaluation of the results of the present studies.

In the dogs given I fibrinogen without isotope, there was a transient fall in the platelet number and a simultaneous rise in the fibrinogen concentration.

The *fibrinolytic activity* in 20 normal dogs was found to be 89 7 μg/ml/hr and the s.d. 19.6.

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Experimental conditions

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From the values for fibrinogen concentration and radioactivity in plasma and fibrinogen at different times the amounts of degraded fibrinogen-fibrin (in III called nonclottable labelled fibrinogen products, NCLP) could be calculated and were expressed as equivalent amounts of fibrinogen (II III) For the thrombin and thromboplastin groups at the time of the plasma peak the degradation products amounted to between 40 and 100 per cent of the fibrinogen decrease up to that point. The true amounts of degradation products are probably larger than the calculated ones, as part of them may escape rapidly from the circulation by excretion and other clearing mechanisms. As indicated from the specific activity (μC/mg) new synthesis of fibrinogen in the dogs given thrombin began soon after the start of the infusion. At 4 hrs after that point the de novo synthesis seemed to account for up to 50 per cent of the circulating fibrinogen.

As to the dose response relations it was found for both thrombin and thromboplastin that with increasing doses, the fibrinogen concentration was more depressed and proportionally larger amounts of nonclottable labelled fibrinogen products appeared in the circulation. This indicates an intimate relation between the intensity of the coagulation trauma and a process of fibrinogen degradation.

With respect to comparative aspects the effects obtained with thromboplastin and PPS in general, were similar to those produced by thrombin. In dogs infused with thrombin during 1 hr the initial plasma radioactivity decrease was followed by an increase, with a maximum at 2 hrs after the start of the infusion. Although in the experiments with thrombin, no significantly increased fibrinolytic activity could be demonstrated in the circulating blood there was evidence that the subsequent plasma radioactive increase could have been caused by a fibrinolytic process, probably induced secondarily to intravascular coagulation. Evidence for this is given by the demonstration of fibrinogen degradation products in separation studies and the immunological behaviour of serum (V) as well as by the effects of simultaneously administered fibrinolysis inhibitors (II III V) This is also in accordance with the suggestions of Kowalski et al. (1963) The thromboplastin- and PPS-doses were given within half a minute and the maximum reactions occurred earlier and more dramatically than in the dogs given thrombin. In contrast to the findings in the thrombin experiments, an elevated fibrinolytic activity was recorded in the circulating blood. Because of the shorter injection time of thromboplastin, the coagulation trauma was probably more intense. This might also explain why a fibrinolytic process could be more intensively evoked and therefore demonstrable in the circulating blood.

I v injections of PPS caused a drop in blood pressure from about 160 to around 60 mm Hg, the pressure returned to near initial values within 10 to 20 min The femoral arterial blood flow showed a subsequent decrease in most instances to zero Infusions into the femoral artery did not produce any appreciable changes in the local blood flow indicating that PPS has no direct action on the peripheral blood vessels, and was not contaminated with vasoactive material.

Effects of thrombin and PPS in heparinized dogs

In dogs given an i.v. injection of heparin in a dose corresponding to 1 000 units per kg b.w. before thrombin infusion or PPS-injection, respectively most of the changes induced in the groups mentioned were prevented. A slight to moderate decrease in the platelet number was observed early after the start of the thrombin infusion. Heparinized animals injected with PPS showed a fall in blood pressure from 140—180 mm Hg to 90—150 mm Hg. The pressure reached 90 per cent of the preinjection value in 1—6 min (IV).

According to Hardaway (1966) the immediate arterial hypotension after i.v. administration of procoagulants is caused by occlusion of the microcirculation with subsequent decrease in cardiac return and therefore also in cardiac output. The decrease in blood pressure following injections of PPS also in the heparinized animals, indicates that part of the hypotensive effect may be due to other mechanisms than intravascular coagulation. It is reasonable to conclude that part of the hypotension is secondary to activation of the plasma coagulation system, with thrombin formation and subsequent peripheral vasodilatation (Delin et al. 1967, Olsson et al. 1969—see IV).

Summary

Intravenous administration of bovine thrombin, dog brain thromboplastin and a procoagulant from human postpartum serum (PPS) provoke similar effects. There is a decrease in platelet count, fibrinogen concentration and, as followed only after PPS a decrease also in the concentration of prothrombin. After injection of ^{131}I dog fibrinogen there is a difference between the radioactivity of plasma and that of thrombin-coagulated fibrinogen of around 10—15 per cent. This is explained by the fact, that by thrombin-coagulation around 15 per cent of the radioactivity is released into the clot supernatant. Thus, when determined as fibrin, a corresponding amount of radioactivity has been lost from the fibrinogen. Soon after the start of thrombin infusions or after short time injection of the other procoagulants, there is a rapid fall of the radioactivity in both plasma and fibrinogen. A following secondary increase of the plasma radioactivity is suggested as reflecting the appearance of degradation products of fibrinogen or fibrin, obtained through a fibrinolytic process. Most of the effects are inhibited if the dogs are heparinized before administration of procoagulants. The amounts of degradation products can be calculated from the radioactivity values and the specific radioactivity of the fibrinogen before the experiments. A strong capacity for resynthesis of fibrinogen after partial defibrination is demonstrated. With short time injections of thromboplastin and PPS more drastic effects are obtained with respect to symptoms of intravascular coagulation than those obtained with thrombin infusion during 1 hr. Also, after the more rapid coagulation traumata an increased fibrinolytic activity in vitro can be demonstrated, which is not the case after thrombin. The effects upon aortic blood pressure and

femoral arterial blood flow studied only after PPS-injections, demonstrate a pronounced decrease of the pressure returning to near the normal within 10—20 min. The femoral arterial blood flow decreased usually the zero. In heparinized animals the effect upon the blood pressure was smaller PPS-injections in the femoral artery did not produce major changes in the local blood flow indicating that PPS has no direct action on the peripheral blood vessels and is not contaminated with vasoactive material acting in this region.

INFLUENCE OF FIBRINOLYSIS INHIBITORS ON FIBRINOGEN TRANSFORMATION AND DEGRADATION IN EXPERIMENTAL INTRAVASCULAR COAGULATION

Review of other investigation

With a growing interest in fibrinolysis on the whole and not least in its pathological manifestations, a great number of studies have been made on the effects of increased fibrinolysis in vitro and in vivo. Because there seems to be in clinical fibrinolysis close connections between coagulation and fibrinolysis, it is important to reveal if after defibrination with procoagulants, fibrinogen products appear as a consequence of primarily thrombin action or if they are unrelated to the fibrinogen-fibrin transformation.

The conversion of fibrinogen to fibrin is initiated by thrombin through the release of peptides A and B from the fibrinogen molecule. Aggregation of "activated" fibrinogen molecules (fibrin monomers) proceeds by end to-end and side to-side aggregation. As a consequence of this process fibrin is formed. A suggestion was made by Hammarsten (1876) of the existence of a soluble intermediate in the fibrinogen-fibrin transformation. Blombäck and Laurent (1958) concluded that, during the action of thrombin on fibrinogen, mainly peptide A is released in the initial stage. They found that purified fibrinogen could loose as much as 40 per cent of its A peptide before visible coagulation occurred. Abildgaard (1965) calculated that in plasma only 6 per cent of fibrinogen from which peptide A had been removed was necessary to start aggregation.

As to the implication of occurrence of fibrin intermediates in clinical states, several observations have been made on such components in various thrombotic disorders (Cummine and Lyons 1948, Korst and Kratochvil 1955, Campbell 1959, Kalbfleisch and Bird 1960, Boros et al. 1962). Godal and Abildgaard (1963) demonstrated a good clinical effect of heparin in a patient having in plasma a substance which according to the authors most likely represented a mixture of fibrinogen and fibrin.

Degradation of fibrinogen-fibrin by plasmin gives rise to degradation products of varying size and behaviour (Fletcher et al. 1962, 1966, Fisher et al. 1967). Concerning the states of pathological fibrinolysis there have been much conflicting views on all aspects as underlying mechanisms, diagnosis, importance as a cause of bleeding as well as its treatment. With respect to especially the connection between coagulation and fibrinolysis important contributions were given by Schneider (1951, 1952) and Müllertz (1957). They found that when tissues are damaged by trauma or anoxia both systems are activated or accelerated by the release of tissue thromboplastin and/or fibrinolytic tissue activator. Similar effects were obtained after trauma by Bergentz and Nilsson (1961), after thromboplastin by Izak (1965) and after thrombin by Girolami et al. (1966). Weiner (1953) suggested that fibrinolytic activity could be released from the vascular endothelium as a result of intravascular clotting. Both phenomena do not, however, always develop in parallel.

Important for the understanding of the pathogenesis of fibrinolysis was the finding made in 1957 independently by Triantaphyllopoulos, Niewiarowski and Kowalski as well as of Stormorken that spontaneously lysed fibrinogen besides losing its thrombin clottability acquires in addition anticoagulant properties. The fraction responsible for this phenomenon was called by Triantaphyllopoulos AFIF (anticoagulant fraction of incubated fibrinogen) and antithrombin VI by the Polish authors. Later similar products of fibrinogen, also showing an antithrombin effect, were found responsible for a coagulation anomaly termed "defective fibrin polymerization" (Fletcher et al. 1962 Fisher et al. 1967) Besides their action upon the later stages of fibrin formation fibrinogen degradations products have also effects upon the platelets. Kowalski et al. (1964) found that aggregation, viscous metamorphosis as well as adhesivity in vitro were influenced by FDP the effects being dependent on the concentrations of split products and of activating agents (ADP thrombin, connective tissue extract) The authors discussed the possible significance of the described findings for the hemostatic functions, referring to investigations by Salmon (1961) and Salmon et al. (1962) which had been in favour of a relationship between enhanced fibrinolysis, platelet function and bleeding These investigators had noticed that bleeding only appeared when fibrinolysis and thrombocytopenia were observed together

With respect to connections between intravascular coagulation and fibrinolysis the findings of Lipinski et al. (1967) are interesting. These authors demonstrated that fibrinogen with intermediates and degradation products may form soluble complexes, unclottable by thrombin. They found that paracoagulation, i.e. formation of a clot upon protamine sulphate addition to partially digested fibrinogen, unclottable with thrombin (Derechin 1935 Derechin and Szuchet 1956) occurs when defibrination is caused by excessive intravascular coagulation. On the other hand, when proteolytic digestion of fibrinogen is predominant, paracoagulation occurs after combined action of thrombin and protamine sulphate. Lipinski et al. suggested that formation of such complexes may explain the occurrence of cryofibrinogens in various pathological states

Based on suggestions of a secondary compensatory fibrinolysis after coagulation trauma, studies have been made on the influence of fibrinolysis inhibitors administered simultaneously with procoagulants It has been demonstrated that EACA given together with thrombin, promotes the development of the generalized Shwartzman phenomenon in rabbits (Lee 1962) and also the persistence of fibrin in the glomeruli of the rat. Beller et al. (1967) tested the effects of several different proteolysis inhibitors together with thrombin in rabbits and found all of the inhibitors to result in fibrin deposition in the glomeruli of rabbit kidney However as earlier mentioned, Studer and Lorez (1965) found that administration of EACA did not influence upon the fate of pulmonary emboli, obtained by i.v. thrombin infusion into rabbits.

Present investigation

The aims of these studies were 1 to find out if substances with antifibrinolytic action would influence upon the secondary changes obtained after i v administration of procoagulants, and 2. to investigate, whether an anticoagulant effect demonstrated in vitro for Trasylol (a kallikrein inhibitor with antifibrinolytic action) could be obtained also in vivo after injection of procoagulants.

Experimental conditions

Dogs were treated and coagulation parameters followed as described earlier with the addition that antifibrinolytic drugs were administered together with procoagulants. Four groups of animals were given simultaneous infusions of 1 thrombin and epsilon aminocaproic acid (EACA) 2. thrombin and Trasylol, 3 thromboplastin and EACA, and 4 *thromboplastin and Trasylol. The dose range was for* EACA 0 ? to 0.8 g per kg b.w and for Trasylol 6,500 to 100 000 K.I U (kallikrein inhibitor units) per kg b w The number of dogs in each group varied between 5 and 11 In 3 dogs, not included in the series mentioned, the radioactivity and immunological behaviour of serum during simultaneous infusions of EACA and thrombin were followed (V)

Results and discussion

With the largest doses of EACA and Trasylol the secondary plasma radioactivity increase, obtained after thrombin and thromboplastin (II III) was abolished and the amount of calculated fibrinogen degradation products was reduced. However although a fibrinolytic activity seemed to be inhibited, still significant amounts of degradation products were calculated. This is in accordance with the findings made by Barnhart (1966) who identified and quantitated by means of immunological methods degradation products after combined administration of EACA and thrombin in dogs. The author concluded that a dose of EACA large enough to inhibit effectively a general plasma fibrinolytic activity could not block a local fibrinolysis going on in microthrombi in the circulation. In the present study a correlation was obtained between the dose of the antifibrinolytic drugs and the effect upon the induced fibrinolytic activity With medium and smaller doses there was a secondary plasma radioactivity increase but it was delayed like also the increased plasma fibrinolytic activity appearing after thromboplastin. The effect of 10,000 K.I U of Trasylol was greater than that of 0 2 g of EACA, which implies that on a molar basis, Trasylol is a more effective fibrinolysis-blocking substance than EACA This is in accordance with the observations by Dubber et al. (1968) finding the molar potency of Trasylol as an inhibitor of fibrinolysis to be 1,000 times that of EACA.

The evidence obtained of a secondary fibrinolysis after procoagulants was further

strengthened by the information obtained in studies performed by means of gel filtration of plasma and immunoelectrophoresis of serum in connection with thrombin infusions (V) As presented later a clear effect of EACA upon fibrinogen degradation products was noticed.

Trasylol, besides its antifibrinolytic effect, has also been demonstrated to possess an anticoagulant activity in vitro. This latter effect, however is directed against the initial phase of coagulation and not towards thrombin (Amris 1964 Blombäck et al. 1964) In the present study no effect on the disappearance of fibrinogen in dogs given thrombin was found (II) which is in conformity with the information obtained from the in vitro studies. A comparison with respect to early changes after thromboplastin injection alone and combined with EACA and Trasylol, respectively indicated a slightly less decrease of fibrinogen radioactivity and concentration in the Trasylol-thromboplastin group if all dogs surviving 15 min were considered (III) This could speak for a slight anticoagulant effect of Trasylol during the conditions of this study However in those dogs surviving 4 hrs only small differences between the groups were observed. Our results do not contradict those of Dubber et al. (1968) which authors from studies in humans concluded that Trasylol has an anticoagulant effect.

Among the dogs given thrombin as procoagulant, the mortality rate was highest in the Trasylol thrombin group (3 of 8) One of 6 dogs given only thrombin died and none in the EACA-thrombin group With thromboplastin, the highest mortality (6 of 8) was in the group given only procoagulant. Half of the dogs in the Trasylol-thromboplastin group died and 1 of 6 in the EACA-thromboplastin group. As to the higher mortality rate in Trasylol than in EACA treated dogs, one might speculate that the difference could be due to the fact that EACA does not, as Trasylol, directly inhibit plasmin. Thus, with respect to the influence upon thrombi formed after procoagulants, Trasylol is probably sometimes more unfavourable because of its stronger antifibrinolytic effect. An inhibiting effect by EACA upon fibrin polymerization (Godal and Helle 1963 Godal and Theodor 1965) might also have contributed to the protective action by this substance. Studying the effects of Trasylol in dogs injected with thromboplastin, Mammen and Poucho (1966) suggested that the protective action of Trasylol in decreasing thrombus formation was also by interference with the cross-linking of fibrin monomers rather than by its antithromboplastic activity

An interesting finding was, that administration of EACA resulted in a temporary increase of the hematocrit. Cummings and Welter (1966) found that large doses of aminocaproic acid evoked a syndrome resembling dehydremic shock. They suggested that this was possibly an effect of histamine, release by catecholamines through the action of aminocaproic acid.

In separate studies (Zetterqvist, Carlson, Liljedahl 1968) it was demonstrated, that indwelling polyethylene catheters for 8 hrs may double the turnover rate of iodine-labelled fibrinogen. As earlier mentioned, such an effect would not, however influence upon the evaluation of the results of the present studies.

Summary

After intravenous administration of procoagulants to dogs a "secondary fibrinolysis" seems to appear The effects obtained by large doses of fibrinolysis inhibitors epsilon aminocaproic acid (EACA) and Trasylol support this assumption. On a molar basis the antifibrinolytic effect of Trasylol is larger than that of EACA. When these drugs are administered simultaneously with thrombin no anticoagulant effect by either is detectable. Trasylol seems to exert some anticoagulant action when injections of thromobplastin are given As indicated from studies in dogs in clinical practice antifibrinolytic drugs should be given in combination with anticoagulants when fibrinolytic bleeding is suspected to be secondary to intravascular coagulation. Like in earlier studies an increasing hematocrit after administration of EACA is observed

SEPARATION BY GEL FILTRATION OF FIBRINOGEN AND DEGRADATION PRODUCTS IN THROMBIN INDUCED INTRAVASCULAR COAGULATION

Previous investigations

In later years clinical experience and laboratory findings have brought evidence for a connection between intravascular coagulation and fibrinolysis The differential diagnosis of these states of abnormal coagulation is, however difficult.

Valuable information for diagnosis and treatment has been obtained through a number of investigations devoted to the mechanisms of abnormal conversion as well as pathological degradation of fibrinogen-fibrin. Already Hammarsten (1876) suggested the existence of a soluble intermediate in the formation of fibrin from fibrinogen. In the previous chapter several observations on such intermediates, occurring in vitro or in clinical thrombotic disorders have been mentioned.

Activation of fibrinolysis has been demonstrated after trauma (Schneider 1951 1952, Müllertz 1957 Bergentz and Nilsson 1961) and also after administration of procoagulants (Kowalski et al. 1963 Izak 1965 Nordström and Zetterqvist 1966 Leandoer and Nilehn 1969 and others) Following the discovery of the anti thrombin effect of spontaneously lysed fibrinogen (Niewiarowski and Kowalski 1957 Stormorken 1957 Triantaphyllopoulos 1957) a similar in vivo effect exerted by split products was demonstrated by Fletcher et al. (1962) During pathological degradation of fibrinogen-fibrin a wide variety of products may appear (Fletcher et al. 1962 Nilehn and Nilsson 1964 Fisher et al. 1967 Nilehn 1967) In experimental studies with thrombin infusions to dogs a variety of degradation products (Leandoer and Nilehn 1969) as well as the appearance of fibrinopeptide A (Teger Nilsson 1969) have been demonstrated.

As mentioned above, complexes between fibrinogen and fibrin monomer or between these and fibrinogen degradation products are also involved in the pathophysiological phenomena observed during intravascular coagulation and fibrinolysis (Shainoff and Page 1962, Copley and Luchini 1964 Lipinski et al. 1967) Such products have been suggested to have relations to cryofibrinogens.

Present investigation

In earlier studies (II—IV) on the elimination of iodine labelled fibrinogen after procoagulants, a secondary rise in plasma radioactivity was demonstrated, following an initial decrease in the radioactivity of both plasma and fibrinogen. This finding was postulated to reflect a secondary fibrinolysis.

The aim of this investigation was to try to identify by means of ^{131}I-fibrinogen, fibrinogen degradation products and possibly also complexes after thrombin infusions into dogs. This was made by studying: 1 the radioactivity distribution in

plasma, filtered through Sepharose columns, before and after administration of thrombin, 2. the molecular size distribution and immunological behaviour of labelled components, and 3. the influence of epsilon aminocaproic acid (EACA) administered simultaneously with thrombin.

Experimental conditions

Nine dogs were injected with ^{125}I fibrinogen. After complete distribution at steady state catabolism, the animals were anesthetized and catheters inserted into the leg veins. Three dogs in each of 2 groups were given 1. thrombin infusion during 1 hr and 2. thrombin infusion together with epsilon aminocaproic acid (EACA). Before, during and after the thrombin infusions blood samples were taken for determination of radioactivity in plasma, fibrinogen and serum as well as fibrinogen concentration, platelet count and hematocrit. On the serum samples immunological studies were also performed.

For separation of fibrinogen and degradation products on Sepharose columns, plasma from only 3 dogs was used. Plasma and clot supernatant of thrombin-coagulated plasma obtained before thrombin infusion as well as plasma from the point of maximum secondary plasma radioactivity increase, were filtered through the column. As a control radioactive fibrinogen was added to normal plasma and filtered through the same column. The distribution patterns for one dog given only thrombin and for one given thrombin together with EACA were compared. Immunoelectrophoresis was performed on eluate fractions obtained at different effluent volumes. For further separation of corresponding fractions of low molecular weight products obtained with Sepharose, Sephadex G-25 columns were used. Finally after Sephadex filtration, thin layer electrophoresis was performed on the fractions, which from the radioactivity profile and effluent volume were expected to contain fibrinopeptide B.

Results and discussion

Radioactivity elimination and immunological behaviour of serum during experiments — The radioactivity curves for plasma and fibrinogen were similar to those in earlier studies (II—IV) described in the previous chapters. During infusion of thrombin alone an initial steep slope of both curves was followed by a secondary rise of the plasma curve. When EACA was given simultaneously with thrombin the curves diverged only slightly after the initial phase. The mean value for the initial serum radioactivity in 9 dogs was 25 %. In the dog given only thrombin the serum curve rose to around 50 % at the time of maximum plasma value thereafter running parallel with the plasma curve. In the dog given thrombin and EACA the serum radioactivity did not change during the first 2 hrs, then it rose slowly to a value about 5 % above the initial.

Immunoelectrophoresis demonstrated faint precipitates in both dogs before the infusions. In the dog given only thrombin strong arches were obtained with serum taken from 1 to 6 hrs after the start. On the following day there was still a faint precipitate. In the dog given EACA in addition to thrombin there were precipitates, weaker and appearing later than in the dog given only thrombin. Thus after thrombin infusions increasing amounts of products containing the antigenic determinants of fibrinogen seem to appear

Radioactivity distribution before thrombin infusions — Of the total radioactivity 87 % was recovered in thrombin-coagulated fibrinogen and 21 % in serum, obtained after spontaneous coagulation. After Sepharose filtration 61—64 % of the radioactivity was recovered in a fraction (peak I) corresponding to fibrinogen, 14 % in a fraction (peak II) corresponding to material with lower molecular weight and 17 % in a region between the two mentioned. This showed that fibrinogen degradation products had been produced in the dogs during the time required to obtain "steady state" catabolism. The serum radioactivity was higher than could be expected from the figure for radioactivity recovered in the clot formed by addition of thrombin to plasma. This was explained as due to a higher extent of copolymerization or trapping of high molecular weight degradation products during the rapid fibrin formation in the presence of thrombin. This is supported by the finding, that in the clot supernatant after thrombin coagulation of a corresponding plasma sample, much smaller amounts of "high molecular weight" degradation products like those demonstrated in plasma were observed An alternative explanation, though less likely could be that enzymes other than plasmin have been acting during the time of formation of serum.

Radioactivity distribution after infusion of thrombin.—At 2 hrs after the start of thrombin infusion, 71 % of the radioactivity was recovered in plasma, 52 % in serum and 16 % in coagulable fibrinogen. After Sepharose filtration 30 % was found in the fibrinogen fraction (peak I) This value was higher than that for fibrinogen in plasma. The discrepancy might indicate the presence of labelled, non-clottable high molecular-weight fibrinogen derivatives. The radioactivity in material with lower molecular weight had increased, indicating that smaller degradation products had appeared. The radioactivity of the intermediary fraction was essentially unchanged. The amount of radioactivity confined to material of lower molecular weight, together with that of the intermediary region and of non-clottable products in the fibrinogen peak, corresponded fairly well with the value for serum radioactivity

Radioactivity distribution after infusion of EACA and thrombin. — At 2 hrs after the start of the infusion 32 % of the radioactivity was in plasma, 26 % in serum and 4 % in fibrinogen. After filtration, 5 % was in the fibrinogen fraction, 12 % in the peak of relatively lower molecular weight material (peak II) In the intermediary region the radioactivity was reduced in comparison with that before the experiment

It was concluded that the EACA treatment had inhibited the appearance of degradation products following thrombin infusion.

Immunoelectrophoresis on eluate after Sepharose filtration—Eight to nine fractions from every filtration experiment were tested. Material with the antigenic determinants of fibrinogen was recovered in samples corresponding to effluent volumes of fibrinogen and intermediary region but not in material with lower molecular weight. There were positive tests in these regions in samples obtained both before and 2 hrs after start of thrombin infusion. In the dog given EACA in addition to thrombin only material within the intermediary region gave faint precipitates. Thus, like the rise of serum radioactivity the immunological behaviour of the eluate indicates the appearance of increasing amounts of fibrinogen degradation products after thrombin infusion and also an inhibition of these by EACA. These findings confirm that the suggestion of a secondary fibrinolysis, made from the results of the earlier studies (II—IV) is valid

Gel filtration of lower molecular weight material—When pooled fractions of the radioactive material eluted at about the total volume of the Sepharose column and containing no antigenic determinants of fibrinogen, were filtered through Sephadex G-25 two radioactivity peaks were obtained. With respect to the fractionation range of the column, the radioactive material eluted in between the void and total volumes of the column could be expected to contain fibrinopeptide B Electrophoresis of this material failed, however to demonstrate the presence of fibrinopeptide B. It might be that this peptide is released at a slow rate or rapidly degraded to smaller fragments escaping detection with the methods used. Spots with mobility close to that of fibrinopeptide A were found. However as they were present in both plasma and serum obtained before thrombin infusion, the significance of this observation is doubtful. It seems reasonable to assume that the radioactivity in the fraction containing low molecular weight material (peak II) is bound to material released as a result of common catabolism of fibrinogen.

Summary

After thrombin infusion to dogs, injected with ^{125}I fibrinogen, there is first a rapid decrease of the radioactivity in plasma and fibrinogen followed by an increase of plasma and serum activity The initial amount in serum of 20—25 per cent of the total plasma radioactivity is about the double 2 hrs after the start of thrombin infusion. With large doses of fibrinolysis inhibitor the secondary rise in plasma and serum radioactivity is almost blocked Immunological studies indicate the appearance after thrombin infusion of increasing amounts of products with the antigenic determinants of fibrinogen. This effect is almost completely abolished in the presence of the fibrinolysis inhibitor The radioactivity distribution after gel filtration indicates the occurrence of fibrinogen degradation products of varying size Except those of

the lowest molecular weight, the products contain antigenic determinants of fibrinogen. Degradation products have been produced in the dog even before thrombin infusion but appear in increased amounts after defibrination. No products with larger molecular weight than that of fibrinogen are observed after thrombin. This speaks against the appearance in our experiments of complexes between fibrinogen and fibrin or degradation products. Fibrinopeptides could not be demonstrated during the conditions of the experiments.

Other investigations

In the fibrinopeptide B of fibrinogen from several species there are tyrosine residues (Blombäck et al 1966) capable of being iodinated. It has been demonstrated for some fibrinogens that labelling of this peptide can be obtained (Nordström et al. 1966 Regoeczi and Walton 1967) From their results Regoeczi and Walton suggested that labelling of fibrinopeptide B was depending on the occurrence of non esterified tyrosine residues.

Present investigation

In connection with studies on the elimination of ^{131}I dog fibrinogen after i.v administration of procoagulants (II—IV) it was consistently observed that fibrin formed from iodinated fibrinogen by thrombin coagulation had a considerably lower specific radioactivity than fibrinogen. This difference could not be accounted for on the basis of impurities present in the fibrinogen preparations used for iodination. It was assumed that the difference might at least partly be due to iodination of fibrinopeptides split off from fibrinogen during clotting. Therefore, after ^{131}I labelling of dog fibrinogen, trials were made to isolate the dog fibrinopeptide B which because of its content of tyrosine residues would be capable of being iodinated.

Isolation and location of fibrinopeptides

Dog fibrinogen with a coagulability of 88—99 per cent was labelled as described for human fibrinogen (I A) Between 0.3 and 0 9 atoms of iodine were introduced per mole of fibrinogen. After thrombin coagulation of the labelled fibrinogen the clot supernatant contained around 16 per cent of the radioactivity introduced into the fibrinogen.

Gel filtration on Sephadex of clot supernatant was performed in some experiments. The supernatant contained ninhydrin-positive material, which was retained by the gel. In this material 50—60 per cent of the original radioactivity in the clot supernatant was recovered in a distinct peak. Paper electrophoresis of this fraction demonstrated the presence of 2 main components with the same mobility as authentic fibrinopeptides A and B_1 The radioactivity was almost exclusively associated with material having the same mobility as fibrinopeptide B

Chromatography of clot supernatants on a Dowex 50×2 1 mm revealed several ninhydrin-positive peaks. On the basis of chromatographic behaviour and electro-

phoretic mobility 2 main peptide fractions obtained (peaks II and III) identified with fibrinopeptides B_1 and A. Most of the radioactivity about 4 per cent of the original in fibrinogen, was recovered in a fraction (peak IV) which was only slightly ninhydrin-positive The latter was somewhat more retarded on the column than A and considerably more than fibrinopeptide B_1 Electrophoresis of the peak IV material mixed with reference peptides A and B_1 showed that the radioactivity completely overlapped with fibrinopeptide B_1

In order to obtain further evidence for the view that peak IV contained a radioactive, labelled fibrinopeptide B_1 an attempt was made to produce peak IV material from fibrinopeptide B_1 by iodination with ^{131}I according to the monochloride method (McFarlane 1958) Per mole of peptide 0 0022 atoms of iodine were introduced. A low label was aimed at to reduce the chance of obtaining more than one iodinated peptide derivative. This fibrinopeptide B_1 showed the same chromatographic and electrophoretic behaviour as its analogue obtained by iodination of fibrinogen.

For location of iodine incorporated in fibrinopeptide B the labelled fibrinopeptide B_1 was digested with pronase. Thin-layer chromatography revealed that most of the radioactivity was associated with material having the same Rf value as monoiodotyrosine. After alkaline hydrolysis a small radioactive component with a Rf value similar to that of monoiodohistidine was demonstrated by thin-layer chromatography Therefore it may be that histidine residues are to some degree iodinated in the peptide.

Discussion

The present study shows that ^{131}I-labelling of dog fibrinogen gives rise to incorporation of the isotope in the dog fibrinopeptide B By chromatography of the clot supernatant obtained after thrombin coagulation of the labelled fibrinogen, both the analogues of fibrinopeptide B (B_1 and B_2) present in dog fibrinogen, can be isolated. They differ only with respect to the degree of sulphation In B_1 only one, in B_2 both tyrosine residues are sulphated. The iodine isotope is incorporated in B_1 (but not in B_2) As to the chromatographic behaviour of the labelled B_1 it is clearly retarded on a Dowex 50 × 2 column in relation to its non-labelled counterpart. However the electrophoretic mobility of the two is the same. The fact that fibrinopeptide B_1 labelled in vitro on chromatography as well as in electrophoresis shows the same behaviour as the labelled peptide isolated from the iodinated dog fibrinogen, is further proof that the incorporation is really in B_1 As to the attachment of the label in the peptide mainly monoiodotyrosine is formed but some degree of iodination also of histidine residues can not be excluded.

The amount of radioactivity released into the clot supernatant after coagulation of the native protein with thrombin was 16 per cent of the activity in the fibrinogen the amount found by Regoeczi and Walton was somewhat lower The radioactivity content of the clot supernatant explains the greater part of the difference between the specific activity of fibrin and that of fibrinogen. As after Sephadex filtration

50—60 per cent of the radioactivity in the clot supernatant is confined to material which identifies with fibrinopeptide B_1 roughly 9 per cent of the total radioactivity in fibrinogen is incorporated in this peptide. The radioactivity in the supernatant that can not be accounted for by the label in fibrinopeptide B may derive from over iodinated fibrinogen or impurities present in the fibrinogen preparation. Our results suggest that iodination of fibrinopeptide B does not render the fibrinogen unclottable as the iodinated peptide is released from the molecule during the coagulation process. The labelled fibrinogen seems not either to be more rapidly eliminated from the circulation in vivo as the specific activity in fibrin in per cent of that in fibrinogen remains roughly constant during the whole time of distribution and steady state catabolism. These findings are in conformity with the results obtained by Regoeczi (1967) in studies on fibrinogen from the goat. This author suggested that the in vivo behaviour of a labelled fibrinogen does not depend on whether or not the fibrinopeptide B is labelled.

In investigations on the effect of thrombin coagulation upon the label in iodinated fibrinogen Regoeczi and Walton (1967) studied the protein from eight species. They showed that fibrinopeptides B from ox and rabbit, containing each one tyrosine-O-sulphate residue could not be iodinated. In goat and sheep fibrinogens, having one of two tyrosines sulphated, they found that the amount of iodine, released into the supernatant after thrombin coagulation, on a molar basis was only half of that obtained when dog fibrinogen was coagulated. This finding made them suggest that the fibrinopeptide B from dog contains two unesterified tyrosine residues. However as the bulk of dog fibrinopeptides contains at least one tyrosine-O-sulphate residue (Blombäck et al. 1966) this interpretation is not valid. It seems as if the iodination is not dependant only on the occurrence of free tyrosine residues but steric factors are also operative. In six out of eight fibrinogens studied by Regoeczi and Walton, tyrosine-O-sulphate is present in the fibrinopeptide B. They noticed that no label was introduced into those peptides having all tyrosine residues esterified with sulphate and suggested that labelling of sulphated tyrosine residues is not favoured. In the present study no incorporation was obtained in the fibrinopeptide B_3 where both tyrosine residues are sulphated.

The information obtained in the present study is important for interpretation of results obtained in studies on the turnover of fibrinogen in species, in which the fibrinopeptide B is labelled. As presented elsewhere (II) the loss of radioactivity from fibrinogen after thrombin coagulation in among other the dog protein must be taken into account. Release of radioactivity into serum might occur from fibrinopeptide B during coagulation in vivo and from the degradation of fibrinogen and fibrin. As presented (V) there was, under such conditions no evidence that radioactivity from fibrinopeptide B had been released as a consequence of coagulation in vivo.

Summary

When iodine labelled dog fibrinogen is coagulated with thrombin, around 16 per cent of the total radioactivity is released into the clot supernatant. From the latter fibrinopeptides A, B_1 and B_2 can be isolated by appropriate procedures. The B_1 peptide contains about 9 per cent of the original radioactivity in fibrinogen in the B_2-peptide no label can be demonstrated The part of radioactivity in the super natant not incorporated in fibrinopeptide B_1 might be contained in non-clottable, overiodinated fibrinogen or in impurities. On iodination the clottability of the fibrinogen is reduced by some 3 per cent. A constant difference in vivo between plasma and fibrinogen radioactivity indicates that the non-clottable contaminants are not screened out but show the same turnover as the clottable protein.

GENERAL SUMMARY

The half life time of iodine labelled fibrinogen in normal humans was around 4.5 days, which is in accordance with the results of most other studies. Like in previous investigations, patients with various coagulation factor deficiency states were found to have a normal fibrinogen elimination. This speaks for that the short half life time of fibrinogen as compared to that of other plasma proteins, is not to a considerable degree explained by consumption of fibrinogen in a continuous intravascular coagulation. An increased turnover rate of fibrinogen was found in some disorders, where hypercoagulability has been suggested to occur. Results obtained in some patients with polycythemia vera and in some with liver cirrhosis indicate that in these disorders intravascular coagulation with consumption of fibrinogen and other coagulation factors may occur.

Intravenous administration of procoagulants into dogs results in a consumption coagulapathy accompanied by a secondary fibrinolysis. This fibrinolysis can be effectively blocked by simultaneous treatment with large doses of fibrinolysis inhibitors.

A "spontaneous" fibrinolysis in the dog is suggested by the demonstration in plasma of fibrinogen degradation products in animals, subjected only to anesthesia and insertion of catheters. After i.v. administration of procoagulants degradation products of varying molecular size appear in increased amounts.

When dog fibrinogen is labelled with iodine around 9 per cent of the total iodine in fibrinogen is incorporated in the fibrinopeptide B. The possibility of labelling of fibrinopeptide B must be taken into consideration in connection with studies on the turnover of fibrinogen in several species.

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EXTRINSIC NERVOUS CONTROL OF GASTRIC MOTILITY

An experimental study in the cat

By

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This summary is mainly based on studies reported in the following papers

I Vago-vagal reflex relaxation of the stomach in the cat. Jansson, G Acta physiol scand. 1969 *75* 245—252

II Elicitation of reflex relaxation of the stomach from pharynx and esophagus in the cat. Abrahamsson, H and G Jansson Acta physiol. scand. 1969 In press.

III Studies on the ganglionic site of action of sympathetic outflow to the stomach. Jansson, G and J Martinson Acta physiol. scand. 1966 *68* 184—192

IV On adrenergic influence on gastric motility in chronically vagotomized cats. Jansson, G and B Lisander Acta physiol. scand. 1969 In press.

V Hypothalamic control of adrenergic outflow to the stomach in the cat. Jansson G., B Lisander and J Martinson. Acta physiol. scand. 1969 *75* 176—186

VI Effects of reflexes of somatic afferents on the adrenergic outflow to the stomach in the cat. Jansson, G Acta physiol scand. 1969 In press.

These papers are referred to in the text by their Roman numerals.

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CONTENTS

	Page
INTRODUCTION	5
1 General considerations	5
2. Arrangement of the gastric smooth muscle coat	5
3 Neural elements in the gastric wall	6
4 Extrinsic nerve supply to the stomach	7
a. Cholinergic fibres	7
b Vagal relaxatory fibres	8
c. Adrenergic fibres	9
d. Afferent fibres	10
5 Reflex and central nervous influences	10
AIM OF THE PRESENT STUDIES	12
METHODOLOGICAL CONSIDERATIONS	14
RESULTS AND COMMENTS	17
1 Gastric motility in intact anesthetized animals (balloon technique)	17
2 Gastric motility after abdominal surgery (direct recording technique)	18
3 Effects on gastric motility by direct stimulation of the efferent nerve supply	20
a. Vagal stimulation	20
b Splanchnic stimulation	21
4 Reflex activation of the extrinsic nerve supply to the stomach	23
a. Reflex activation of vagal excitatory fibres	23
b Reflex control of the vagal relaxatory fibres	24
Mode of reflex excitation	24
The arrangement of the reflex arcs	26
Extent of gastric reflex response	27
Relaxation of the stomach in connection with swallowing	29
Functional significance of the vagal relaxatory fibres	29
c. Extrinsic gastric control mediated by the adrenergic supply	30
Intestino-gastric inhibitory reflex	31
Influences on adrenergic fibre activity by hypothalamic and somatic afferent stimulation	33
Mode of adrenergic fibre influence on gastric motility	35
5 Vagally mediated gastric relaxation *versus* inhibition produced by adrenergic fibres	36
SUMMARY	37
ACKNOWLEDGEMENTS	39
REFERENCES	40

INTRODUCTION

1 General considerations

The stomach subserves three important motor functions. First, it provides an adjustable reservoir by adapting quickly and markedly in volume in connection with food intake, without development of excessive pressure rises. Secondly gastric contents are mixed with gastric secretion. Thirdly the gastric contents are gradually passed into the intestinal tract for final digestion and absorption at a rate which is suitable for these processes.

As early as 1764 Haller observed alternating contraction and relaxation of the stomach (see Cannon 1898) Mainly on the basis of studies of intestinal motility starting with the classical investigation by Bayliss and Starling (1899) it has been generally accepted that intestinal motility is reciprocally controlled by vagal and sympathetic fibres, a concept that has been accepted to hold for the whole gastro-intestinal tract.

Studies on gastric motility responses, elicited by vagal and splanchnic stimulation also received wide attention during the first decades of this century Thus, Cannon and Lieb (1911—12) coined the term "receptive relaxation" for the suppressive effect on gastric motility which they observed in connection with deglutition. In a survey of the literature and by own observations of the nervous control of gastric motility McSwiney (1931) stressed that both vagal and splanchnic stimulation could produce excitatory responses if prevailing gastric tone was low while both types of stimulation tended to cause inhibitory responses if gastric tone was initially high This view has since been largely accepted in textbooks and reviews though the exact nature of these responses has not been fully understood (e g Youmans 1952, 1968 Kuntz 1953 Thomas and Baldwin 1968)

2. Arrangement of the gastric smooth muscle coat

From early morphological studies (e g Jefferson 1914—15 for ref see McSwiney 1931 Schofield 1968), it was clear that the arrangement of the gastric smooth muscles is a complex and differentiated one, which suggests that the muscle coat cannot be regarded as a simple functional unit. However the relation between gastric motor function and the arrangement of the smooth muscle cover is in several respects still not clear

The gastric muscle layers are less distinctly separated into complete sheaths than in most other parts of the gastro-intestinal tract. Thus, an incomplete outer layer of longitudinal fibres extends from the esophageal smooth muscles and forms well-developed bundles along the curvatures while only scattered fibre streaks are present in other parts of the gastric wall. The only sheath of muscle which is complete throughout is formed by the middle circular layer which increases progressively in thickness towards the pylorus. The internal layer of the gastric smooth muscle spreads from the fundus in a fan-like fashion over the anterior and posterior walls of the stomach where it connects with the middle circular coat. The functional implications of this arrangement of the muscle layers and their interactions are largely unknown An exploration by means of electro-physiological methods *in situ* may here prove fruitful.

3 Neural elements in the gastric wall

Gastric motility is certainly in part based on myogenic activity but co-ordinated gastric function calls for specific local and extrinsic nervous control mechanisms However the relationship between inherent smooth muscle activity and superimposed nervous control is still not well understood.

The stomach wall contains *ganglion cells* linked by fine nerve fibres in plexiform networks. The complicated anatomical arrangement of the smooth muscle layers of the stomach wall however makes the division of intramural nervous structures into different layers less distinct in the gastric wall than in the intestine (Schofield 1968)

Most ganglion cells in the gastric wall are reported to stain intensely with dyes for acetylcholine-esterase activity (Jacobowitz 1965) indicating that they are cholinergic. The majority of these cells evidently form the postganglionic link mediating extrinsic parasympathetic influences and intrinsic peristaltic reflexes.

The specific histochemical fluorescence technique for identification of adrenergic neurons and nerve fibres developed by Hillarp and Falck (see Falck 1962) enabled a widening of knowledge of the adrenergic innervation of the gastro-intestinal tract. No ganglion cells situated in the wall were found to display the specific catecholamine fluorescence (see Schofield 1968) thus, removal of the coeliac ganglia with subsequent degeneration of the axons of the ganglionic cells results in the disappearance of all catecholamine fluorescence in the cat's stomach (Jacobowitz 1965) This suggests that the cell bodies of all the adrenergic neurons are situated in

the sympathetic ganglia outside the stomach wall proper Cell bodies containing 5-HT in the gastric wall do not seem to exist as judged from studies on mouse by Gershon and Ross (1966)

Results by Norberg (1964) Jacobowitz (1965) and Schofield (1968) have shown that most of the adrenergic ramifications of these neurons terminate in close contact with the intramural ganglion cells. This strongly indicates — but is not *per se* unequivocal evidence of — a synaptic contact with these neurons. In contrast to this dense network of adrenergic fibres around the intramural ganglion cells, relatively few adrenergic nerves seem to penetrate into the smooth muscle layers. The majority of these fibres seem to innervate intramural blood vessels rather than the gastric smooth muscles proper (Norberg 1964 Jacobowitz 1965 Schofield 1968) Nerve endings containing 5-HT observed in the gastric wall of the mouse, also seem to terminate on intramural ganglion cells rather than on the smooth muscle cells (Gershon and Ross 1966)

To summarize, there is at present no evidence of monoaminergic intramural neurons in the stomach. Most of these neurons appear to be cholinergic. However it is possible that some of them utilize still another type of a so far unknown transmitter It is, for instance, not known how the vagal "relaxatory" fibres (see below) which appear to be neither adrenergic nor cholinergic in their *final* action on the gastric smooth muscles, exert their powerful effect on the stomach.

4 Extrinsic nerve supply to the stomach

The stomach has an extrinsic supply of nerve fibres running in the vagal and splanchnic nerves. These nerves contain both *efferent* fibres, emerging from extramural ganglion cells, and different sets of *afferent* nerve fibres emerging from the gastric wall

By means of the extrinsic efferent nerves, gastric motility is adjusted either reflexly from the gastro-intestinal tract itself and other parts of the body or from central autonomic structures of the brain. Present knowledge of the functional significance of the extrinsic nervous supply to the stomach is incomplete, especially with respect to the nervous mechanisms responsible for suppressive effects on motility (for ref see Youmans 1952 Thomas and Baldwin 1968)

a. Cholinergic fibres

As early as 1858 Hartung described gastric motor fibres in the vagal nerves. This excitatory fibre type has been extensively studied. Jansson

and Martinson (1965) and Martinson (1965 c) showed that the excitatory cholinergic fibres, whose responses are selectively blocked by atropine, are relatively low threshold and can therefore be stimulated separately from the vagal relaxatory fibres described below — In addition it has been found that the splanchnic nerves contain some cholinergic excitatory fibres to the stomach but their functional significance is unknown (see Thomas and Baldwin 1968)

b. Vagal relaxatory fibres

It has been much debated in what way the vagal nerves suppress gastric motor function (for ref see McSwiney 1931 Thomas and Baldwin 1968) According to Greff *et al* (1962) and Paton and Vane (1963) the vagal supply to the stomach contains adrenergic inhibitory fibres. Martinson (1965 c) has clearly shown that the vagal nerves contain a group of high-threshold efferent fibres. These fibres can produce a manifold increase in gastric volume (Jansson and Martinson 1965) an effect which is largely confined to the corpus-fundus region and mediated by a non-adrenergic transmitter mechanism (Martinson 1965 b) This "relaxatory" vagal path way is blocked by hexamethonium but not by atropine, guanethidine (Martinson 1965 a) bretylium or by pretreatment of the animals with reserpine (Martinson unpublished observations) These findings have been largely confirmed in studies of the stomach *in vitro* by Campbell (1966) Also on the basis of *in vitro* experiments, Bülbring and Gershon (1967) suggest that acetylcholine and 5-hydroxytryptamine may be involved as transmitters for this relaxatory pathway to the gastric smooth muscle cells. However the transmission at the final neuro-effector junction for this pathway remains unknown.

It should be emphasized that there is apparently no true *antagonism* between the vagal relaxatory fibres and the cholinergic excitatory ones with respect to gastric motor control. Thus, Martinson (1965 c) considered the excitatory gastric response to be facilitated by the action of the relaxatory fibres, which widens the reservoir part of the stomach It was suggested by Jansson and Martinson (1965) that the vagal relaxatory fibres may be involved in the "receptive relaxation" of the stomach. However no definite information was then available concerning their physiological significance, though their pronounced effects on gastric volume indicated a functional involvement closely associated with food intake

c. Adrenergic fibres

The splanchnic supply to the stomach contains nerve fibres which unlike the abovementioned vagal relaxatory fibres, are blocked by guanethidine (Martinson 1965 a) and bretylium (Campbell 1966) McSwiney and Wadge (1928) observed inhibition of gastric motility upon splanchnic nerve stimulation at high frequencies (10 to 60 imp/sec) In the acutely vagotomized stomach, splanchnic nerve stimulation at physiological frequencies (below 8—10 imp/sec) produced only a weak and slowly developing inhibition of the stomach after a latency of 10—30 sec, a response which became more substantial at "supraphysiological" frequencies of stimulation (Martinson 1965 a)

In the aforementioned study by Martinson (1965 a) comparisons were also made between the responses obtained by direct stimulations of the vagal relaxatory fibres and of the adrenergic inhibitory fibres. Martinson distinguished clear differences in effects by the two types of nerve fibres. First, already low frequency stimulation of the vagal relaxatory fibres elicited pronounced gastric relaxation and, secondly this relaxation appeared after a far shorter latency than that characterizing the weak and sluggish inhibitions obtained on the acutely vagotomized stomach which was produced by splanchnic stimulation Principally similar results were obtained on the stomach *in vitro* by Campbell (1966)

The high stimulation frequencies needed and the delayed and sluggish inhibitory responses to sympathetic stimulations in acutely vagotomized preparations, suggest some type of indirect mechanism (*cf* Celander 1959 Martinson 1965 a, c) such as diffusion of transmitter to the gastric smooth muscle from nerve endings around ganglionic cells or in vascular walls By contrast, the atropinized gastric smooth muscle responds promptly to the vagal relaxatory fibres. It should be stressed that in the results described above, cholinergic nervous activity was largely abolished either by acute vagotomy or by administration of atropine.

However if the adrenergic fibres exert their action on the ganglionic relay of the cholinergic system, the effect of adrenergic activity can only be clearly demonstrated when a cholinergic nervous activity is present. It should be remembered that the histochemical studies, referred to above, suggest that the majority of the adrenergic fibres take synaptic contacts with the intramural cholinergic neurons, which form the final link in the vagal excitatory control of gastric motility (Norberg 1964 Jacobowitz 1965 Norberg 1967 personal communication, Schofield 1968) This by no means denies the fact that considerable regional as well as species

differences in the pattern of innervation may occur. In conclusion, the mode of adrenergic influence on gastric motility and the functional significance of the central and reflex patterns, which involve the adrenergic fibres is not fully understood.

d. Afferent fibres

Afferent nerve fibres from the stomach run in both the vagal and splanchnic nerves, together with afferents from other visceral organs. The dominance of afferent fibres in the vagal nerve is noteworthy. Agostoni *et al.* (1957) have shown that in the cat about 90 % of the 31,000 vagal nerve fibres at the diaphragmatic level are afferent; thus, only about 3,000 fibres are efferent at this level. Paintal (1954) and Iggo (1955, 1957—58 a, b) have shown that afferent vagal fibres from the stomach and intestine can be activated from mechano- and chemoreceptors which are sensitive to *e. g.* stretch and pH-changes. Furthermore, Andrew (1957) has described sensory endings that cause discharge in vagal afferents upon esophageal distension. It appears as if most of the vagal afferents are "specific" ones, *i. e.* non-nociceptive. On the other hand, most — but not all — of the afferent fibres in the sympathetic supply seem to be nociceptive, *i. e.* signalling pain or discomfort as a result of distorsion, ischaemia, strong distension etc. (*cf.* Balchum and Weaver 1943).

5. Reflex and central nervous influences

Extensive literature describes reflex patterns affecting gastric motor function; these patterns also include suppressing influences, in the efferent direction conveyed both *via* the vagal and splanchnic nerves (for ref. see Youmans 1952, 1968, Thomas and Baldwin 1968). Reflex suppressive effects on gastric motility by stimuli emanating from the intestine have been studied (*e. g.* Brunemeier and Carlson 1914—15, Thomas and Mogan 1931, Lalich, Meek and Herrin 1936, Daniel and Wiebe 1966). Reflex effects of stimuli induced in the esophagus (Veach 1926) or as a result of electrical stimulation of afferent vagal nerve fibres have also been investigated (Harper, Kidd and Schratcherd 1959).

It appears, however, as if no experimental analysis has been undertaken to differentiate and characterize the reflex motility responses, with particular reference to the different sets of efferent nerve fibres involved and their peripheral interactions as outlined above. Most of the conflicting interpretations of pathways involved in the earlier literature can be ascribed to the fact that it was not until recently clearly shown that there

exist *two* distinct sets of efferent fibres producing suppressive effects on gastric motility. However, they appear to do so in very different ways and, presumably, in quite different response patterns. The vagal relaxatory fibres and their distinct differences in effects in contrast to adrenergic fibres require a re-classification of the peripheral nervous mechanisms and justify a re-evaluation of reflex and central response patterns.

With respect to central nervous structures and their influence on gastric motility, both augmentative and inhibitory influences have been shown to be conveyed *via* both the vagal and the splanchnic nerves (*e. g.* Eliasson 1952). However, it is not known whether the splanchnic effects can all be ascribed to adrenergic mechanisms, and then in which way the effects are brought about. It is known that *e. g.* hypothalamic stimulation can produce both an increase and decrease in adrenergic discharge to the cardiovascular system; for example, stimulation of the "defence area" produces excitation (Eliasson *et al.* 1951, Abrahams *et al.* 1960) and the cortical and hypothalamic "sympatho-inhibitory" structures produce inhibition (Folkow *et al.* 1959, Löfving 1961). In accordance herewith it is known that also different somatic afferents produce either a reflex inhibition of sympathetic tone ("somatic depressor" fibres) while activation of afferent C-fibres increases sympathetic discharge to the cardiovascular system ("somatic pressor" fibres) (see Johansson 1962). It appears likely that these central nervous structures and somatic afferents affect gastrointestinal adrenergic control as well, but in different directions.

It should in this connection be stressed that a widening of the stomach, evoked by extrinsic efferent nerves, may be due to an activation of the vagal relaxatory fibres or the sympathetic adrenergic fibres, as well as by inhibition of prevailing activity in the vagal excitatory fibres or in the little known splanchnic cholinergic fibres. Thus, many types of responses can be distinguished that — with respect to functional importance and significance of the response patterns involved — may be widely different. Likewise, augmentation of gastric tone and motility may be due to excitation of the two aforementioned sets of cholinergic fibres or to inhibition of a prevailing activity in the vagal relaxatory fibres or the adrenergic fibres. Again, these are responses that may be of widely different physiological significance, which must be thoroughly analysed to be fully understood.

AIM OF THE PRESENT STUDIES

As mentioned, earlier studies from this department (Martinson and Muren 1963, Jansson and Martinson 1965, Martinson 1965 a, b, c) have shown two types of efferent vagal fibres to the stomach affecting its motility. The low-threshold excitatory fibre group can be separately activated because of its particular stimulation threshold. This fibre group enhances gastric motility by a cholinergic mechanism and is identical with the "conventional" vagal excitatory fibres, while the high-threshold relaxatory fibres produce marked corpus-fundus relaxation by a non-adrenergic mechanism.

In the vagally decentralized stomach, the effect of the relaxatory fibres is far more prominent and immediate in onset also at low frequency stimulation than are the inhibitory responses produced by sympathetic stimulation (Martinson 1965 a). Such quantitative differences are, however, first fully revealed when isotonic volume recordings are used at low transmural pressures, as inhibitory neurogenic responses are then more easily recorded than when intragastric pressure recordings are used, as was the case in most earlier investigations. It has for such reasons been difficult to interpret the often varying gastric responses obtained upon vagal (for ref. see Martinson 1965 c) and splanchnic stimulation (for ref. see McSwiney 1931).

The question arises as to what is the physiological significance and involvement in reflex and central nervous patterns of the high-threshold vagal relaxatory fibres to the stomach and what is their relationship to cholinergic excitatory effects on stomach motility, as compared with the inhibitory effects produced by the adrenergic fibres.

In order to study these questions a modification of the method described by Jansson and Martinson (1965) was worked out by which gastric motility could be studied without such disturbances as are produced by the "unphysiological" reflex activity in connection with abdominal surgery. *The present investigation was primarily concentrated on the reflex involvement of the vagal relaxatory fibres and their significance for gastric motility control, as contrasted to the role played by the adrenergic fibres.*

The first two studies (I, II) deal with the reflex activation of the vagal relaxatory fibres to the stomach, either produced by electrical stimulation

of vagal afferents (I) or by "adequate" receptor stimulation in the esophagus and pharynx (II).

Studies III—VI deal with the mode of local action and the reflex and central engagement of the adrenergic fibres to the stomach, as compared with the effects mediated by the vagal relaxatory fibres. As mentioned in the introduction, it appears as if the inhibitory effect produced by the adrenergic fibres calls for prevailing cholinergic activity in order to be fully displayed; if such activity is not present the adrenergic fibre effects on gastric motility seem to be feeble, considerably delayed and sometimes even absent. Attempts were therefore made to explore the influence of the adrenergic fibres, not only when directly stimulated (III, IV) but also when reflexly (III, IV, VI) and centrally engaged (V), both when vagal cholinergic activity was present and largely absent.

By such studies it proved possible to obtain some information about principal differences in the mode of local action of the aforementioned three sets of efferent fibres to the stomach and to reveal some aspects of their functional significance and involvement in central and reflex response patterns.

METHODOLOGICAL CONSIDERATIONS

Gastric motility was, as mentioned briefly above, assessed from measurements of the volume of the stomach at constant low intragastric pressure ("isotonic" recording). This recording principle was considered to mimic the physiological situation far better than the conventional recording of intragastric pressure at constant volume ("isometric" recording). The original method was described in detail by Jansson and Martinson (1965) and is outlined also in III.

The isotonic recording of volume is especially suitable for evaluation of gastric relaxatory responses. Manifold increases of gastric volume may be induced, which with "isometric" recording technique may even escape detection, as only slight pressure reductions may ensue thanks to the particular pressure-volume characteristics of the stomach.

In attempts to reflexly activate the vagal relaxatory fibres, the original "direct" method involving recording from a pyloric cannula (III) proved to be less suitable in many situations, as it requires extensive abdominal surgery and handling of the stomach. This may *per se* cause marked reflex activation of the vagal relaxatory fibres and of the adrenergic fibres as well. It may then be difficult to produce further reflex widening of the stomach due to activation of the vagal relaxatory fibres.

In an attempt to eliminate these drawbacks of abdominal surgery, a modification of the original method was devised (see I). The principle of recording gastric volume at isotonic conditions is largely the same, but is instead achieved by introducing a large thinwalled ballon *via* the esophagus, leaving the abdominal cavity intact. Recordings of gastric volume by means of this balloon technique, proved to adequately reflect gastric relaxation, at the same time as disturbances by means of reflex effects induced from the abdominal cavity were avoided. Also excitatory responses could be adequately recorded by this method (I, II, IV, VI). The presence of even a thinwalled balloon may to some extent induce reflex or local changes in gastric motility. However, when such changes are present, they may be considered to be more "physiological" in nature and in all likelihood less extensive than in recording techniques which involve a greater interference with the tissues.

It is furthermore obvious that the volumetric recordings of gastric

motility will hardly reveal *all* forms of local contractions, but only net changes in total gastric volume, where those of the essential reservoir section, the corpus-fundus regions, will dominate. No doubt some types of local contractions of great functional importance, *e. g.* in the antral-pyloric sections, may here produce regional reductions in volume which may be largely offset by a simultaneous slight relaxation in other, more voluminous, parts of the stomach so that no net change in *total* volume is obtained. The present experimental analysis therefore preferentially deals with the nervous control of the reservoir function of the stomach and hardly with such motility that is more directly involved in *e. g.* antral-pyloric control.

Excitations of the various sets of extrinsic nerves to the stomach always produced clear-cut responses in terms of net volume changes (I—VI). Therefore, the recording principle used, was considered to serve its purpose for the present investigation, *i. e.* to record both suppressive and augmentative effects on motility in the main reservoir part of the stomach, as produced by experimental activations of the various sets of extrinsic nerves.

One great advantage of the recording principle used is the possibility of assessing the level of gastric tone. Such an assessment was shown to be necessary for the correct interpretation of whether a fibre set has been reflexly involved or not. For example, an already relaxed stomach cannot be further relaxed, and an excitatory motor influence cannot be properly demonstrated if the stomach is already strongly contracted. Pressure recordings alone will hardly be able to adequately reveal the level of gastric tone. Further, the effects of the vagal relaxatory fibres are poorly reflected by a conventional pressure-recording balloon, despite the fact that excitatory effects of the vagal cholinergic fibres are reasonably well recorded (Martinson and Muren 1963, Martinson 1965 b). It can be implied that open-tip tubes or small pressure transducers must provide still more incomplete information about effects produced by *e. g.* the vagal relaxatory fibres, despite the fact that these fibres may produce manifold increases of gastric volume. These shortcomings of the conventional techniques for recording gastric motility appear, in fact, to be one of the main reasons why the vagal relaxatory fibres and their function were not clearly defined long ago.

The following drugs were used as experimental aids for pharmacological blocks of some of the efferent fibre sets to the stomach. For blocking the vagal cholinergic excitatory fibres, atropine (Atropine sulphate, Merck)

was used while adrenergic fibre effects were blocked by guanethidine (Ismelin®, CIBA). Further, the α-receptor blocker phenoxibenzamine (Dibenzyline®, Smith, Kline & French) was sometimes used to minimize the vasoconstrictor fibre effects on gastric blood supply. In addition, intra-arterial infusion of acetylcholine was used in III to initiate gastric contractions by a direct action on the gastric smooth muscles. Lastly, some animals in each series were studied after treatment with gallamine triethiodide (Flaxedil®, May & Baker) to exclude the possibility that skeletal muscle effects had interfered with the recordings of gastric motility.

RESULTS AND COMMENTS

1. Gastric motility in intact anesthetized animals (balloon technique)

In anesthetized and tracheotomized but otherwise intact animals, the balloon technique often reveals the presence of large oscillations in volume (*e. g.* Fig. 1, upper left part). When present, such waves are usually superimposed on a fairly constant level. At times, the rhythmic contractions are absent so that only the steady level, for convenience called "*gastric tone*",

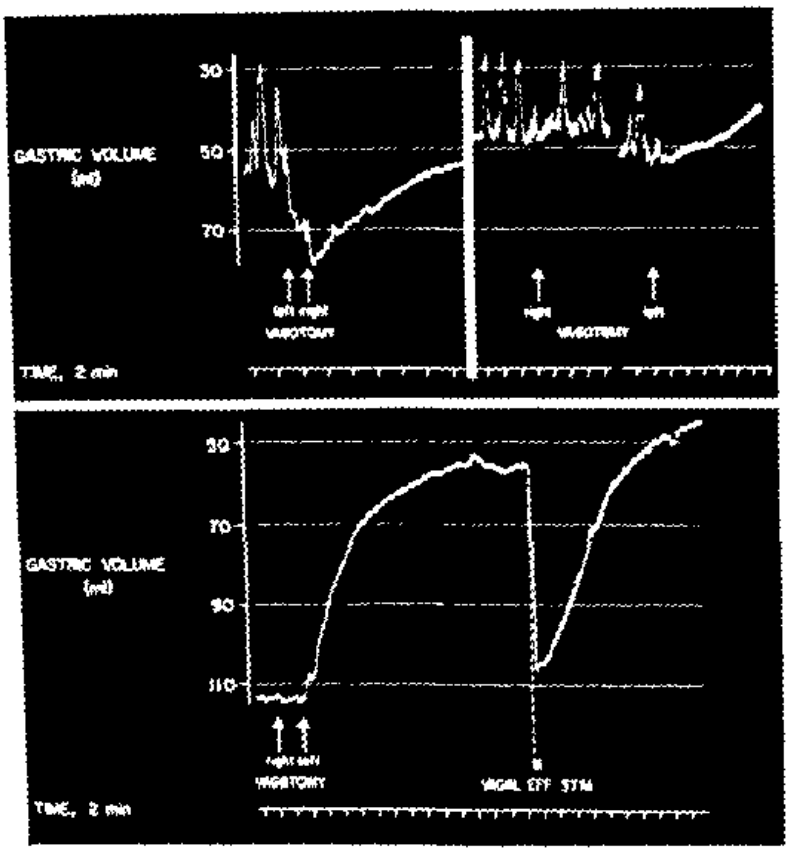


Fig 1. Effects of acute cervical vagotomy on gastric motor function. *Upper panel, left part.* Cat 3.2 kg. Recording with a large balloon introduced *via* esophagus (intragastric pressure 4.5 cm H_2O). Disappearance of gastric contractions on cervical vagal transection. *Upper panel, right part.* Cat 2.6 kg. Recording with the "direct" intragastric volume method (intragastric pressure 4.5 cm H_2O). The splanchnic nerves transected. Note the disapperance of gastric contractions on cervical vagotomy followed by a volume decrease. *Lower panel* Cat 3.0 kg. "Direct" intragastric recording (intragastric pressure 5 cm H_2O). Atropine 0.5 mg/kg i.v.; artificial respiration; Flaxedil® administered i.v. in repeated small doses. Cervical vagotomy results in a pronounced volume decrease. Electrical stimulation of the cut distal ends of the vagal trunks (8 imp/sec, 3 msec, 7 V) elicits gastric relaxation.

is reflected in the volume recording. However, direct observation of the stomach in such experiments in which the abdomen was opened usually revealed a faint rhythmic activity, which does not show up in the volume recording.

The large oscillations vanish after administration of atropine (II) or upon vagotomy (Fig. 1, upper left panel) indicating that they are dependent upon the vagal cholinergic excitatory fibres. The gastric contractions can be closely mimicked by stimulation of the cholinergic vagal excitatory fibres (I, *cf.* Jansson and Martinson 1965). Chronically vagotomized animals showed re-appearance of similar contractions, which represents synchronized movements induced and integrated by the intramural cholinergic neurons (IV). These rhythmic contractions are abolished by administration of atropine.

2. Gastric motility after abdominal surgery (direct recording technique)

The experimental approach, necessitating abdominal surgery and manipulation of the abdominal organs, was used in many experiments and some observations concerning gastric motility in this situation will be briefly outlined. Fig. 1, lower panel, shows a recording of gastric volume *via* a pyloric cannula. Atropine had been administered to block the vagal excitatory fibres. Gastric volume is here large with few signs of rhythmic activity. However, sectioning of the vagal nerves leads to a marked *decrease* of gastric volume, *i. e.* gastric tone becomes markedly increased. A brief stimulation of the peripheral ends of the vagal nerves leads to a phasic increase of volume to the level present before the vagal section. These results indicate that the vagal relaxatory fibres were tonically active in this preparation. This was often the case whenever abdominal organs had been manipulated and suggests an activation of gastro-intestinal and/or other types of abdominal receptors. Thus, the presence of "background discharge" in the vagal relaxatory fibres could be revealed in such situations by cooling or cutting the vagal nerves. However, such tonic activity could sometimes also be observed in animals where the balloon technique was used, thus without any abdominal surgery (II) though the activity was generally far less pronounced in this situation. Moreover, on plotting intragastric pressure *versus* volume of fluid introduced into the stomach in experiments similar to the one illustrated in Fig. 1 (lower panel), it was noted that the organ was "hypotonic" before vagotomy, but "hypertonic" after vagotomy (Jansson and Martinson unpublished

observations). This observation might suggest that mere distension of the stomach by its contents can produce reflex activation of the vagal relaxatory fibres, then presumably initiated by mechanoreceptors in the stomach wall. Such reflex effects seem to be greatly enhanced when the stomach is manipulated in connection with abdominal surgery.

On recording of gastric motility *via* a pyloric cannula ("direct" recording) (III) after splanchnicotomy, rhythmic contractions of the stomach were often seen (Fig. 1, upper right panel). Vagal section (Fig. 1, upper right panel), atropine, as well as low frequency splanchnic stimulation (Fig. 2) abolishes such rhythmic gastric motor activity, showing that it is dependent on the vagal cholinergic excitatory fibres and that adrenergic discharge can suppress their effect. The rhythmic waves can be re-induced by stimulation of the vagal nerves in preparations where these nerves had been cut but where atropine had not been given. After vagotomy, however, such rhythmic waves are seen only in chronically decentralized preparations where sufficient time has elapsed after vagal section. In such preparations the rhythmic activity appears to emanate from the cholinergic ganglion cells in the stomach wall, which upon chronic denervation display a more vivid activity (IV).

Comments: Cooling or section of nerve trunks usually has only negligible effects unless the nerves convey some background activity, though nerve section may itself produce a brief fibre stimulation and a transient effect. The two types of vagal efferent fibres affecting gastric motility are in many situations tonically active, producing their characteristic effects which are easily detected by the recording techniques used. In the upper left panel of Fig. 1 (balloon recording) the cholinergic excitatory fibres appear to be most active, since vagotomy eliminates the rhythmic contractions. In addition, nerve section *per se* appears to have caused a brief excitation of the relaxatory fibres, to judge by the immediate transient volume increase after nerve section, whereupon gastric tone returns to the level present before vagotomy. On the other hand, it is possible that some slight tonic discharge of the relaxatory fibres was present as well, largely balanced by that in the vagal excitatory fibres.

Such background discharge in the different fibre sets to the stomach often seems to be due to reflex activation, particularly associated with the experimental manipulations themselves, such as abdominal surgery, distension of abdominal organs etc. Such manipulations may produce considerable reflex activations of especially the vagal relaxatory fibres,

which may explain the low level of gastric tone shown in Fig. 1, lower panel.

Further, surgical trauma could cause considerable reflex activation of the adrenergic fibres as well (IV). Elimination of the adrenergic fibre influence on the stomach leads to increased gastric motility due to the withdrawal of the inhibitory influence of these fibres on the cholinergic excitatory mechanisms (Fig. 2). It is well-known that peritoneal irritation causes generalized suppression of gastro-intestinal motility (*e. g.* Cannon and Murphy 1906, Douglas and Mann 1941, Perret and Hesser 1960). Furthermore, spinal anesthesia (Markowitz and Campbell 1927) as well as splanchnicotomy or splanchnic anesthesia (Douglas and Mann 1941) usually prevent intestinal inhibitions.

If pronounced, reflex tonic discharge of the adrenergic inhibitory fibres and of the vagal relaxatory ones can make it impossible to elicit additional reflex responses in any of the efferent fibre sets supplying the stomach. The stomach may already be exposed to nearly maximal activities of the vagal relaxatory and adrenergic inhibitory fibres (I, IV).

From these considerations it seems clear that — during recordings of gastric motility in preparations with an intact nervous supply — it may be impossible to assess the "normal" level of discharge of the various efferent fibre sets to the stomach, since a series of uncontrollable factors may influence the level of this activity if great caution is not exercised.

3. Effects on gastric motility by direct stimulation of the efferent nerve supply

a. Vagal stimulation

Recording of gastric motility with the balloon modification of the isotonic volume upon electric stimulation of the cut vagal nerves (I), confirm Martinson's findings (1965 c) that the vagal supply to the stomach contains two types of efferent nerve fibres. The excitatory cholinergic fibres could be selectively activated if comparatively weak stimuli were used (I). Excitatory vagal responses were more easily elicited and stronger when gastric motility was measured with the balloon method, as this does not require abdominal surgery thus avoiding undue reflex sympathetic activation. It was observed that gastric contractions, produced by vagal stimulation, were briefly suppressed by an abdominal wall incision; handling of the intestines resulted in prolonged and intense suppression of the vagal excitatory effects. Administration of guanethidine, which

blocked the adrenergic influence, enhanced the effects of the vagal excitatory fibres (III, V).

If the strength of vagal electrical stimulation was increased, the high-threshold vagal relaxatory fibres were activated as well (I, III). The characteristic effect of this fibre type on the stomach was fully revealed after blocking the cholinergic excitatory fibres with atropine; vagal stimulation of sufficient strength then produced prompt gastric relaxation (Fig. 1, lower panel).

b. Splanchnic stimulation

The effects of direct sympathetic stimulation on gastric motility was studied particularly in respect to the effect of various frequencies of stimulation and the latency of the responses. Such stimulations were performed at different levels of activity in the intramural cholinergic neurons and when their influence was blocked.

In the acutely vagotomized and adrenalectomized cat, a mixture of excitatory and inhibitory responses was usually observed as a result of splanchnic nerve stimulation (III). The *excitatory* motor response to splanchnic stimulation, which was blocked by atropine, was not regularly obtained, but when present, it was seen at low frequency stimulation (1—4 imp/sec). After an adrenergic fibre block (guanethidine) higher stimulation frequencies also produced excitatory responses. The splanchnic nerves evidently carry some excitatory fibres to the stomach, but their site of action and functional significance is not known (*cf.* Thomas and Baldwin 1968).

The nature of the *inhibitory* effects of sympathetic stimulation varied greatly in magnitude as well as in latency with the presence or absence of prevailing activity of the intramural cholinergic neurons. Thus, prompt and usually pronounced inhibitions were even produced at low frequencies of sympathetic stimulation when the vagal excitatory fibres were active (III). This is illustrated in the left panel of Fig. 2, where in an animal with vagal nerves intact, gastric contractions were promptly inhibited by low frequency splanchnic stimulation. Gastric contractions induced by spontaneous intramural cholinergic neurons in chronically vagotomized cat were also inhibited (IV).

In sharp contrast to these prompt inhibitory responses to splanchnic stimulation, only retarded and weak inhibitory responses were obtained in acutely vagotomized or atropinized animals and mostly high frequencies (8 imp/sec or above) had to be used, in agreement with Martinson's

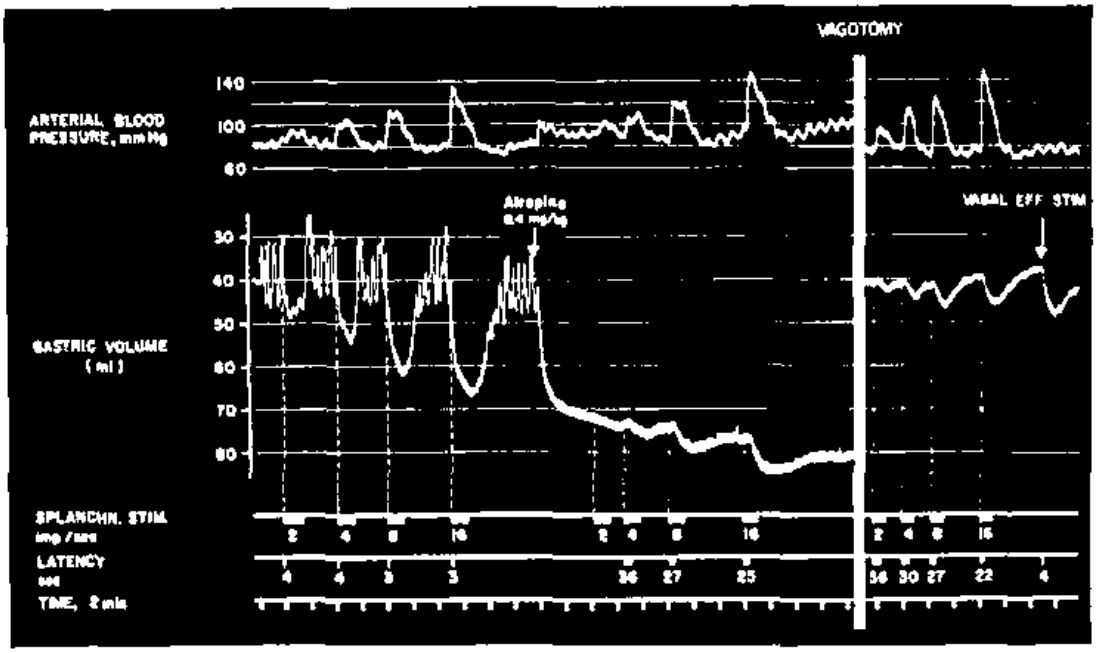


Fig. 2. Cat 2.3 kg. "Direct" recording of gastric volume by means of a pyloric cannula. Adrenalectomized. Splanchnic nerves bilaterally transected. Efferent electrical stimulation (2 msec, 6 V) of right splanchnic nerve elicits prompt inhibition of gastric contractions as long as cholinergic activity is present (left panel). Note the marked increase of latency of the response to splanchnic stimulation after administration of atropine (left panel) *cf.* the short latency of a comparable gastric relaxatory response to vagal efferent stimulation (2 imp/sec, 2 msec, 6 V) (right panel). (Intragastric pressure left panel 6 cm; right panel 4 cm)

findings (1965 a). Occasionally retarded inhibitory responses could be seen at lower frequencies as well (Fig. 2). It was characteristic, however, that the latency of these inhibitory responses could be as long as 15—40 sec but never shorter than 10 sec. This long latency should be compared with the short latency (3—4 sec) for the vagal relaxatory responses or with that obtained upon sympathetic stimulation when the vagal cholinergic excitatory fibres were active, which was also 3—4 sec (Fig. 2). Confirming Martinson's results (1965), all the sympathetic inhibitory responses mentioned above were blocked by guanethidine (IV), while the effects of the vagal relaxatory fibres remained after administration of this drug (Fig. 7, upper panel).

In summary, the prompt inhibitory responses to sympathetic stimulation, obtained when the intramural cholinergic neurons were active, were strikingly different from the considerably retarded and usually feeble inhibitions seen when the postganglionic link of the cholinergic supply to the gastric smooth muscle cells had been blocked by atropine. However, in this same situation the vagal relaxatory fibres still produced immediate

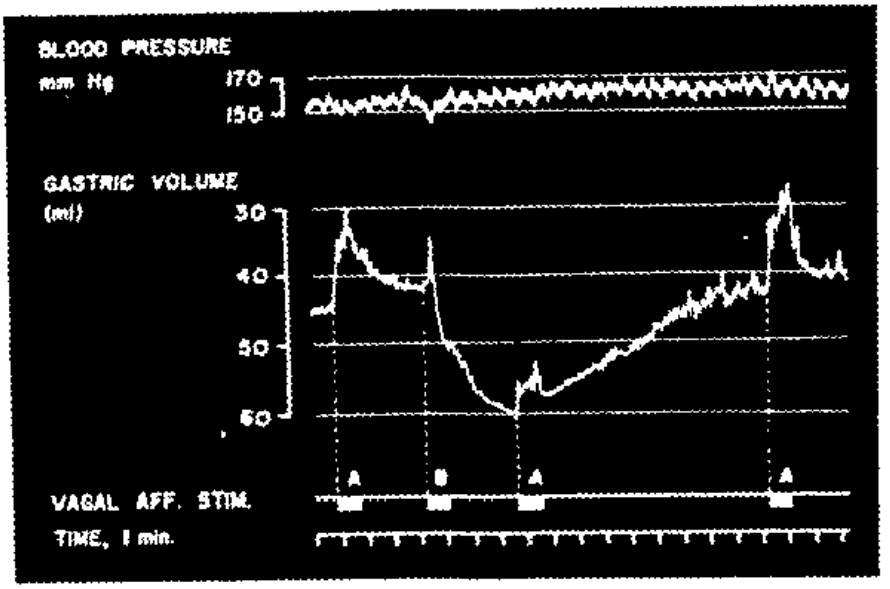


Fig. 3. Cat 3.2 kg. Balloon recording of gastric volume at an intragastric pressure of 4.5 cm H_2O. Continuous artificial respiration; repeated small doses of Flaxedil® were given intravenously. Electrical stimulation in afferent direction of a small vagal branch just cranial to the diaphragm. Stimulation A (10 imp/sec, 0.2 msec, 8 V) elicited an excitatory gastric response. Stimulation B (10 imp/sec, 1 msec, 8 V) elicited an initial excitation followed by relaxation.

and marked increase of gastric volume due to a strong suppression of gastric tone in the corpus-fundus section. These observations indicate that the sympathetic inhibitory influence is preferentially — possibly solely — exerted on the cholinergic intramural neurons, at least in the corpus-fundus sections. When these neurons are blocked, only weak and retarded sympathetic inhibitions were obtained and mainly by high frequency stimulation, suggesting an overflow of adrenergic transmitter to the gastric smooth muscles.

4. Reflex activation of the extrinsic nerve supply to the stomach

a. Reflex activation of vagal excitatory fibres

Harper, Kidd and Schratcherd (1959) reported gastric contraction on stimulation of afferent vagal fibres, effects which appeared to be conveyed also in the efferent direction by the vagal nerves. In accordance with these findings the present investigation showed that weak stimulation of vagal afferents just cranial to the diaphragm could produce weak excitatory motor responses (Fig. 3) which persisted after a sympathetic block (guanethidine 3 mg/kg), but were blocked by atropine. In the present study, such reflex excitatory effects produced by vagal afferents originating from abdominal structures have not been further analysed since the investigation

was mainly concentrated upon the reflex engagement of the relaxatory fibres and the sympathetic inhibitory fibres. The present results and those of Harper, Kidd and Scratcherd (1959) indicate, however, that the vagal excitatory fibres can also be reflexly activated by means of afferents of abdominal origin.

b. Reflex control of the vagal relaxatory fibres

The results reported in Papers I and II are mainly concerned with the reflex control of the vagal relaxatory fibres to the stomach. These nerve fibres, first clearly defined by Martinson (1965 c), have dramatic effects on corpus-fundus volume and the question arises as to what is their reflex involvement and general functional significance.

Since the existence of vagal fibres with such profound effects on gastric volume was unknown until recently, it is evident that considerable discrepancies in interpretation of recorded gastric responses exist in the earlier literature, not only with respect to the effects of efferent vagal stimulation (for ref. see Martinson 1965 c) but also concerning the nature of reflex changes of gastric motility (*e. g.* Harper, Kidd and Schratcherd 1959).

Paintal (1954), Andrew (1957) and Iggo (1955, 1957—58 a, b) have described increased discharge in vagal afferents on stimulation of mechano- and chemoreceptors in the esophagus, stomach and intestine, receptors which presumably are involved in reflex adjustments of the gastro-intestinal tract. Harper, Kidd and Scratcherd (1959) directly stimulated vagal afferents and recorded a reduced gastric tone with superimposed contractions, measured by recordings of gastric pressure; these gastric effects were abolished by vagotomy. Although their results show the existence of a vago-vagal inhibitory gastric reflex, the efferent pathways involved were not analysed. Further, considering the large number of different types of afferent fibres in the vagal nerves, the physiological significance of the described reflex was unclear. However, Andrew (1957) showed afferent vagal discharge upon esophageal distension and Veach (1926) described that such distension can produce a relaxation of the stomach, suggesting a reflex suppression of gastric tone.

As it was not clear from earlier studies exactly which efferent fibres conveyed the reflex responses studied, it was thought to be of interest to eliminate the activity of all efferent fibres to the stomach other than the vagal relaxatory ones. It would then be easier to define the circumstances in which these fibres are reflexly activated, and what their functional significance is.

Mode of reflex excitation. In I it is shown that electrical stimulation of afferent fibres at different levels along the vagal nerves produce reflex activation of the vagal relaxatory fibres to the stomach. However, the vagal nerves contain so many afferent fibres from many different organs that it is difficult to assess the physiological significance of the results of such electrical stimulations alone (*cf.* Thomas and Baldwin 1968). This type of afferent stimulation of the proximal end of a cut vagal nerve, has also the disadvantage that many of the efferent fibres normally involved in such reflexes have then been cut as well. Preparation of fine vagal filaments just above the diaphragm, calls for extensive surgery with thoracotomy, implying disturbance of respiration and circulation during prolonged experiments.

In search of a receptor mechanism that allowed reflex excitation of the vagal relaxatory fibres, it became evident that esophageal distension was suitable (II). This type of afferent fibre activation by means of the receptors is also of advantage because no surgical interference is needed and only weak distension proved sufficient to elicit pronounced and well graded reflex relaxations of the stomach. Furthermore, no nerves had to be cut and well reproducible reflex responses to graded distension could be elicited over a long time of study. The different ways in which the vagal relaxatory fibres could be reflexly activated are schematically illustrated in Fig. 4.

The esophageal receptors inducing reflex relaxation of the stomach were not only sensitive to even weak distension, but seemed to be rather slowly adapting, since prolonged steady distension caused a well sustained reflex gastric relaxation. Whether the receptors involved were sensitive only to distension or to contractions of the esophageal wall as well (Andrew 1957) was not settled.

Different types of mechano- and chemoreceptors in the gastric and intestinal wall have earlier been explored electrophysiologically (for ref. see Paintal 1963). Electrical stimulation of vagal afferents, presumably originating from such receptors, can produce reflex excitation of the relaxatory fibres (I). Further, abdominal surgery, involving handling of the stomach and intestines elicits a marked and often quite prolonged reflex discharge of the vagal relaxatory fibres (Fig. 1, lower panel), sometimes to the extent that it is difficult to produce significant additional vago-vagal reflex relaxation. However, little is known about the nature and specificity of the receptor mechanisms involved in such situations, though investigations are in progress to explore these problems. Thus, an

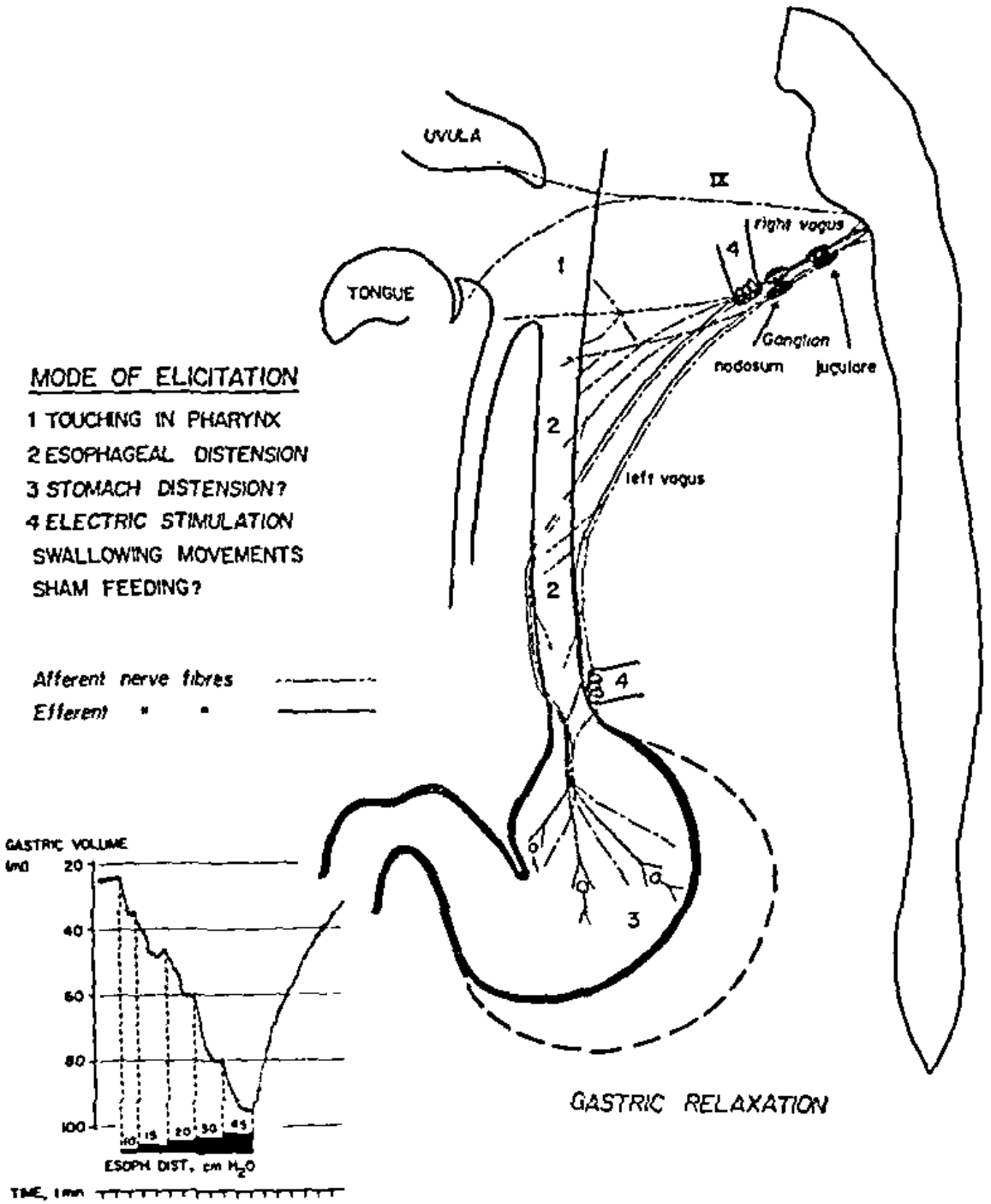


Fig. 4. Schematic drawing of different modes of eliciting gastric relaxation *via* the vagal relaxatory fibres. The lower left part of the figure illustrates a recording of effects produced by the vagal relaxatory fibres when reflexly activated by graded esophageal distension in an anesthetized atropinized cat. Complete disappearance of reflex relaxation after vagotomy.

attempt is being made to define which stimuli in the stomach and intestine that produce reflex excitation of the vagal relaxatory fibres in the form of either intestino-gastric or gastro-gastric vago-vagal relaxatory reflexes (*Abrahamsson and Jansson* to be published).

The arrangement of the reflex arcs. In most of the experiments presented in I, the proximal end of the cut cervical vagal trunk was used for afferent electrical stimulation, leaving the opposite vagal nerve intact for efferent discharge to the stomach. Furthermore, electrical stimulation of thin vagal nerve filaments, situated just cranial to the diaphragm, induced reflex excitation of the vagal relaxatory fibres; thus far, only vagal afferents have been studied.

The experiments presented in II suggest that vagal fibres constitute the major afferent pathway for eliciting reflex excitation of the vagal relaxatory fibres, at least when thoracic parts of the esophagus are distended. On the other hand, pharyngeal stimulation may also involve the glossopharyngeal and trigeminal nerves as afferent pathways (*II*; Doty 1968).

The location of the central relays involved in the vago-vagal gastric relaxatory reflex was not studied in detail in the present series of experiments. However, the reflex relaxation produced by afferent vagal stimulation persisted after intercollicular decerebration and spinal cord transection at C1—C2 (unpublished observations), but vanished after cutting the vagi cranial to the nodose ganglia; furthermore, afferent vagal stimulation on one side elicits reflex relaxation *via* the other vagal nerve. These observations indicate that the vago-vagal relaxatory reflex is conveyed *via* the vagal nuclei at the brain stem level, though it is likely that still higher autonomic centres may influence the bulbar relay station.

Definition of the vago-vagal relaxatory reflex presupposes that vagal relaxatory fibres constitute the efferent pathway; other efferent pathways had been excluded as possible routes. The described type of reflex relaxation was not due to any inhibition of a prevailing activity of the cholinergic vagal excitatory fibres, since the response remained after administration of atropine which totally blocks the cholinergic fibres. Further, an adrenergic block by guanethidine did not affect the reflex relaxation and moreover, the response persisted after cervical spinal transection. Gastric relaxation could be reproduced after vagotomy in all experiments when the peripheral ends of the vagal nerves were electrically stimulated at adequate strength (*e. g.* Fig. 3 in II).

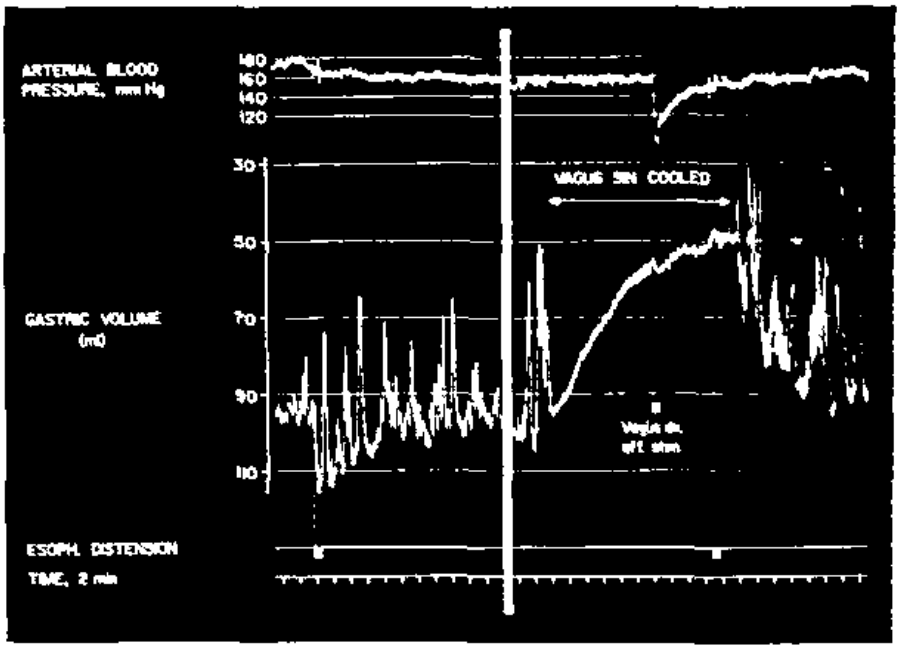


Fig. 5. Cat 3.4 kg. Balloon recording of gastric volume at an intragastric pressure of 5 *cm* H_2O. *Continuous artificial respiration; repeated small doses of Flaxedil®. Effect* of cooling the left cervical vagal nerve, the right one being cut. Note that vagal cooling leads to disappearance of pronounced gastric contractions and to a volume *decrease*. Rewarming of the nerve leads to reappearance of contractions and to gastric relaxation. Vagal afferent stimulation 8 imp/sec, 2 msec, 8 V; reflex activation of vagal fibres by esophageal distension (balloon distended 10 ml).

Extent of gastric reflex response. Reflex activation of the vagal relaxatory fibres elicited pronounced gastric relaxation in all experiments, often of the magnitude of 50—100 ml; sometimes implying a manifold increase of volume. The latency of the reflex response was about 5—7 sec; after termination of afferent stimulation, the stomach gradually regained the prestimulatory volume during a period of 3 to 20 min.

The magnitude of the reflex gastric relaxations varied considerably from one experiment to another, due mainly to varying degrees of reflex "background discharge" in the vagal relaxatory fibres. Such background discharge was caused by usually unidentified stimuli associated with the experimental conditions. Efforts were made, however, to standardize these conditions by avoidance of extensive surgery and traumatic recording techniques. Nevertheless, it appears from II that the receptor mechanisms producing reflex relaxatory fibre discharge are so sensitive that the introduction of the thinwalled recording balloon into the stomach may also produce some reflex background discharge. This was sometimes fairly pronounced despite cautious preparation of the animals; thus deliberately induced reflex relaxations of the stomach were correspondingly less pronounced. As illustrated in Fig. 5, the presence of such a background reflex

discharge was evident by the obvious but gradual volume decrease that sometimes followed vagotomy or vagal cooling (II).

During the balloon recordings of gastric volume, regular contractions of the stomach were often seen. These were evidently dependent upon activity of the vagal excitatory fibres since they disappeared after atropine and upon vagal cooling. However, their presence was by no means any prerequisite for the demonstration of pronounced reflex relaxatory responses (Fig. 1—3 in II); such responses were pronounced also after atropine.

In summary then, graded reflex excitations of the vagal relaxatory fibres to the stomach were elicited either by afferent vagal stimulation or by weak esophageal distension. The receptor mechanisms are so sensitive that even weak esophageal distension elicits well graded reflex relaxation of the stomach; this relaxation is caused by an efferent nervous discharge in the high-threshold relaxatory fibres of the vagal nerves.

Relaxation of the stomach in connection with swallowing. As early as 1911—12, Cannon and Lieb observed that gastric relaxation, dependent on the vagal nerves, was produced during sham-feeding in cats (*cf.* Lind *et al.* 1961). In a patient with esophageal atresia and gastrostomy, Carlson (1916) showed that swallowing movements with only saliva, thus not necessarily reaching the stomach, produced a suppressive effect on gastric motility. However, the nervous mechanisms involved in these earlier studied suppressive effects on gastric motility were not clearly revealed; the relaxatory fibres were not known and a reflex inhibition of the vagal excitatory fibres might have been involved.

The results presented in II indicate that reflex gastric relaxation induced *via* the vagal relaxatory fibres is regularly obtained by pharyngeally elicited swallowing, also in the anesthetized cat. Repeated swallowing in the atropinized cat produced repeated reflex relaxations of the stomach which, if occuring in a rapid sequence, were mostly superimposed on each other until a profound gastric relaxation was obtained (II). After vagal transection at low cervical levels, the swallowing movements could still be produced but no gastric relaxation followed.

It may be questioned whether the induced swallowing movements produce reflex gastric relaxation from a pharyngeal site proper, relayed *via* the bulbar structures, or if more peripherally situated receptors are activated in the esophagus. Experiments on unanesthetized cats with esophagostomy and gastrostomy (Jansson and Lisander, to be published) show that prompt and intense gastric relaxation may occur upon sham-

feeding and in such a preparation the reflex probably emanates from the pharynx. Therefore, both pharyngeal and esophageal receptors seem to produce reflex activations of the vagal relaxatory fibres.

Functional significance of the vagal relaxatory fibres. It seems clear that even slight physiological stimuli in pharyngeal and esophageal sections of the gastro-intestinal tract elicit reflex relaxation of the stomach. As shown in II, repeated swallowing and esophageal distension produce a relaxation of the stomach in a manner that implies a graded increase of the gastric lumen for the accomodation of increasing amounts of swallowed food. Such a widening of the reservoir conveyed *via* the vagal relaxatory fibres thus occurs without any increase in intragastric pressure, producing true "receptive relaxation". Furthermore, pronounced rhythmic contractions of the stomach dependent upon cholinergic neurons are often present and obviously involved in the mixing of food with gastric secretion and subsequent transport of the gastric contents from the stomach. These contractions may occur simultaneously with the pronounced reflex vagal relaxation of the corpus-fundus section of the stomach (Fig. 6).

Such a response pattern appears to be highly useful for the execution of the main motor functions of the stomach, *i. e.* that of "receptive relaxation" with graded accomodation of the corpus-fundus part for storing of the food, at the same time as enhanced rhythmic activity of the gastric wall produces a mixing of the contents with the gastric secretion and ultimately an emptying of the reservoir.

Furthermore, it is of interest to note the generally long-lasting relaxation followed by a slow progressive decrease in volume, often superimposed by rhythmic contractions as shown in Fig. 6. Such a slow and gradual regain of gastric smooth muscle tone superimposed by contractions, might also be physiogically meaningful in the process of *expelling* gastric contents into the intestine. If it can be shown that these mechanisms can be modified from duodenal-jejunal receptors, an important link in the understanding of gastric emptying would have been found. It is known that gastric emptying is modified by *e. g.* osmolarity, pH-changes and fat in the intestine (for ref. see Hunt and Knox 1968). The existence of duodenal chemo- and mechanoreceptors eliciting a spike discharge in afferent vagal nerve fibres has been shown (for ref. see Paintal 1963); further, electrical stimulation of afferent vagal nerve fibres from the abdomen elicits a vago-vagal relaxation (I). It is therefore suggested that the gastric response pattern shown in *e. g.* Fig. 6 (see also II) with the long-lasting relaxation and progressive

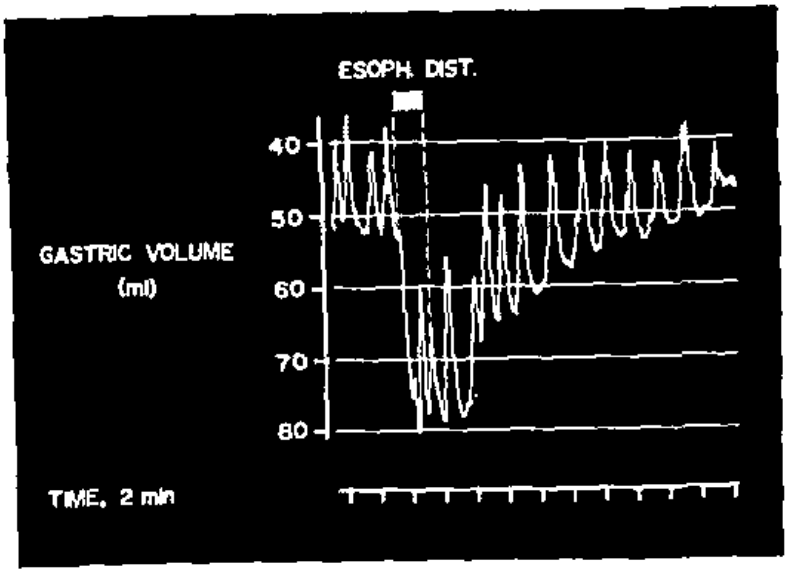


Fig. 6. Cat 2.9 kg. Balloon recording of gastric volume at an intragastric pressure of 6 cm H_2O. Vagal nerves intact. Reflex gastric relaxation produced by esophageal distension (40 cm H_2O). Note that the rhythmic contractions not only persisted but became more pronounced during the reflex relaxation of the stomach.

volume decrease together with simultaneous contractions may form an integral part of normal gastric emptying.

c. Extrinsic gastric control mediated by the adrenergic supply

Studies III—VI are mainly concerned with the extrinsic influence of the adrenergic outflow to the stomach.

Intestino-gastric inhibitory reflex. A reflex adrenergic discharge was not only present in the form of a "background discharge" as a result of abdominal surgery *per se* (IV), but could also be deliberately elicited from the intestine. In the present study, the "intestino-gastric inhibitory reflex", termed by Morin and Vial (1934), is used to designate a reflex elicited from different parts of the intestine which by means of splanchnic afferents and efferent adrenergic fibres affect the stomach (compare the intestino-intestinal inhibitory reflex; Youmans 1944, Johansson and Langston 1964). In III and IV it is shown that prompt reflex inhibitions of gastric motility (latency 3—4 sec) could be induced *via* the adrenergic fibres by graded distensions of either duodenal, jejunal or ileal loops, *provided that a cholinergic influence on gastric smooth muscles was present.* Similar reflexes could be induced by graded electrical stimulations of thin afferent nerve filaments around the superior or inferior mesenteric artery (III, IV).

However, when the stomach was not under cholinergic influence, intes-

part in the adjustment of the rate and extent of gastric emptying to the capacity of the intestine to receive food. Further studies are needed to elucidate these problems.

Influences on adrenergic fibre activity by hypothalamic and somatic afferent stimulation. The demonstration in III and IV of prompt reflex and pronounced adrenergic inhibitions of gastric motility — provided that the cholinergic fibres to the stomach were active — raises the question of how suprabulbar nervous structures and afferent fibres of somatic origin may influence such adrenergic mechanisms. For this purpose separate studies of the hypothalamic control of the adrenergic outflow to the stomach (V) and of the reflex effects of "somatic pressor" and "depressor" afferents (VI) were performed.

From the hypothalamus some well defined response patterns of known localization can be induced by topic stimulation *e. g.* the defence reaction (see Abrahams, Hilton and Zbrozyna 1960) and the sympatho-inhibitory response (Folkow, Johansson and Öberg 1959). In studying the adrenergic effects on gastric motility, blood pressure and skeletal muscle blood flow were also recorded for a functional identification of the responses.

Stimulation of the "defence area", or surrounding "pressor areas", produced — besides the characteristic circulatory effects — a prompt inhibition of gastric motility mediated *via* the adrenergic fibres to the stomach, which suppressed vagally elicited gastric contractions. Similar effects were obtained on stimulation of somatic "pressor" afferents (VI). On the other hand, topic stimulation of the hypothalamic sympatho-inhibitory area elicited an *increased* gastric motility; similar responses were obtained by stimulation of somatic "depressor" afferents. Evidence was presented to show that these augmentations were due to an inhibition of a tonic adrenergic discharge to the stomach (V, VI), thus releasing the excitatory influence of prevailing vagal cholinergic activity from suppression. The tonic adrenergic discharge was in this situation mainly the result of a spinal reflex induced by abdominal surgery (IV), a reflex discharge that was suppressed by the hypothalamic sympatho-inhibitory area (V) or by the somatic "depressor" afferents (VI). Thus hypothalamic structures, as well as the somatic afferents mainly mediating pain (see Johansson 1962), can affect adrenergic activity to the stomach in both directions; the adrenergic discharge acting in turn mainly — possibly only — by counteracting cholinergic effects on the gastric smooth muscles.

The hypothalamic defence area has, however, at least one other type of action. It appears also to inhibit the vagal preganglionic neurons in the

bulb, to judge from experiments on animals with a transected spinal cord at the cervical level (*Jansson and Lisander, unpublished observations*).

An enhancement of gastric motility mediated *via* the spinal cord was seen as a result of stimulation of the sympatho-inhibitory area in the vagotomized cat when electrical vagal stimulation produced a tonic cholinergic discharge, which in turn was counteracted by an adrenergic influence. Thus, guanethidine administration and interruption of the vagal stimulation or atropinization abolished the enhanced gastric motility produced by this type of hypothalamic stimulation. Had only the effects of splanchnic nerve section and atropinization been tested, the effect might easily have been mistaken for a hypothalamic activation of splanchnic cholinergic fibres.

Mode of adrenergic fibre influence on gastric motility. As outlined in Introduction, neuro-histochemical methods suggest that there are no adrenergic neurons in the ganglionic plexa of the gastro-intestinal wall; most of these intramural neurons appear to be cholinergic. The abundance of adrenergic fibres in the gastric wall, which predominantly seem to make contact with the intramural neurons, have their cell bodies in the abdominal sympathetic ganglia outside the stomach. Very few of these adrenergic ramifications penetrate into the smooth muscle layers and most, possibly all, that do not terminate on intramural ganglionic cells, innervate blood vessels rather than the gastric smooth muscles proper (Norberg 1964, Norberg, personal communication 1967, Jacobowitz 1965, Schofield 1968).

The close approximation of the adrenergic ramifications and the intramural neurons, with few — if any — such ramifications in contact with the gastric smooth muscles, suggest that the adrenergic fibres may affect gastric motor function by modulating cholinergic ganglionic activity rather than affecting the smooth muscles directly. From the results described in III and IV it is clear that the adrenergic fibres to the stomach act *antagonistically* to cholinergic effects with respect to gastric motility. It further appears from the motility studies in III and IV that the adrenergic fibres may affect gastric motility by primarily inhibiting the intramural cholinergic ganglion cells, rather than directly affecting the gastric smooth muscles; thus, the functional studies are in good agreement with morphological findings. Adrenergic fibre stimulation had comparatively weak inhibitory effects on gastric tone in the corpus-fundus in the acutely vagotomized and atropinized animal. Such inhibitory effects were, moreover, considerably delayed (latency 10—40 sec), suggesting an indirect mode of action, in sharp contrast to the prompt (latency 3—4 sec) adrenergic inhibition of

cholinergic neuronal activity even at low frequency stimulation and the rapid (latency 3—4 sec) pronounced suppression of gastric tone produced by the vagal relaxatory fibres.

Such a long latency of nerve action is hardly compatible with the presence of a close junction between adrenergic fibres and those gastric smooth muscles which are responsible for the pronounced gastric tone described in III. Only when comparatively high frequencies of sympathetic stimulation were used, did the suppression of gastric tone become significant in size although the latency was still remarkably long. In this situation it is known that overflow of the adrenergic transmitter takes place and that considerable portions also reach the blood stream.

It can be argued that the weak, or absent, adrenergic inhibitory responses after blocking of the cholinergic intramural neurons might still be compatible with a direct adrenergic fibre influence on gastric smooth muscles. Such directly innervated muscle cells, however, may display any activity that can be inhibited adrenergically *only* if they are excited by the cholinergic intramural neurons. If so, they are certainly not identical with the smooth muscles in the stomach that are responsible for the pronounced gastric tone seen in acutely vagotomized and atropinized animals. This possibility, however, is also unlikely since gastric contractions, induced by the muscarinic effect of an intra-arterial infusion of acetylcholine, were unaffected by such adrenergic nerve activation that promptly inhibited *vagally* induced contractions of largely similar magnitude and appearance (III).

It appears difficult to harmonize these observations with any other view than the suggestion that the *major*, possibly only, site of inhibitory action of the adrenergic fibres of the stomach is localized to the intramural cholinergic neurons, at least with respect to the nervous control of the reservoir function of the stomach. It is, of course, possible that the arrangement of the nervous control might be different in other, specialized parts of the stomach, such as the sphincteric regions, which largely escape exploration in this type of study.

5. Vagally mediated gastric relaxation versus inhibition produced by adrenergic fibres

Martinson (1965 c) considered the excitatory gastric response induced by the low-threshold vagal fibres to be facilitated by the action of the vagal relaxatory fibres, a view strongly supported by the present findings. On efferent vagal stimulation with increasing intensities, pronounced, or

even enhanced, rhythmic contractions of the stomach were also recorded when the vagal relaxatory fibres were simultaneously activated. Furthermore, in animals with an intact vagal nerve supply, pronounced gastric contractions were often seen simultaneously with a considerable background discharge in the vagal relaxatory fibres (Fig. 5); as mentioned earlier, the presence of such a background discharge could easily be revealed by vagal cooling.

Lastly, when esophageo-gastric vago-vagal relaxatory reflexes were elicited, gastric contractions occurred simultaneously with the pronounced relaxatory responses. In the experiment illustrated in Fig. 6, gastric contractions not only persisted during an activation of the vago-vagal relaxatory reflex, but they even became more pronounced. There is thus no evidence of any suppressing influence from the relaxatory fibres on such gastric contractions that are dependent on the vagal cholinergic influence. The two vagal fibre sets thus appear to act jointly in promoting the changes in gastric motility associated with food intake; the reservoir becomes widened and exhibits at the same time increased rhythmic activity. This apparent lack of any direct competition between vagal excitatory and relaxatory influence in the effector organ might be explained by an action of the two sets of vagal fibres on different smooth muscle layers in the stomach wall; one with high myogenic activity (responsible for gastric tone) that is suppressed by the relaxatory fibres, and one that is excited by the cholinergic excitatory fibres.

In contrast to the facilitating effects between the two vagal fibre sets, the adrenergic fibres, exerting their main inhibitory action by suppressing the intramural cholinergic neurons, thus act *antagonistically* to the vagal excitatory influence on gastric motility.

The mode of action of the relaxatory and the adrenergic fibres and the situations when they are activated are thus completely different. The relaxatory fibres appear to form part of the feeding response and prepare the stomach for the reception and digestion of food; the adrenergic fibres appear to suppress gastro-intestinal function in general.

Summary

Extrinsic nervous activity controlling gastric motility was studied in anesthetized cats with special reference to the influence exerted by the vagal relaxatory fibres and the splanchnic adrenergic fibres.

On the basis of the observations made, the following conclusions were drawn:

1. The existence of a vago-vagal relaxatory reflex to the stomach is shown by electrical stimulation of vagal afferents at different levels. The vagal high-threshold relaxatory fibres form the efferent pathway of this reflex. These efferent vagal fibres operate by a non-adrenergic neuro--effector transmission and seem to facilitate the cholinergic influence of the vagal excitatory fibres on gastric motility. The gastric response to activation of the relaxatory fibres consists of a marked and prolonged relaxation, primarily affecting the corpus-fundus sections which can increase manifold in volume.

2. Receptor mechanisms sensitive to even slight esophageal distension are shown to reflexly activate the vagal relaxatory fibres, forming an "esophageo-gastric vago-vagal relaxatory reflex". Also in connection with pharyngeally elicited swallowing, reflex gastric relaxation is induced *via* the vagal relaxatory fibres.

These results suggest that the gastric relaxation patterns described, play a dominant role in "receptive relaxation" of the stomach during deglutition.

3. Activation of the adrenergic supply to the stomach elicits prompt gastric inhibition by counteracting the vagal excitatory influence on gastric smooth muscles. The results suggest that this type of neurogenic inhibition is due mainly to an adrenergic suppression of the cholinergic intramural neurons.

4. It is shown that even slight distension of different parts of the small intestine causes reflex activation of the adrenergic supply to the stomach. Hypothalamic sympatho-excitatory structures (the defence area) produce gastric inhibitory responses by means of an activation of the adrenergic supply to the stomach. Activation of the hypothalamic sympatho-inhibitory area suppresses prevailing adrenergic discharge to the stomach. Similar modulations of a prevailing adrenergic discharge to the stomach can be reflexly elicited *via* somatic pressor and depressor afferents, respectively.

These results suggest that the adrenergic fibres to the stomach are involved in such central and reflex patterns of sympathetic discharge that involve a generalized suppression of gastro-intestinal activity. However, it is not excluded that the adrenergic fibres might also be more directly involved *e. g.* in normal gastric emptying.

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SUPRASPINAL AND SEGMENTAL CONTROL OF STATIC AND DYNAMIC γ-MOTONEURONES IN THE CAT

BY

STEN GRILLNER

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to Lena

CONTENTS

GENERAL INTRODUCTION 5

METHODOLOGICAL CONSIDERATIONS 7

REFLEX EFFECTS FROM PRIMARY AFFERENTS 8

A. Group I afferents 8

B. Group II Afferents (Muscle) 9

C. Cutaneous afferents and group III muscular and joint afferents 10

D. Recurrent inhibition 12

E. α—γ linkage? 12

SUPRASPINAL CONTROL VIA TWO FAST DESCENDING SYSTEMS 14

THE "NORADRENERGIC RETICULOSPINAL SYSTEM" 17

A. The resting discharge and the identification of static and dynamic γ-motoneurones 17

B. The effect of DOPA on the reflex discharge evoked through short latency reflex paths in static and dynamic γ-motoneurones 19

C. The late reflex discharge 20

D. The stretch reflex 20

GENERAL DISCUSSION OF THE PROPERTIES OF THE γ-MOTOR SYSTEM WITH PARTICULAR REFERENCE TO THE SYSTEMS INVESTIGATED IN PAPERS I—V 22

A. Properties of the stretch reflex loop 22

B. The functional role of the "γ-loop" 25

1. During posture 26

2. During movement 27

3. What is the functional role of the resting discharge? 27

ACKNOWLEDGEMENTS 29

REFERENCES 30

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II. Bergmans, J. and S. Grillner, Changes in dynamic sensitivity of muscle spindle primary endings induced by DOPA. Acta physiol. scand. 1968. 74. 629-636.

III. Bergmans, J. and S. Grillner, Reciprocal control of spontaneous activity and reflex effects in static and dynamic γ-motoneurones revealed by an injection of DOPA. Acta physiol. scand. 1969. In press.

IV. Bergmans, J. and S. Grillner, Monosynaptic control of static γ-motoneurones from the lower brain stem. Experientia 1968. 24. 146-147.

V. Grillner, S., The influence of DOPA on the static and dynamic fusimotor activity to the triceps surae of the spinal cat. Acta physiol. scand. 1969. In press.

GENERAL INTRODUCTION

The "final common path" for motor regulation, i. e. the α-motoneurone (Sherrington 1904) is strongly influenced from segmental reflex paths originating from various kinds of receptors (cf. Creed, Denny-Brown, Eccles, Liddell and Sherrington 1932). Of particular importance is the muscle spindle which has large afferent fibres with monosynaptic excitatory effects to motoneurones supplying its own muscle (cf. Lloyd 1943, Granit 1950). These afferents provide the afferent path for the well-known stretch reflex (Liddell and Sherrington 1924).

Onanoff's finding (1890) of an efferent nerve supply to the muscle spindle gave this receptor a unique position among muscle receptors. The subsequent analysis by Leksell (1945) and Kuffler, Hunt and Quilliam (1951) showed that the muscle spindle of the cat indeed has a motor supply of its own via nerve fibres of small calibre, the γ-efferents. The central control of the γ-motoneurones is exerted via different segmental reflex paths (Hunt 1951, Kobayashi, Oshima and Tasaki 1952) and from various supraspinal structures (Granit and Kaada 1952, Eldred, Granit and Merton 1953, Granit and Holmgren 1955); parallel effects could often be evoked in α- and γ-motoneurones but often with lower threshold in the latter. The γ-control determines the discharge rate in the muscle spindle afferents during dynamic and static conditions in the muscle and is thereby one important factor determining the excitability level of the α-motoneurones (cf. Granit 1955, 1968, Matthews 1964).

In 1962 it was shown that the γ-efferents are of two kinds (Jansen and Matthews 1962a, Matthews 1962), one type controlling mainly the dynamic sensitivity of the primary endings (Ia afferents) and the other the discharge rate, and presumably also the static sensitivity of the primary endings (Jansen and Matthews 1962a, Brown, Lawrence and Matthews 1969) and in addition the activity in secondary endings (group II afferents) (Appelberg, Bessou and Laporte 1966).

Rather selective effects on static and dynamic γ-motoneurones can be produced from different central nervous structures. Thus, stimulation of the anterior lobe of the cerebellum gives a depression of the activity mainly in dynamic γ-motoneurones (Jansen and Matthews 1962 a). A selective activation of dynamic γ-motoneurones to certain muscles can

be evoked from the mesencephalic tegmentum (Appelberg 1963, Appelberg and Emonet-Denand 1965), and from the pyramidal tract (Vedel 1966). Effects on static γ-motoneurones are obtained from the caudate nucleus (Vedel and Paillard 1965) and from certain regions of the cerebral cortex outside the motor area (Baudevin and Vedel 1967).

Certain differences between the two kinds of γ-motoneurones appear to exist also at the segmental level (Jansen 1966). Thus in the spinal cat, dynamic but not static γ-motoneurones are spontaneously active and reflexly excitable (Alnaes, Jansen and Rudjord 1965). On the other hand, after an injection of DOPA, which is assumed to liberate noradrenaline from descending noradrenergic fibres in the spinal cat, static γ-motoneurones to flexors acquire a resting discharge and can be reflexly activated (Grillner, Hongo and Lundberg 1967). The cited studies on the selective control of dynamic and static γ-motoneurones have all been obtained by indirect methods (recording of muscle spindle afferents) and it is difficult to draw conclusions regarding the synaptic linkage of various systems influencing these neurones.

The detailed investigations of the control of γ-motoneurones have been greatly handicapped by the difficulty in obtaining intracellular records or applying a monosynaptic test technique. In the present studies γ-motoneurones have been investigated by intra- or extracellular recordings in the spinal cord or by the activity in single efferents in peripheral nerve filaments and also indirectly through spindle afferents. The aim has been to elucidate some aspects of

I. The segmental control of static and dynamic γ-motoneurones via different reflex paths.

II. The descending control of static and dynamic γ-motoneurones via

a) two fast conducting descending systems from the brain stem. i. e. the vestibulospinal tract and fibres coursing in the medial longitudinal fascicle (MLF) presumably of reticular origin and

b) the "noradrenergic reticulospinal system"

METHODOLOGICAL CONSIDERATIONS

Papers I—V have been concerned with a study of γ-motoneurones supplying the left hindlimb of the cat. The detailed methodological descriptions are given in the different papers.

In papers I, III—V the control of single γ-motoneurones has been investigated and only one point of general interest will be considered here, the identification of γ-motoneurones.

In paper I the γ-motoneurones intracellularly recorded could be identified by the following criteria

1) antidromic invasion from one muscle nerve should occur without graded prepotentials,

2) the conduction velocity of the efferent responsible, should be within the range 20—45 m/sec (cf. Eccles, Eccles, Iggo and Lundberg 1960),

3) the location of the cell should be within the appropriate motor nucleus (Eccles et al. 1960, Nyberg-Hansen 1965),

4) no monosynaptic excitation should be evoked from group Ia afferents (Hunt and Paintal 1958, Eccles et al. 1960),

5) the threshold for the efferent fibre, should be several times above that for group Ia afferents or α-efferents (Leksell 1945).

A few cells were recorded when the ventral roots were cut and then the identification rested on criteria 1, 3—5. Particular attention was then paid to criterion 4, and the lack of Ia EPSP was checked by stimulation of the dorsal roots. In the extracellular material the activation of a cell with a fixed latency was taken to indicate that it was antidromically activated; criteria 2—5 could then be applied. If such a cell was later impaled it was always found to be a γ-motoneurone by the above criteria.

In papers III—V the γ-motoneurones had to be investigated for a long time and therefore the γ-efferents were recorded in peripheral nerve filaments. By stimulation of the intact ventral root(s) the number of γ-efferents in a filament could be determined; the isolation of γ-efferents did not depend on the presence of a resting discharge. The identification of an efferent as being of the "γ-type" rested on criteria 2, 4, 5. The isolation of only one, or sometimes two easily distinguished γ-efferents, was performed by making successive lesions in the ventral root until only one (two) γ-efferent was in connexion with the spinal cord.

be evoked from the mesencephalic tegmentum (Appelberg 1963, Appelberg and Emonet-Denand 1965), and from the pyramidal tract (Vedel 1966). Effects on static γ-motoneurones are obtained from the caudate nucleus (Vedel and Paillard 1965) and from certain regions of the cerebral cortex outside the motor area (Baudevin and Vedel 1967).

Certain differences between the two kinds of γ-motoneurones appear to exist also at the segmental level (Jansen 1966). Thus in the spinal cat, dynamic but *not static γ-motoneurones are spontaneously active* and reflexly excitable (Alnaes, Jansen and Rudjord 1965). On the other hand, after an injection of DOPA, which is assumed to liberate noradrenaline from descending noradrenergic fibres in the spinal cat, static γ-motoneurones to flexors acquire a resting discharge and can be reflexly activated (Grillner, Hongo and Lundberg 1967). The cited studies on the selective control of dynamic and static γ-motoneurones have all been obtained by indirect methods (recording of muscle spindle afferents) and it is difficult to draw conclusions regarding the synaptic linkage of various systems influencing these neurones.

The detailed investigations of the control of γ-motoneurones have been *greatly handicapped by the difficulty in obtaining intracellular records* or applying a monosynaptic test technique. In the present studies γ-motoneurones have been investigated by intra- or extracellular recordings in the spinal cord or by the activity in single efferents in peripheral nerve filaments and also indirectly through spindle afferents. The aim has been to elucidate some aspects of

I. The segmental control of static and dynamic γ-motoneurones via different reflex paths.

II. The descending control of static and dynamic γ-motoneurones via

a) *two fast conducting descending systems from the brain stem. i. e. the* vestibulospinal tract and fibres coursing in the medial longitudinal fascicle (MLF) presumably of reticular origin and

b) the "noradrenergic reticulospinal system"

the decerebrate cat. In a subsequent study, Hunt and Paintal (1958) could not confirm these results in the spinal cat. However, these two preparations differ in two respects: 1) static γ-motoneurones are spontaneously active in the decerebrate (Jansen and Matthews 1962 a, b) but not in the spinal preparation, in which only the dynamic have a resting discharge (Alnaes, Jansen and Rudjord 1965, paper II); 2) a tonic stretch reflex occurs in the decerebrate but not in the spinal preparation (Liddell and Sherrington 1924). The different findings on decerebrate and spinal cats would be explicable if the autogenetic inhibitory effects were exerted only on static γ-motoneurones. If the group I inhibition (paper I) is responsible, it is relevant that of four γ-motoneurones tested for descending effects, all received monosynaptic EPSPs, which suggests that they are of the static type (paper I, IV, V). It is also relevant that the group Ib afferents have a high sensitivity for active, but not for passive tension (Jansen and Rudjord 1964, Houk and Henneman 1967). The absence of autogenetic inhibition in the spinal, but its presence in the decerebrate preparation may be correlated with the fact that a stretch reflex occurs in the latter but not in the former state (Liddell and Sherrington 1924). Accordingly it may be possible to explain the findings by Hunt (1951) and Hunt and Paintal (1958) without any postulate regarding a selection in destination to static and dynamic γ-motoneurones.

B. Group II afferents

Stimulation of muscle nerves at strength of 2.5 to 8 times threshold of the most excitable fibres activates the fibres conducting in the group II range (Eccles and Lundberg 1959). At this stimulation strength excitatory effects could be evoked in flexor γ-motoneurones classified as static and dynamic (paper III) and also in gastrocnemius γ-motoneurones excitatory or inhibitory reflex effects could be revealed (paper V). These effects could be evoked from nerves to flexors and extensors working at different joints. In this unanaesthetized spinal preparation corresponding effects can occasionally be evoked in α-motoneurones (cf. paper V). Although the majority of group II fibres probably have secondary endings from the muscle spindles (cf. Hunt 1954) the presence of other fibres of this diameter has been shown by Paintal (1960) and Barker (1967). Thus the presence of "group II effects" does not necessarily imply that they are caused by secondary endings from the spindle. However, Laporte and Bessou (1959) have shown that in a spinal preparation, in which conduction in group I afferents had been blocked, inhibitory effects are evoked on muscle stretch in extensor and excitatory effects in flexor α-motoneu-

rones. Although reflex effects from smaller diameter fibres cannot be entirely excluded the most likely explanation is that these reflex effects are due to activation of secondary endings, which hence are assumed to give effects conforming to the flexor reflex pattern (Sherrington 1910). These results are in accordance with the results obtained with electrical stimulation of peripheral nerves (Laporte and Lloyd 1952, Eccles and Lundberg 1959). However, excitatory effects to extensors can also sometimes be encountered (Holmqvist and Lundberg 1961, Wilson and Kato 1965). Since the secondary endings are under efferent control and have a signal lineraly related to muscle length, these seemingly nonspecific reflex connexions (cf. Matthews 1964) to α- and presumably also γ-motoneurones are rather puzzling (cf. also page 24). However, it might be pertinent to note that the frog, which has no specific motor supply to the spindles (Katz 1949), only has one type of afferent corresponding presumably to the primary ending. The significance of the secondary endings in the cat might not be related to a delicate spinal reflex pattern but in mediating information related to muscle length and static γ-bias to supraspinal structures.

C. Cutaneous afferents and group III muscular and joint afferents

In Hunt's (1951, cf. also Kobayashi, Oshima and Tasaki 1952) original description of the reflex activation of γ-motoneurones he reported that these efferents as a rule followed the pattern of flexor-crossed extensor reflexes of α-motoneurones (Sherrington 1910). Numerous exceptions to this pattern were, however, found in a subsequent study by Hunt and Paintal (1958). Their conclusion that the reflexes to α- and γ-motoneurones differ in several respects deserves some further discussion.

In confirmation of several previous studies, γ-motoneurones to flexors (paper III) were found to receive excitation from ipsilateral nerves. All γ-efferents studied could be reflexly activated by single volleys, dynamic γ-motoneurones more effectively than static (for identification see page 17). The analysis of their reflexes was aided by the comparison of effects before and after DOPA. An injection of DOPA decreased the reflex activation of dynamic and increased the reflex activation of static γ-motoneurones (paper III). These opposite effects may be a consequence of the corresponding changes in the resting activity; an alternative possibility is that the effects were mediated by two different short latency reflex pathways (cf. Fig. 10 paper III). Reflex effects have previously only been demonstrated in a part of the extensor γ-motoneurone population (Hunt and Paintal 1958), but with the aid of DOPA it was [illegible] to demonstrate

short-latency reflex effects in all gastrocnemius γ-motoneurones investigated. Hence it is postulated that both static and dynamic γ-motoneurones can be influenced. The detailed analysis of these reflex effects is difficult since both excitatory, inhibitory and mixed effects could be evoked from ipsilateral nerves. There were some discrepancies between intra- and extracellular findings, which rather would be expected (paper I). Since ipsilateral excitatory effects were common in extensor γ-motoneurones (I, V), it can be concluded that the reflex pattern is more complex than the classical flexor-crossed extensor reflex pattern. This confirms the previous results by Eldred and Hagbarth (1954), Hunt and Paintal (1958), Alnaes, Jansen and Rudjord (1965) and Jansen and Rudjord (1965).

However, the reflex effects to α-motoneurones from these afferents also diverge from this reflex pattern (Creed, Denny-Brown, Eccles, Liddell and Sherrington 1932, Hagbarth 1952); after Hunt and Paintal's (1958) report detailed information concerning these effects have been obtained. The crossed reflex actions to α-motoneurones in the spinal unanaesthetized cat are excitatory to extensors in about 50 % of the preparations, in the remainder they are inhibitory or mixed (Holmqvist 1961). From ipsilateral high threshold muscle afferents excitatory effects can often be encountered in extensor α-motoneurones (Lundberg, personal communication, Holmqvist and Lundberg 1961, Wilson and Kato 1965). From cutaneous afferents excitatory or mixed effects are generally evoked in α-motoneurones (triceps surae) supplying fast motor units, while inhibitory effects dominate in slow motor units (Bruggencate, Burke and Jankowska, unpublished). The complex organization and the fact that there are two types of α-motoneurones with different reflex effects within the same muscle renders a conclusion regarding an α-γ-linkage in reflex effects to the gastrocnemii very difficult. Reflexes to foot muscles from the pad can also diverge from the flexor reflex pattern (Engberg 1964) in a similar way to fusimotor reflexes to these muscles (Hunt and Paintal 1958).

The analysis of reflex effects evoked from repetitive nerve stimulation at different frequencies were of particular interest because of the previous findings (with indirect methods) by Alnaes, Jansen and Rudjord (1965) that reflex activation of dynamic but not static γ-motoneurones could be obtained in the spinal cat by repetitive nerve stimulation (40 pps). γ-motoneurones with a resting discharge were further accelerated with increasing rate of stimulation. On the other hand, units with no spontaneous activity could often be discharged at low stimulation frequencies (paper

III, V), but at higher rates they were inhibited. With the frequency employed by Alnaes, Jansen and Rudjord (1965) only γ-efferents with a resting discharge were influenced and not the other group (paper III, V). These findings are relevant in relation to the problem of whether or not the short-latency reflex effects to static and dynamic γ-motoneurones are mediated by the same reflex pathway (cf. paper III), and were also an important criterion in the classification of cells as dynamic or static (cf. below).

Adequate activation of the skin and underlying tissues could, in the present low spinal preparation (paper III, V), sometimes reflexly influence γ-motoneurones from a receptive field extending from the level of the spinalization to the most caudal part of the tail (paper V) including afferents from the skin, muscle tissues (activated by pressure or pinching of the muscle), the periosteum and the bladder; thus by unspecific stimuli from a very large receptive field that is unexpected for a system dealing with motor control. Increasing evidence has, however, accumulated that the descending control of α-motoneurones from e. g. the pyramidal and the rubrospinal tract (Lundberg and Voorhoeve 1962, Hongo, Jankowska and Lundberg 1969) is exerted through a facilitation or inhibition of the interneurones of reflex arcs providing an excitatory or inhibitory impingement of the cells. The functional significance of these large receptive fields might be to provide a background for the descending control, which will "rule" through an inhibition or facilitation of the reflex paths.

It should be emphasized that even if the above explanation is correct it does not exclude the presence of specific and very delicate spinal reflex pathways to γ-motoneurones.

D. Recurrent inhibition

In four of nine extensor γ-motoneurones, tentatively classified as being of both the dynamic and the static type (paper V), recurrent inhibitory effects were revealed in confirmation of the reports of Ellaway (1968) and Brown, Lawrence and Matthews (1968 a). The important problem whether the recurrent inhibitory effects are exerted in parallel in α- and γ-motoneurones remains to be solved.

E. α—γ linkage?

What is now the position with respect to an α-γ-linkage in the organization of spinal reflex pathways? The only firmly established difference between connexions to α- and γ-motoneurones is the lack of monosynaptic

excitatory connexions from group Ia afferents in the latter (Hunt and Paintal 1958, Eccles et al. 1960). The effects from group I afferents offer a particularly interesting problem. It has not yet been established if the group I IPSPs in γ-motoneurones are evoked from muscle spindles or Golgi tendon organs. Should the latter receptor turn out to be responsible it still remains to be shown if the linkage is limited to static γ-motoneurones.

The complex reflex pattern evoked from cutaneous and high threshold muscle afferents makes it difficult to compare the detailed effects to α- and γ-motoneurones. Further investigations are required but at present there is no strong evidence to disprove the hypothesis of an α-γ-linkage of reflex effects from these afferents. The finding that short-latency reflex effects can be evoked both in dynamic and static γ-motoneurones raises the question of a linkage between α-motoneurones and both types of γ-motoneurones. Effective reflex activation of the two types of γ-motoneurones occurred under different experimental conditions. It is not known if the effects to static and dynamic γ-motoneurones are transmitted via different reflex pathways or whether the differences in reflex transmission mainly depends on a differential excitability in the two types of cells due to other factors (cf. below and paper III).

SUPRASPINAL CONTROL VIA TWO FAST DESCENDING SYSTEMS

The presence of monosynaptic connexions to lumbosacral α-motoneurones from descending fibres were demonstrated by Eide, Lundberg and Voorhoeve (1961). These fibres have subsequently been found to be largely derived from two supraspinal regions. One is the Deiters' nucleus that gives supraspinal effects to knee and ankle extensors (Lund and Pompeiano 1968, Grillner, Hongo and Lund 1969) and the other can be activated within a very limited region of the ipsilateral medial longitudinal fascicle (MLF) (Grillner and Lund 1968) by fibres that were postulated to originate in the lower pontine reticular formation. This descending system gives monosynaptic effects to knee and ankle flexors as well as hip and some toe extensors. In the analysis of the origin of these effects to α-motoneurones chronic lesion experiments were employed, as well as a correlation of the threshold for evoking the monosynaptic effects with the location of the Deiters' nucleus and MLF as indicated by the antidromic field potential evoked on stimulation of the lower thoracic ventrolateral funicle. The location of the stimulation electrode was always checked histologically.

In paper I it is shown with intracellular technique that lumbosacral γ-motoneurones also receive monosynaptic excitation from descending fibres activated at the lower thoracic level and coursing in the ipsilateral ventral quadrant. EPSPs have been considered as monosynaptic when occurring with a latency of 0.4—0.9 msec, from the peak of the positivity in the descending volley recorded at cord dorsum of the segment, to the onset of the EPSP. This peak can be assumed to signal the arrival of activity in the fastest descending fibres (Lund and Pompeiano 1968, Grillner and Lund 1968, Grillner, Hongo and Lund 1969). The "segmental latency" includes the conduction time in the arborizing terminals of the descending fibres and the actual synaptic delay.

To analyze the supraspinal origin of these effects stimulating electrodes were located in the Deiters' nucleus and the MLF respectively with the same criteria as used in the studies on α-motoneurones (cf. above). Since only the ipsilateral ventral quadrant was left intact the risk for coactivation of other descending systems is decreased; furthermore since only

monosynaptic effects evoked by fast conducting fibres (100 m/sec) were studied, the risk for trans-synaptic activation of other descending systems can be neglected. Monosynaptic EPSPs could be evoked with low threshold in lumbosacral γ-motoneurones from either of the two regions. γ-motoneurones belonging to a given motor nucleus received monosynaptic excitation with low threshold from the same region as the α-motoneurones did (paper I). In each experiment also α-motoneurones were recorded and they could be monosynaptically excited from one of the two regions with the same low threshold. Since only 13 γ-motoneurones (2 not included in paper I) with descending monosynaptic effects have been studied, great caution should be applied in interpreting these results. However, they do allow the suggestion that the two descending supraspinal systems exert parallel effects on α- and γ-motoneurones of the same motor nuclei.

Since not all γ-motoneurones received the descending monosynaptic EPSP the question was raised whether the monosynaptic control was exerted only on one type of γ-motoneurone. To test this hypothesis the γ-efferents studied in paper III and V were also tested for descending effects (IV and V) by stimulation of the ipsilateral ventrolateral funicle. These γ-efferents could be classified as static or dynamic (though less certainly to extensors, see below), and indeed monosynaptic effects were revealed in static but never in dynamic γ-motonerones. This does not necessarily imply that there is no monosynaptic effects in dynamic cells, but if so, this effect was not strong enough to discharge the cells although they were tested against a high level of background activity. Since the size of the descending monosynaptic EPSP evoked from supraspinal regions in paper I was of similar amplitude to the one obtained on stimulation of the descending fibres at lower thoracic level, the results indicate that the descending EPSPs evoked in the γ-motoneurones investigated are largely of supraspinal origin and that strong effects are exerted on static γ-motoneurones.

It is of some interest that driving of primary endings can be elicited from the "fast descending system" of Granit and Holmgren (1955). Driving seems to be an exclusive action of static γ-motoneurones (Crowe and Matthews 1964 b, Appelberg, Bessou and Laporte 1966). Although this system is activated from the contralateral tegmentum its fibres descend in the ventral quadrant of the ipsilateral side, hence this system could be identical with the systems investigated in the present study.

In Fig. 1 the descending effects to motoneurones acting at the knee joint are summarized, demonstrating the parallel effects on α- and γ-moto-

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THE "NORADRENERGIC RETICULOSPINAL SYSTEM"

Descending fibres in the spinal cord containing large amounts of noradrenaline were described by Carlsson, Falck, Fuxe and Hillarp (1964); subsequently Dahlström and Fuxe (1965) demonstrated that these fibres originate from cell bodies located in the reticular formation of the lower brain stem. No neurones with their cell bodies located in the spinal cord were found to contain noradrenaline (Carlsson et al. 1964). The injection of DOPA, i.e. a precursor of noradrenaline, has been postulated to cause an increased synthesis and liberation of noradrenaline from descending noradrenergic fibres (Andén, Jukes, Lundberg and Vyklicky 1966 a, Andén, Jukes and Lundberg 1966 b). The evidence relating the injection of DOPA to the formation and liberation of noradrenaline has been summarized in paper III. *On the basis of this postulate the effects of the descending noradrenergic system can be studied in the acute spinal cat after an intravenous injection of DOPA.*

The injection of DOPA causes a profound change in reflex transmission in the spinal cord. Thus the short-latency effects evoked from cutaneous, high threshold joint, group II and III muscular afferents (i.e. FRA) to α-motoneurones and ascending neurones are depressed (Andén et al. 1966 a), but, on the other hand, late reflex effects evoked with long central delay occur in α-motoneurones and in the terminals of Ia afferents (Andén, Jukes, Lundberg and Vyklicky 1966 c, Jankowska, Jukes, Lund and Lundberg 1967).

A. The resting discharge and the identification of static and dynamic γ-motoneurones

In paper III it was found that the single efferents to flexors could be subdivided in two groups, one with a resting discharge that was decreased after DOPA, the other with no resting discharge initially, but which acquired a spontaneous activity after DOPA. Identification of these two groups of γ-motoneurones in terms of dynamic and static can be made by a comparison with results obtained with indirect methods. Thus,

Alnaes, Jansen and Rudjord (1965) found evidence for a resting discharge only in dynamic γ-efferents in the spinal cat and Grillner, Hongo and Lundberg (1967) showed that static γ-motoneurones are accelerated after DOPA. For the identification it was also necessary to know whether the activity in dynamic γ-motoneurones changed after DOPA. In paper II the response of primary endings to linear stretch at different velocities was studied; the dynamic sensitivity, the peak and the mean frequencies during stretch were decreased to lower values after DOPA, although there was evidence for an increased static fusimotor bias. The possibility of a tonic slow α-activity was excluded, since there was no EMG activity in the tenuissimus muscle before DOPA. These findings can only be interpreted as a decreased dynamic γ-motor drive to the spindles after DOPA. This evidence is further supported by the previously discussed reflex effect evoked from repetitive nerve stimulation (page 11). At low frequencies neurones classified as both static and dynamic γ-motoneurones could be activated; at a stimulation frequency of 40 pps only neurones classified as dynamic were activated, which was the frequency employed by Alnaes, Jansen and Rudjord (1965) when they found reflex activation of only dynamic γ-motoneurones. Since the type of γ-motoneurones obtained during the isolation procedure is largely random it is of relevance that 7 γ-motoneurones could be classified as dynamic and 15 as static, corresponding approximately to the ratio of the numbers of dynamic and static γ-efferents, which is 1 to 3 (Bessou and Laporte 1966).

Hence the two types of γ-efferents to flexors (paper III) could be identified in terms of dynamic and static. Accordingly the noradrenergic descending system exerts a reciprocal control of dynamic and static γ-motoneurones to flexors.

γ-efferents to extensors (gastrocnemius) were almost invariably accelerated after DOPA (paper V). 4 out of 15 had a resting discharge before DOPA. The identification of these neurones in terms of dynamic and static is more difficult than was the case with flexor cells. On the basis of a comparison with the results of Alnaes, Jansen and Rudjord (1965) the tentative suggestion was made that the four neurones with a resting discharge before DOPA were of the dynamic type and those without a resting discharge were of the static type. This was again supported by the effects of repetitive nerve stimulation with the same findings as discussed above for flexors. Since all γ-efferents (except one) were accelerated after DOPA, it can be inferred, even if the above tentative identification should prove to be wrong, that both dynamic and static γ-efferents to extensors are accelerated after DOPA.

The results thus indicate that the descending noradrenergic system creates a situation with a static fusimotor activity to flexors and a dynamic and a static activity to extensors. The functional implications of this will be discussed below (cf. page 27).

The generation of the resting activity in the spinal cat is largely dependent on the activity in the dorsal roots (Hunt 1951), although some activity may persist after secion of the dorsal roots (Voorhoeve 1960, Lindsley and Eldred 1960). Also the resting activity in neurones spontaneously active after DOPA is greatly reduced after transection of the dorsal roots of the neighbouring segments (paper III, V). Thus it can be concluded that the afferent inflow in tonically discharging afferents, presumably from a large variety of receptors, maintains this activity; in this connexion it is of particular interest that the receptive fields from which the γ-motoneurones can be influenced is very large (paper V). It is likely that the resting activity in γ-motoneurones spontaneously discharging before DOPA is maintained through the short-latency reflex paths to γ-motoneurones. A more complex situation exists after DOPA when both short-latency pathways and the pathways mediating the late discharge are open. A more detailed discussion of different factors of importance is given in paper III.

B. The effect of DOPA on the reflex discharge evoked through short latency reflex paths in static and dynamic γ-motoneurones

The short-latency transmission from the flexor reflex afferents (FRA) to certain ascending pathways and to α-motoneurones is depressed after DOPA, very likely due to a depression at an interneuronal level (Andén et al. 1966 a).

The reflex discharge evoked in γ-motoneurones from the FRA has been compared before and after DOPA in flexor (paper III) and extensor γ-motoneurones (paper V). In flexor γ-motoneurones a rather consistent pattern was found. The reflex discharge in dynamic γ-motoneurones was smaller after DOPA; the resting discharge, however, also ceased. Static γ-motoneurones on the other hand are more effectively activated after DOPA, though a resting discharge appears. In extensor γ-motoneurones mixed excitatory and inhibitory effects were often elicited; furthermore the excitability in all γ-motoneurones increased after DOPA as reflected by the increased resting discharge. Whether this is due to a facilitation of short latency excitatory pathways or to other factors is not known.

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Hence it is not possible to conclude whether this change of reflex effects reflects only a modified excitability of the γ-motoneurones or if it is due to influences on the short-latency interneuronal pathway.

C. The late reflex discharge

Neuronal systems for alternate activation of the limb muscles do exist in spinal cord, since in the chronic spinal cat stepping movements can occur for long periods (Sherrington 1910). Graham Brown (1911, 1914) showed that it is essentially due to an intraspinal process. In an unanaesthetized (decerebrate) acute spinal cat with its hindlimbs intact, stepping movements can be induced by a variety of adequate stimuli after the injection of DOPA (unpublished observation). The late and longlasting reflex discharge that can be evoked from ipsilateral nerves in flexor motoneurones and from contralateral nerves in extensor motoneurones after an injection of DOPA, can give rise to alternate activation of flexors and extensors similar to "spinal stepping" (Jankowska et al. 1967, cf. paper V, Fig 1). It has been suggested that stepping can be evoked through the interneuronal network responsible for these reflex discharges not only by reflex effects from spinal afferents but also by effects in descending pathways and probably also from autochtonus interneuronal activity (Jankowska et al. 1967).

α-motoneurones and also static and dynamic γ-motoneurones participate in the late reflex discharges to extensors and flexors (paper III, V). In most instances there was a slowing of the resting discharge in extensor γ-motoneurones during the reflex discharge in flexors, which would be meaningful since it decreases the reciprocal Ia inhibition evoked in antagonists (paper V).

The results thus infer that the activation of descending noradrenergic fibres reveals a neuronal system for alternate activation of flexors and extensors. It is of considerable interest that both dynamic and static γ-motoneurones are activated with α-motoneurones in the late discharge. The functional implications of linkage will be further discussed below.

D. The stretch reflex

In the decerebrate preparation a tonic stretch reflex occurs in extensor muscles, but not in the acute spinal preparation (Liddell and Sherrington 1924). After an injection of DOPA, however, a tonic stretch reflex does

occur in the soleus muscle (paper V) as judged from an activity together with an increase of tension on extension. Also in flexor muscles a stretch reflex occurs (unpublished). This effect occurs in parallel with an appearance of a ... in static γ-motoneurones (see above). Since after DOPA change in excitability occurs in α-motoneurones in the s preparation used by Andén et al. (1966 a) with most ipsilate contralateral nerves cut, it is tempting to relate this t reflex to the occurrence of a static fusimotor activity. In a preparation there is a spontaneous activity in static γ-moto sen and Matthews 1962 a, b, Jansen 1966) but in the spin only dynamic γ-motoneurones appear to be spontaneously ac Jansen and Rudjord 1965, cf. paper II). The decerebrate pr been described as "a caricature of standing" (Creed et al. 1 spinal cat after DOPA with its hindlimbs pendent both . extensors are at rest with no sign of EMG activity (unpublished tion). One important difference between the decerebrate prepa... and the "DOPA preparation" is that in the former, practically no ...ing activity occurs in γ-motoneurones to flexors (Jansen and Rudjor ... 1965) whereas the "DOPA preparation" has a resting discharge in static γ moto-neurones to both flexors and extensors (paper III, V), which gives rise to a discharge in afferents from muscle spindles in both types of muscles; the reciprocal Ia inhibitory effects exerted in both directions between flexors and extensors might provide an explanation for the lack of tonus in the "DOPA preparation" at rest (cf. above C).

GENERAL DISCUSSION OF THE PROPERTIES OF THE γ-MOTOR SYSTEM WITH PARTICULAR REFERENCE TO THE SYSTEMS INVESTIGATED IN PAPERS I-V

Papers I—V are mainly concerned with the central control of γ-motoneurones. In an effort to understand the possible role of the γ-loop during posture and locomotion, some of its properties will be considered. The muscle spindle will be treated as a black box; some characteristics of its input-output relations will be discussed and also some quantitative aspects of data relevant to the transfer function of the different elements in the stretch reflex loop. Very little attention will be paid to the inhibitory effects evoked from Ia afferents in antagonist motor nuclei (Lloyd 1946), although it should be emphasized that these effects presumably are of great functional significance in motor control.

A. Properties of the stretch reflex loop

The different input variables are the frequency of impulses in fusimotor fibres and the muscle length and its derivative, the velocity of length change. It has been proposed (Schäfer 1967, Grüsser and Thiele 1968) and subsequently denied (Lennerstrand 1968 b) that the spindle is also sensitive to acceleration. This possibility will not be discussed below. The outputs of the spindle are the primary and the secondary endings. The loop from the primary ending will mainly be considered, i.e. primary *ending — monosynaptic excitation of certain α-motoneurones leading* to a contraction and development of tension and eventual shortening of the muscle.

The transfer functions* between both static and dynamic γ-motoneurones and the primary endings are linear, as they are from static γ-motoneurones to secondary endings within the frequency range used (¼—12 Hz) (Andersson, Lennerstrand and Thodén 1968). With length input there is a linear transfer function with amplitudes less than 100 μ (Poppele and

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Terzuolo 1968, Matthews and Stein 1968). Poppele and Terzuolo (1968) have extended their analysis to the synaptic effect on the α-motoneurone (cf. also Redman Lampard and Annal 1968) and the development of tension induced in the muscle under a given γ-bias in an extensor muscle of the decerebrate cat. There is, at stretches below 100 μ, a linear transfer function for sinusoidal stretch between the length change and the induced change in tension. In the first part of the control loop there is a phase advance until the stage of the EMG activity, but in the last part, i.e. α-efferent (EMG) to tension development in the muscle there is a phase lag (Partridge 1965, Poppele and Terzuolo 1968). For these reasons the tension developed in the muscle followed the applied length changes without appreciable phase shift within the frequency range used (0.1—6.0 Hz). With applied sinusoidal stretching above 100 μ the transfer function between applied length change and tension development is not linear (cf. Lippold, Redfearn and Vuco 1958, Jansen and Rack 1966). It is of considerable interest, however, that the relation between motoneuronal activity and applied length can be linear over a wide range of length changes (Poppele and Terzuolo 1968). Since the increase in tension in the stretch reflex is not primarily due to a frequency modulation but rather to a recruitment of new motor units (Denny-Brown 1929, cf. Poppele and Terzuolo 1968), this implies that either the threshold or the synaptic input to, or the properties of different motoneurones supplying the muscle varies within quite a wide range.

It should be noted that with slow sinusoidal stretches the responses (tension developed) to each cycle are not identical if the experiment is performed on a decerebrate cat. Thus Higgins, Partridge and Glaser (1962) have shown that during the first few cycles (0.5—1.2 Hz) of large amplitude stretching (5 or 8 mm), the tension developed is phase advanced and of larger amplitude, reaching after less than 30 sec a steady state with no phase advance and smaller amplitude. These findings could be explained if the sinusoidal stretching decreased the activity in dynamic γ-motoneurones to the muscle studied. This transient response disappeared if the cerebellum was removed. It can therefore not be assumed that the γ-bias is identical during resting conditions and during sinusoidal stretching, or during vibration of the muscle tendon. Whether changes occur also directly in the α-motoneurones due to a similar mechanism is not known.

The reflex stiffness, i.e. the static gain of the stretch reflex loop of the soleus muscle in the decerebrate preparation, can vary considerably; values of 40—200 g. wt/mm have been given (Matthews 1959). When estimating the gain of the stretch reflex loop it is difficult to know how relevant

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B. The functional role of the "γ-loop"

These results provide a basis for an estimate of what effects the γ-loop could exert, but they do not answer the question of what the γ-loop is actually used for. Merton (1953, cf. also Eldred, Granit and Merton 1953) in his follow up length servo hypothesis postulated that the "γ-loop" constituted an independent route for motor control. Merton (1953) clearly pointed out that the direct activation af α-motoneurones would be a disturbing factor for this kind of servo, and further that the gain of the loop needs to be high enough. Subsequent work of Granit and others has shown that in most cases α- and γ-motoneurones are coactivated although the γ-motoneurones often have lower threshold for excitation, which is why the expression α-γ-linkage was introduced (Granit 1955). The results presented on the segmental (paper I, III, V) and on the descending control provide further evidence for an α-γ-linkage as well as the results obtained during naturally elicited alternating rhythmical movements (cf. below page 27). It has also been argued that the gain of the stretch reflex loop is too low (in soleus 40—200 g. wt/mm) to allow operation of an independent follow-up-length-servo (Matthews 1967). For these reasons and since the evidence used to support Merton's (1953) hypothesis merely shows that the excitability of the α-motoneurones is dependent on activity in muscle spindle afferents (cf. Hunt and Perl 1960 and Matthews 1967), the γ-loop will in the present context not be considered as an independent route for motor excitation. However, the "γ-loop" does have servo properties (cf. Matthews 1964); the primary endings are in fact accelerated instead of decelerated during a "naturally" elicited muscular contraction (cf. below), although α- and γ-motoneurones are coactivated.

It is presumably of importance when considering the function of the "γ-loop" to differentiate between posture and locomotion. In standing there is possibly a rather stable discharge in α- and γ-motoneurones. Under these conditions the muscle spindle with its afferent supply acts to counteract any change in muscle length, i.e. as a negative feedback. These negative feedback properties can be changed considerably by the γ-motor supply, and it has to be divided into a static and a dynamic component. A primary ending extended to a given length and with a resting discharge has

1) under no γ-bias a resting discharge with high dynamic sensitivity to muscle shortening (a decreased discharge, i.e. unloading), and a certain dynamic sensitivity to lengthening (increased discharge) (B. H. C. Matthews 1933);

2) during dynamic fusimotor drive there is, compared to 1), an increased dynamic sensitivity to muscle lengthening but a decreased dynamic sensitivity to muscle shortening. There is still, however, an abrubtly decreased resting discharge on shortening (Crowe and Matthews 1964 a, b);

3) during static fusimotor drive the dynamic sensitivity to both shortening and lengthening is reduced compared to 1) and 2) and the signal will be linearly related to muscle length (Crowe and Matthews 1964 a, b);

4) during a concomitant static and dynamic fusimotor drive there is a high dynamic sensitivity to muscle lengthening but not to shortening when the discharge is proportional to length (Lennerstrand 1968 a, b).

The static fusimotor drive will set the discharge rate of the primary endings (Matthews 1962, Crowe and Matthews 1964 a) and in addition presumably also increase their static sensitivity, i.e. the increased discharge on extension in imp/sec/mm (Jansen and Matthews 1962 a, Brown, Lawrence and Matthews 1969).

1. *During posture*

If during standing there is a dynamic and a static fusimotor bias (as in extensors of the decerebrate cat) a sudden increase in load leading to lengthening will be effectively counteracted by the high dynamic sensitivity. If the static loop gain is high enough the muscle will remain at approximately the same length; if not, supraspinal structures would, during the period in which the high dynamic sensitivity is acting, have time to readjust the excitability of the motoneurones. In this connexion the very high sensitivity of the primary ending for changes in muscle length below 100 or 200 μ should be considered (decerebrate cat, Terzuolo and Poppele 1968, Matthews and Stein 1968). It is then tenfold higher than during larger amplitudes of stretch. In other words, the gain of the stretch reflex loop is much higher in this range and would detect and counteract even very small length changes. Thus, if α- and γ-motoneurones are coactivated and have brought a muscle to a certain length, the stretch reflex will act as an efficient circuit for maintaining the length (cf. Matthews and Stein 1968). It is of interest to make a comparison with the frog that has a slow and a fast (twitch) extrafusal motor system. The slow motor system that presumably is involved in postural activities increases the dynamic sensitivity of the spindle but the fast twitch system increases the static discharge of the spindles (Matthews and Westbury 1965). It should perhaps be noted that the viscoelastic properties of the muscle itself, which differs considerably in the active and passive states, also counteracts lengthening.

2. *During movement*

The muscle spindle presumably has a different role in phasic movemer The primary endings are accelerated during naturally elicited movements such as the respiratory movements (Critchlow and Euler 1963), the lo. motion that can be induced in the mesencephalic cat (Severin, Orlov and Shik 1967) and the reflexly initiated contraction of the jaw mus (Taylor and Davey 1968); efferent recordings have shown that both and γ-efferents participate (Eklund, Euler and Rutkowski 1964, Sl Orlovskii and Severin 1966). Euler and Peretti (1966) have found that dynamic and static γ-motoneurones appear to be coactivated in the respiratory movements. The alternate stepping movements might be the corollary of activity in the neuronal system mediating the late reflex discharges that can be evoked after DOPA (cf. page 20, Jankowska et al. 1967) α- and γ-motoneurones of both types participate in these reflex discharges (paper III, V). How does the fusimotor activity influence movements? The advantage of dynamic fusimotor activity is the same as during standing. However, a muscle spindle with dynamic sensitivity to muscles shortening (decreased discharge) would counteract the contraction. A coactivation of α- and static γ-motoneurones removes this effect, since the primary endings are not unloaded; indeed it has been shown that an acceleration of static γ-motoneurones during a shortening (in contrast to dynamic γ-motoneurones) is able to cause an increased discharge rate in the primary endings (Lennerstrand and Thodén 1968). This would not only compensate for the unloading but also add excitation to the α-motoneurones through the γ-loop, the amount of excitation being dependent on the actual length of the muscle. Thus it can be concluded from the above findings that a static γ-α-linkage indeed occurs under natural conditions and that the activation of static γ-motoneurones gives servo properties to the γ-loop.

3. *What is the functional role of the resting discharge in γ-motoneurones?*

In the spinal state there is a resting discharge mainly in dynamic γ-motoneurones (Alnaes, Jansen and Rudjord 1965, paper II); the preparation is then counteracting any changes of length with its dynamic sensitivity to lengthening and shortening, the overall effect being a general damping of movement in the system (cf. Alnaes, Jansen and Rudjord 1965). After DOPA, i.e. under influence from descending noradrenergic fibres, the resting discharge of γ-motoneurones changes; flexor γ-motoneurones of the static type acquire a resting discharge but the dynamic are depressed; to extensors on the other hand the resting discharge of both types of

γ-motoneurones is increased. The functional significance of this change of the resting discharge is obscure. However, if the very speculative assumptions are made that 1) the descending noradrenergic system activated by DOPA is one functional entity and not several separate descending systems provided with the same transmitter and 2) that the injection of DOPA reveals the neuronal pools employed during stepping (Jankowska et al. 1967, see above), it is relevant to ask if the change of γ-bias to flexors and extensors induced by the noradrenergic system would be purposeful for the act of stepping, i. e. if the adjustment of γ-bias can be looked upon as a preparatory state for locomotion.

During stepping, rapid forceful alternate contractions of flexors and extensors are required. The damping properties, i.e. the dynamic sensitivity to lengthening and shortening is probably not particularly important for flexors, since their function in locomotion is to rapidly flex and bring forward the leg and not to counteract the lengthening. With a γ-bias of only the static type, the spindle has its lowest dynamic sensitivity to both lengthening and shortening. During the late discharge there is a concomitant activation of all three types of motoneurones, including dynamic γ-motoneurones, but since during contraction the discharge of the primary ending is entirely influenced by the discharge in static γ-efferents this does not essentially change the condition. The situation in the extensors during locomotion differs considerably from that of flexors since then the contraction will lead not only to an extension of the leg resulting in the propulsive force for the body but will also support the body, i.e. being antigravitatory. The resting discharge in static and dynamic γ-motoneurones thus provides a situation with no dynamic sensitivity during the contraction, but an eventual lengthening would be counteracted by the dynamic sensitivity to lengthening provided by the discharge in dynamic γ-motoneurones.

Hence it seems as if an activation of the descending noradrenergic system changes the resting discharge in static and dynamic γ-motoneurones towards a γ-bias that would give an appropriate background discharge during locomotion on which the phasic activation of α- and γ-motoneurones can be superimposed.

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SUPPLEMENTUM 328

EFFECT OF PHYSICAL TRAINING ON OXYGEN TRANSPORT SYSTEM IN MAN

by

BJÖRN EKBLOM

STOCKHOLM 1969

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To Ulla

Hildur and Tore

CONTENTS

Introduction 9

Methods 10

Procedures 13

Subjects 16

Chapter 1. The oxygen transport system in athletes 17

Chapter 2. Effect of physical training on circulation during submaximal and maximal exercise of short duration in previously untrained male adults 21

Chapter 3. Effect of physical training on circulation during prolonged severe exercise in previously untrained male adults 27

Chapter 4. Effect of physical training on oxygen transport system of adolescents 29

General discussion 34

Acknowledgements 41

References 42

INTRODUCTION

Physical training increases the body's performance capactiy. This is a well established fact, which is easily confirmed with a measuring-tape and a stop watch. However, a closer analysis of the physiological effect of training has mainly been based on cross-sectional studies where different individuals at different stages of training have been compared. The advantages offered by this method are the great intra- individual disparities in the variables studied. The method has, however, its shortcomings. For instance, it leaves unanswered the question as to what degree the measured differences betwen the untrained and the trained individual are the consequence of the training and to what degree they arise from dissimilar constitutional—that is genetic—backgrounds. Furthermore the amount and quality of training and the age at which training was first started may vary greatly. For these reasons longitudinal studies are necessary, in which the same individual is studied before and after training, thereby constituting his own control.

In the present research project the object has been to study:

1. the oxygen transport system of very successful athletes in endurance sports. By studying these individuals extreme values for human performance capacity are obtained and it can be analyzed how these extreme values are accomplished.
2. the effect of training on the oxygen transport system by way of longitudinal studies.

Chapter 1 refers to well-trained athletes (study III). Chapter 2 and 3 deal with the effect of training on previously untrained 20 year-old men (studies II and V). Chapter 4 is concerned with the effect of training on individuals during the years of adolescent growth (study V).

It may be emphasized that in this paper no attempts have been made to elucidate the general regulatory mechanism behind the circulatory adjustment to exercise. For this topic see review articles of Asmussen and Nielsen (1955), Rushmer and Smith (1959) and Bevegård and Shepherd (1967).

METHODS

Detailed descriptions of the methods used are reported on in the separate papers and are therefore only briefly summarized here.

In order to study the effect of training standardized exercise methods were used. Exercise was carried out on either a motor-driven treadmill, of which the speed and degree of inclination can be varied or on an electrically braked Krogh bicycle ergometer.

Oxygen uptake was determined with the Douglas bag technique. Heart rate was calculated from electrocardiographic (ECG)-recordings. Blood lactate concentration was determined in samples, taken from arterial blood in the experiments with determination of the cardiac output and otherwise in blood taken from a prewarmed finger tip. Cardiac output was measured with the dye dilution technique, using Cardio Green as indicator. Brachial intraarterial blood pressures were recorded with an Elema-Schönander pressure transducer.

Current statistical methods were used. Student's t-test was applied to analyze the significance of differences. The probability level of significance used was $p < 0.05$.

Comments

Oxygen uptake: The error of the method for determination of the oxygen uptake at submaximal work (6—8 min) was calculated from the difference between two consecutive determinations for bicycle (study II) and treadmill (study III) exercise. The error of the method for determination of the maximal oxygen uptake, which includes both methodological errors and biological variations, was calculated from two determinations of the maximal oxygen uptake, made on separate days within a week of each other (study II and V). Only values on maximal oxygen uptake obtained in experiments without catheters have been used. The results are given in TABLE 1.

There was no systematic difference between the first and the second determination of oxygen uptake on either submaximal or maximal level of exercise. The error in the methods are of the same order as reported earlier (Taylor et al. 1955, Mitchell et al. 1958, Åstrand 1960, Åstrand and Saltin 1961).

Oxygen uptake measured at about the 5th min on each work load increases

TABLE 1. Error of the method for a single determination of oxygen uptake and cardiac during submaximal and maximal bicycle and treadmill exercise.

	n	Oxygen uptake liters/min	Error of the method %	n	Cardiac output liters/min	Error of the method %
			Submaximal work			
Bicycle	54	1.95	2.5	14	11.1	3.0
				47	17.1	3.2
				63	21.1	3.5
Treadmill	30	3.38	1.5	26	26.5	5.1
			Maximal work			
Bicycle	19	4.13	2.1	43	23.1	3.3
Treadmill				14	32.0	3.9

with progressively heavier work loads up to a level beyond which any subsequent raising of the work load will not induce any further increase in oxygen uptake ("levelling off") (Herbst 1928). In the present publication this "levelling off" of oxygen in relation to work load and/or a high blood lactate concentration was used as criterion that maximal oxygen uptake was attained.

This "levelling off" of oxygen uptake in relation to work load raises the question of the definition of maximal work. Maximal oxygen uptake can be obtained on several different work loads from point A (in Fig. 1)—this is the point of "levelling off"—to point B. The work load at the point of "levelling off" is the lowest load, which still gives maximal oxygen uptake. This load is here defined as $WLmaxO_2$. Work loads above $WLmaxO_2$ (for instace at point B) are "supermaximal" in regard to the maximal aerobic power.

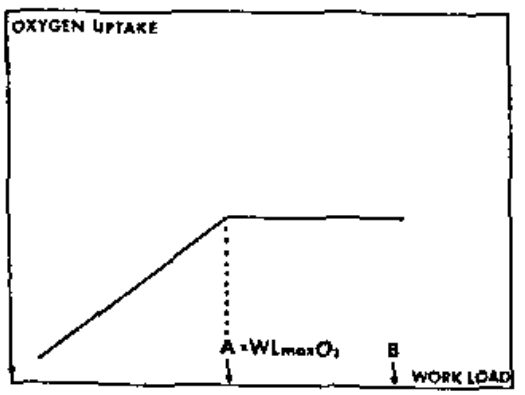


Fig 1. Oxygen uptake in relation to work load. Point A represents the point of "levelling off" defined as $WLmaxO_2$, which is the lowest work load that still gives maximal oxygen uptake To assure that point A represents point of "levelling off", work time should be at least 4 min and blood lactate at least 8 mM. Work loads above this point (for instance point B) are "supermaximal" in regard to maximal aerobic power.

The higher the work load above $WL\text{max}O_2$ the shorter will the work time be, and—as shown in study I—there is a risk that the oxygen uptake at work loads much higher than $WL\text{max}O_2$ is somewhat lower than those obtained at $WL\text{max}O_2$. Furthermore in a recent study from this laboratory (to be published) where heart rate and cardiac output were measured during exhaustive exercise at different work loads all requiring maximal oxygen uptake, it was found that in order to ensure maximal values on heart rate and cardiac output, the work load should be selected so that the work time exceeds 4 min. For these reasons the work loads for the circulatory studies in this publication were chosen from the first part of the plateau just to the right of the point of "levelling off" ($WL\text{max}O_2$) in order to allow for a work time above 4 min.

Cardiac output: The error of the method for the determination of cardiac output during submaximal and maximal exercise on bicycle (study II and V) and treadmil (study III) has been calculated from double determinations in the same experiment. The results are given in TABLE 1. These errors of the method are of the same order—or 3 to 5 per cent—as reported earlier fro mthis and other laboratories (Asmussen and Nielsen 1952, Saltin 1964a., Stenberg 1966).

PROCEDURES

Experimental procedures: A detailed description of the experimental procedure can be found in each individual paper.

The first time the subjects came to the laboratory they familiarized themselves with the test methods used by performing submaximal and maximal work. The data from these very first experiments are not included in the results. In experiments with and without catheters the experimental procedure for submaximal and maximal work of short duration was identical.

When cardiac output was determined the subjects came to the laboratory at 8 a. m. after a light, fat-free breakfast. Two catheters were inserted, one in the brachial artery and one in a deep cubital vein, according to the Seldinger technique (Bernéus et al. 1954). Thereafter the subjects rested for at least 1 hour in supine position, before the exercise was started.

Unless otherwise indicated exercise at submaximal work loads was carried out for 6—8 min. The heart rate was recorded each minute of exercise. The blood pressures were recorded at the 3rd min in the experiments with catheters inserted. Simultaneous determinations of oxygen uptake and cardiac output were made after the 5 th min. Blood samples for determination of lactic acid concentration, hematocrit, hemoglobin and oxygen saturation were taken during the collection of expired air.

All maximal work loads were preceded by a 3 min warming-up spell of work, which loaded the oxygen transport system to about 50 per cent of it's maximum. Immediately thereafter, work at maximal load started and was carried out for 4 to 6 min (see METHODS). The ECG was recorded for 10—15 sec each min of exercise and continuously during the collection of expired air. The blood pressures were recorded at the 2nd min. All other measurements started 2.5 to 3 min after the beginning of the maximal work. At least two simultaneous determinations of oxygen uptake and cardiac output were made.

Training procedure: The same principles for training were applied in all studies, whereas the training volume and pace was adapted to the training status of the individual. Both dash-, interval- and endurance training have been included in the programme.

Dash training consists of full speed preferably uphill running during 30—60 sec and repeated 5—10 times with 2—3 min periods of low activity between each dash. Blood lactate concentration after such a period of exercise exceeds 10 mM and values up to 28—30 mM have been recorded (Ekblom et al. 1967, Karlsson et al. 1967).

In interval training the running speed is chosen so that the running time is about 10 per cent lower than the individual's best time for a given distance (for explanation, see study I). The running time should last 3 to 7 min. Depending on the individual's state of fitness, the intervals are repeated 3 to 7 times with low activity periods of up to 6 to 8 min in between each interval.

Distance training consists of running mostly at submaximal speed for 45 to 75 min. During short periods of increased speed or when running uphill, maximal values for heart rate and oxygen uptake might have been obtained.

The number of training periods per week is specified in each study. The training dealt with in studies IV and V was supervised, but in the remaining cases the subjects conducted their own training according to a predetermined programme.

Comments

Experimental procedure: When performing the same exercise with and without catheters inserted the heart rate did not vary more than what could be expected from day to day variations without catheters indwelled. At the submaximal level, therefore, the catheters do not appear to have caused any specific changes in the circulatory response. This agrees with earlier findings reported (Rowell 1962, Saltin et al. 1968). On the other hand, the maximal oxygen uptake of a number of subjects was lower in the experiments with catheters inserted (in 3 subjects the differences were greater than 10 per cent). The reason for this is not quite clear. It should be mentioned that in connection with these experiments the blood loss in no case was more than 150 to 200 ml.

Training procedure: As was stated under the heading of "METHODS", exhaustive exercise can infer different degrees of load from $\dot{V}O_2max$ and

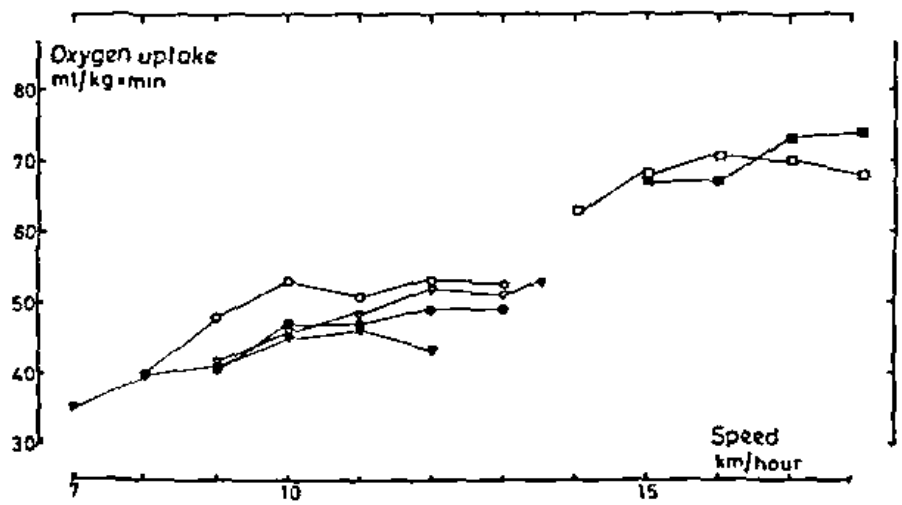


Fig. 2. Oxygen uptake in relation to speed (angle 3°) in 4 untrained and 2 well-trained subjects (PS filled and BE unfilled squares).

higher. This has bearing on the training programme. What speed is sufficient for the attainment of maximal oxygen uptake?

In study I five subjects were studied during submaximal and maximal exercise on the treadmill. Oxygen uptake, heart rate and blood lactate concentration were measured. Firstly tests were made in order to determine the highest speed which the subject could stand for 4 min. Thereafter on different days the subject was tested at speeds slightly below the one which had thereby been established. The duration of the exercise period was regulated so that the same total distance would be covered for each exercise period. The results (Fig. 2) show that the plateau for the "levelling-off" of oxygen uptake in relation to increased speed varied in length for the various individuals. The longest plateau was attained by subject *BE*, i.e. from 15 to 18 km/hour with an inclination of the treadmill of 3° and the shortest by subject *PS*, i.e. from 17 to 18 km/hour with the same inclination. It is worth mentioning that in these two well-trained subjects maximal oxygen uptake was obtained also at 19 and 20 km/hour (with the work time correspondingly shortened). Accordingly, the total plateau for "levelling-off" for subject *BE* was from 15 to 20 km/hour, if the work spells were reduced below 4 min.

As for bicycle exercise the length of the plateau for "levelling-off" (work time $>$ 4 min) is often somewhat shorter than that for treadmill exercise in the same individual. This might depend on that the muscle mass engaged is less in maximal bicycle work than in maximal running; that the proportion of static muscular work is higher during maximal bicycle exercise; that the muscle pump is more effective during running etc.

The indications to which the main results of this investigation point may be summarized by stating that in order to attain maximal oxygen uptake during interval training (work time 3 to 7 min) it is not necessary to maintain the maximal running speed for a given distance. This could indicate that a decrease in speed of 10 per cent may not reduce the training effect of the maximal aerobic power. However, it is still unproved if this reduced speed represents the same training stimulus on the maximal aerobic power, as the training at maximal speed ("supermaximal work"), i.e. if "intensity" can be replaced by "duration". On the other hand the higher the speed the fewer bursts of activity in a given period of time can be performed, since the recovery time is prolonged. For that reason it is highly recommendable to make use of both training methods.

SUBJECTS

Detailed information about the subjects used for this research project are found in each individual study. Since some confusion of definition exists as regards the classification of different subjects with regard to their habitual amount of training, an attempt is made below to establish such categories.

Very inactive—Individuals who for certain reasons, as for instance bed rest, physical or psychic handicaps, were forced to be extremely inactive.

Sedentary—Healthy individuals in occupations which impose no physical demands, who had never taken part in physical training previously, or who had once trained moderately, but not in recent years. Examples—subjects in studies II and V before they started training.

Moderately active.—Individuals who exercise regularly (1—4 times a week) but do not take part in contests. This group also includes individuals in occupations involving heavy manual work or individuals who take part in compulsory exercise during military service or at school. The physical education student belongs to a special sub-group among the moderately active, since the student in physical education trains regularly every day during the term for 2 years in Sweden. However, the intensity of the training cannot be compared with that of the athlete.

Athlete—Individuals who have trained for contests practically every day over the course of several years. Training, for instance in the case of an athlete in endurance events, consists of dash-, interval- and endurance training (for definitions see under heading "METHODS"). The athlete category should be divided into two sub-groups,

1. Top athletes, prominent in international and national events, such as group 1 in study III.
2. Athletes, who in contests cannot be compared with group 1. Examples, group 2 in study III.

In the case of the categories moderately active and athletes information should also be included on the subject's state of training at the time the study was undertaken.

CHAPTER 1

THE OXYGEN TRANSPORT SYSTEM IN ATHLETES

a) Previous investigations

Oxygen uptake during heavy muscular work was measured as far back as in the 1910 decade, but it was not until 1937 that Robinson, Edwards and Dill could present more acceptable data on maximal oxygen uptake in athletes (5.35 liters/min for D. Lash—the world record holder for 2 English miles). Christensen and Högberg (1950) and Åstrand (1955) could confirm these high values for maximal oxygen uptake, and since then many investigations have been reported on maximal oxygen uptake measurements in athletes representing various kinds of sports (Mellerowics and Hansen 1965, and others). In one of the most recent papers Saltin and Åstrand (1967) present the highest measured values heretofore given for maximal oxygen uptake for top athletes in endurance events (highest values 6.17 liters/min and 85.1 ml/kg per min).

In addition to a high maximal oxygen uptake athletes are also characterized by a low heart rate on submaximal loads (Christensen 1931) and large dimensions for the oxygen transporting organs, such as heart volume, total hemoglobin, blood volume and vital capacity (Kjellberg et al. 1949, Roskamm et al. 1966, among others).

In order to throw light on how the large oxygen transport capacity of athletes is coped with, measurements on the cardiac output are of great importance. Rowell (1962), Saltin (1964b) and Åstrand et al. (1964) have presented data on maximal cardiac output (28 to 32 liters/min) maximal stroke volume (150 to 170 ml) and maximal arterio-venous oxygen difference (150 to 190 ml/liter) for subjects with a high maximal oxygen uptake (4.8 to 5.4 liters/min). In addition stroke volumes in the order of 150 to 170 ml have also been measured during submaximal work (Bevegård et al. 1963).

At a submaximal level athletes have a higher oxygen pulse than habitually sedentaries. This is caused to a high degree by a greater stroke volume. There has been some discussion as to whether the well-trained athlete's cardiac output for a given submaximal oxygen uptake is larger, the same or less than that for an untrained person (Bevegård et al. 1963, Hanson and Tabakin 1965). There has also been a lively debate on the size of the change in the athlete's stroke volume when shifting from rest to different degrees of exercise (Rushmer 1959, Bevegård 1962a). Musshof et al. could in 1959 report that the stroke volume increased relatively more in the case of athletes than in non-athletes, whereas

neither Rowell (1962) nor Bevegård et al. (1963) could find any difference between athletes and non-athletes in that respect.

b) Present investigation (study III)

Group 1 consisted of 7 very well-trained top athletes with a very high maximal oxygen uptake— in each case over 70 ml/kg per min. Group 2 comprised 5 athletes who were also well-trained having carried out regular training for many years, but never attained the high maximal oxygen uptake levels which were recorded for the top athletes in group 1.

During maximal exercise the top athletes in group 1 attained an average maximal oxygen uptake of 5.57 liters/min—corresponding to 73.9 ml/kg per min. The highest individual values were 6.24 liters/min and 81.1 ml/kg per min respectively. Average maximal heart rate was 192 beats/min. The cardiac output averaged 36.0 liters/min, the highest individual value being 42.3 liters/min. (Fig 3). The

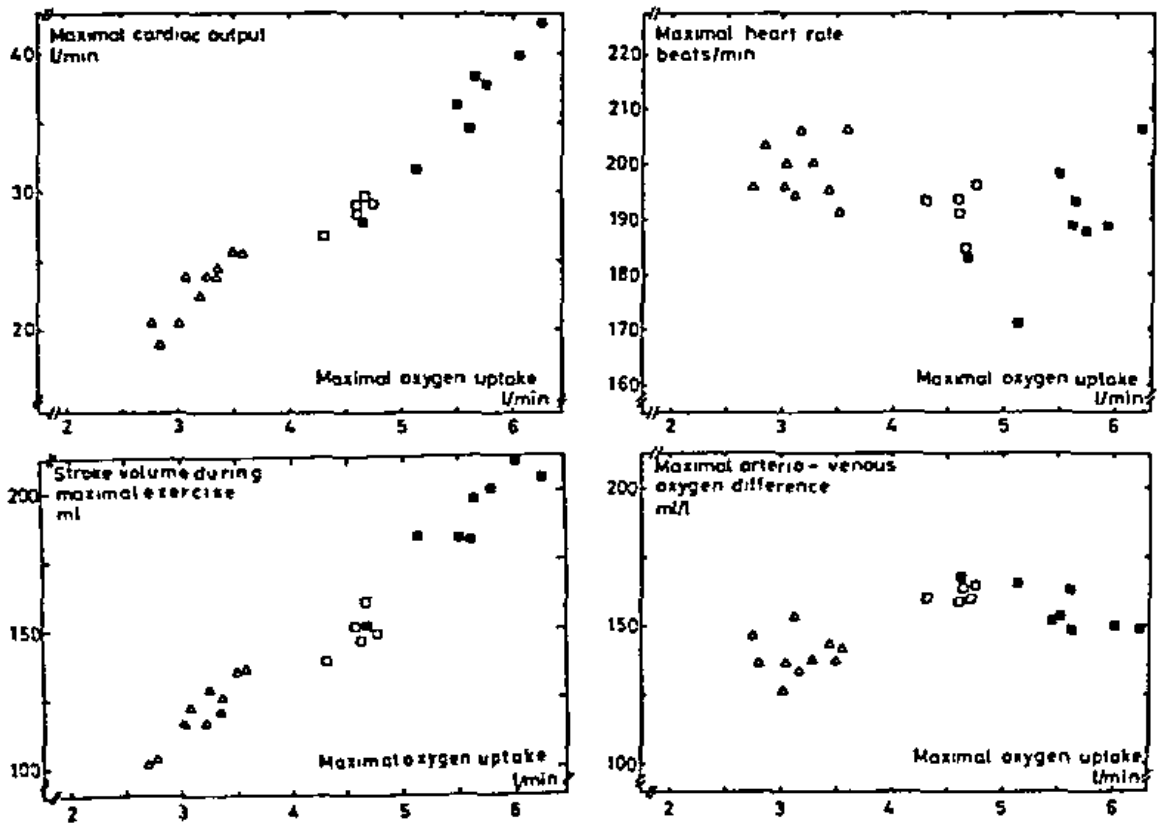


Fig. 3. Heart rate, cardiac output, arterio-venous oxygen difference and stroke volume during maximal exercise in relation to maximal oxygen uptake in top athletes from group 1 (filled squares), athletes from group 2 (unfilled squares) and sedentary from study II (unfilled triangles).

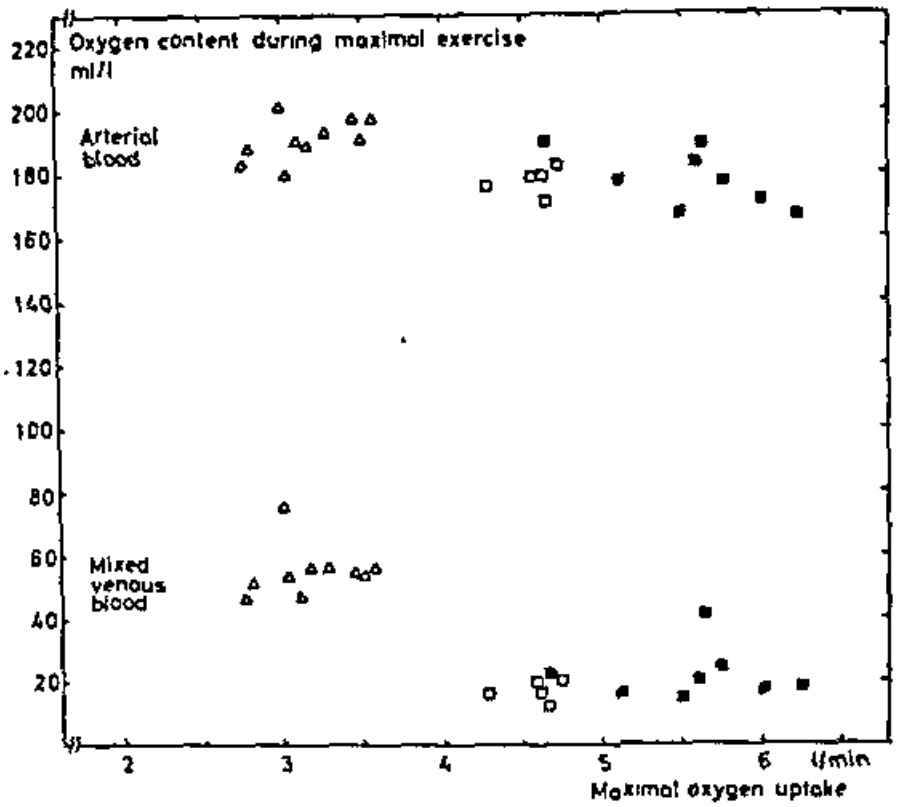


Fig. 4. Oxygen content in arterial blood (measured) and in mixed venous blood (calculated) in relation to oxygen uptake during maximal exercise. Symbols as in Fig 3.

stroke volume during work with the maximal load was on an average 189 ml, the highest individual values being 212, 205 and 201 ml respectively. Maximal arterio-venous oxygen difference was on an average 156 ml/liter. Since the oxygen content of the arterial blood during maximal exercise was on an average 181 ml/liter, the calculated oxygen content of the mixed venous blood was 25 ml/liter

In group 2 the mean maximal oxygen uptake during the time the cardiac output was measured was 4.58 liters/min—corresponding to 64.2 ml/kg per min —the mean maximal heart rate was 191 beats/min and the mean maximal cardiac output 28.4 liters/min with a highest value of 29.4 liters/min (Fig. 3). The mean stroke volume during exercise at maximal load was 149 ml and the mean maximal arterio-venous oxygen difference was 161 ml/liter. The calculated oxygen content of the mixed venous blood was then 20 ml/liter (Fig. 4).

The cardiac output for a given oxygen uptake was the same for the two groups of athletes. The equation for the regression line between cardiac output and oxygen uptake was: $\dot{Q}=4.1+6.09\ \dot{V}o_2$. The mean heart rate for a given submaximal oxygen uptake was lower for the top athletes in group 1 than for the athletes in group 2, which means that the stroke volume was greater for the former group.

The stroke volume of both groups rose as the work was stepwise increased from the lightest loads (c. 30 per cent of maximal oxygen uptake) to a load corre-

sponding to c. 70 per cent of maximal oxygen uptake, after which on the whole it was unchanged up to maximum. (In 9 out of 13 athletes maximal stroke volume was actually measured at the same time as the maximal cardiac output was recorded.)

Discussion

The high maximal oxygen uptake which was observed for the top athletes was made possible by a high cardiac output, whereas the maximal arterio-venous oxygen difference due to the low arterial oxygen content (low Hb concentration), was not greater than 156 ml/liter. The high maximal cardiac output was effected by a very high stroke volume which for most of the subjects was at maximal level even when exercising maximally.

Top athletes in group 1 were distinguished from athletes in group 2 by a higher maximal oxygen uptake. They had a greater cardiac output due to a greater stroke volume, since the maximal heart rate was the same for both groups (Fig. 3). The calculated oxygen content of the mixed venous blood during exercise at maximal load was roughly the same for both groups (Fig. 4). In comparison with habitually sedentaries from study II well-trained athletes have a higher maximal oxygen uptake, partly explained by a higher cardiac output (i.e. the stroke volume) and partly to a more complete extraction of oxygen from the arterial blood, resulting in a lower content of oxygen in the mixed venous blood. It should be pointed out that because of a relatively low hemoglobin concentration in athletes the arterio-venous oxygen difference is not necessarily higher among athletes than among sedentaries.

The body height and weight was 3 cm and 4 kg greater, respectively, for the elite athletes in group 1 than for athletes in group 2. However, this does not explain the differences found in heart volume and red cell volume between the two groups. (For discussion regarding dimensional differences between individuals of different body height, see Chapter III). A difference in the mentioned dimensions of c. 5 per cent could be expected between the groups on the basis of the differences in body height (Asmussen and Heeböll-Nielsen 1955).

The difference actually found in the vital capacity was about 6 per cent, thus corresponding to predicted value. The heart volume and the red cell volume differed, however, much more between the two groups and averaged 19 per cent and 14 per cent, respectively, which was higher than predicted. It should be borne in mind that the individual variation is wide and these static dimensions cannot, therefore, be used for prediction of maximal oxygen uptake. On the other hand, the large dimensions are a precondition for high maximal aerobic power.

CHAPTER 2

EFFECT OF PHYSICAL TRAINING ON CIRCULATION DURING SUBMAXIMAL AND MAXIMAL EXERCISE OF SHORT DURATION IN PREVIOUSLY UNTRAINED MALE ADULTS

a) Previous investigations

Christensen's classical studies in 1931 showed that when heavy muscular exercise was performed over a period of several weeks or months the heart rate decreased at a given load, indicating an improved cardiovascular function. The increase in the maximal oxygen uptake by training in fairly untrained individuals was, however, first demonstrated by Robinson and Harmon (1941) and by Knehr et al. (1942). It was not until about 1960 that an analysis was made of how the increased oxygen uptake in connection with maximal exercise came about. Rowell (1962) established that an increase in the maximal oxygen uptake from 3.47 liters/min to 3.93 liters/min (15 per cent) was a consequence equally of an increase in the cardiac output, where the stroke volume was the factor which increased, and a widening of the arterio-venous oxygen difference. A subsequent study by Saltin et al. (1968) where the maximal oxygen uptake increased up to 43 per cent after 55 days of training performed by 3 previously untrained subjects, agreed as a whole with the findings of Rowell's investigation.

Circulatory adaptation to submaximal work after training has been the subject of several investigations (for reference see Åstrand 1956). A reduced heart rate for a given submaximal load following training has been shown in many studies to depend on an improved mechanical efficiency (Gemmill et al. 1930, Robinson and Harmon 1941, Cotes and Meade 1959). Common to all these investigations has been that also the O_2 pulse has increased during training. It is an open question whether the cardiac output for a given submaximal oxygen uptake is modified by training and in this regard varying assumptions have been presented.

The training effect on the different static dimensions has varied from study to study. The roentgenological heart volume may increase with training, as also the blood volume and the total hemoglobin (Holmgren et al. 1960, Roskamm et el. 1966). Most observers agree (see Åstrand 1965) that the vital capacity remains unchanged in adults (for adolescents, see Chapter III).

b) Present investigation (study II)

In this investigation 8 subjects were studied before and after 16 weeks of physical training. The oxygen uptake, heart rate and cardiac output during sub-

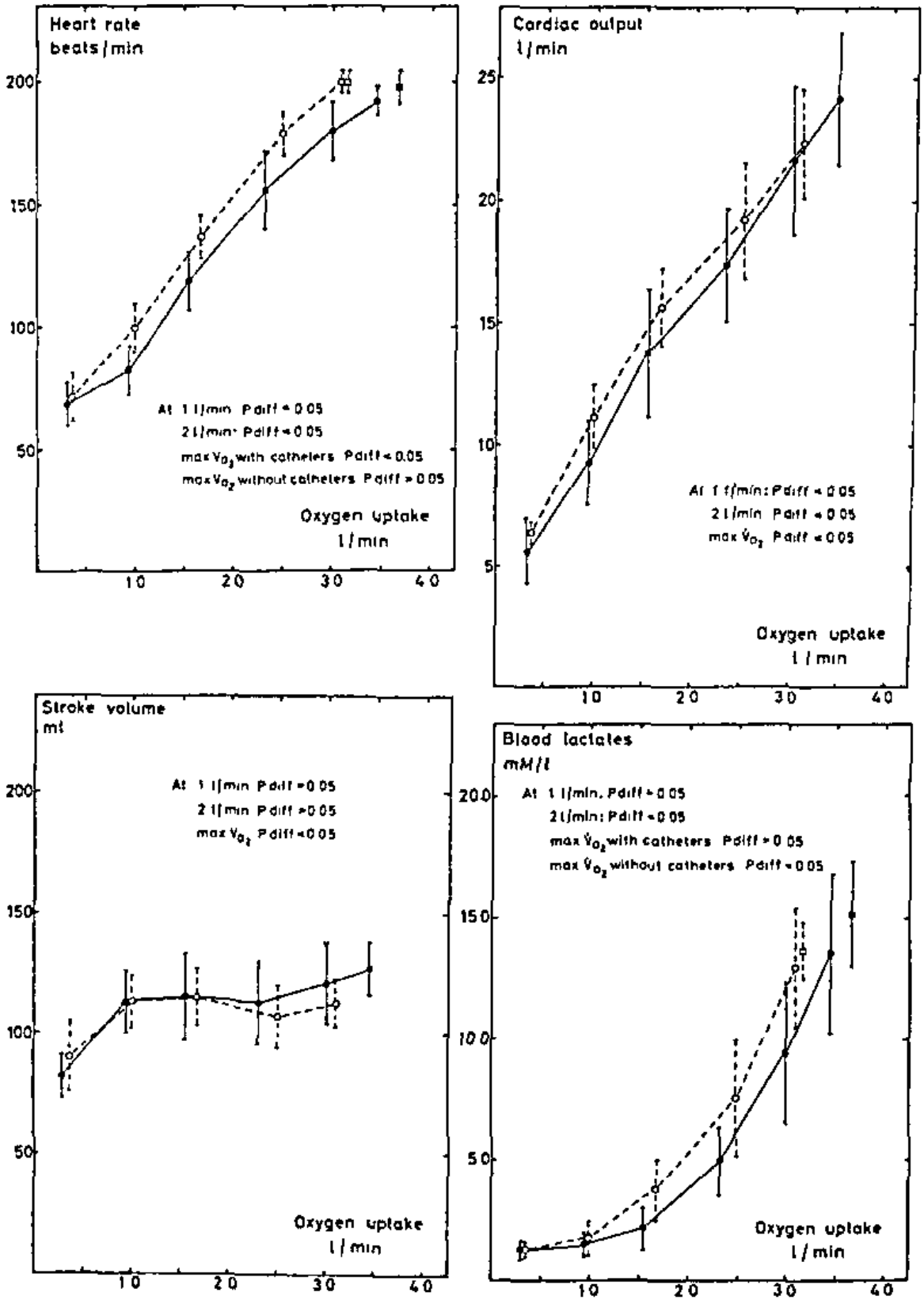


Fig. 5. Mean values (n=8) of heart rate, cardiac output, stroke volume and blood lactates in relation to oxygen uptake during submaximal and maximal exercise before (dotted line) and after (solid line) 16 weeks of training. Squares indiciate values obtained without catheters indwelled. *Pdiff* refers to the difference in ordinate values before and after training at a given abscissa value

maximal and maximal cycling were measured. For details, see study II. Some results are illustrated in Fig. 5.

The maximal oxygen uptake rose from 3.15 liters/min to 3.68 liters/min (16 per cent). The maximal heart rate and the heart volume remained unchanged during the training period. The greater maximal oxygen uptake was accomplished partly by a higher cardiac output—i.e. an increase from 22.4 liters/min to 24.2 liters/min, the stroke volume accounting entirely for the increase (from 112 ml to 127 ml)—and partly by a wider arterio-venous oxygen difference.

For a given submaximal load the heart rate was some 25 beats/min lower after training. This depended partly on an improved mechanical effiency ($p < 0.05$) and partly on a widened arterio-venous oxygen difference ($p < 0.05$) (hemoglobin concentration unchanged). The stroke volume did not change with training at the lower submaximal levels, but was somewhat higher after training at the highest submaximal levels.

The arterial blood pressure tended to be higher during submaximal exercise after training, but the differences were not significant. However, with maximal exercise the average intra-arterial diastolic, mean and systolic blood pressures were higher ($p < 0.05$) after the subject had trained.

The concentration of blood lactate was lower during exercise with a given oxygen uptake. At maximal work loads the blood lactate concentration was significantly higher, however, after training.

At regular intervals after that this study was finished (16 weeks) the subjects were re-examined (a complete report thereon will be published elsewhere). Two

TABLE 2. Circulatory data in subject LM and IS before and after short-term and long-term training.

Time for measurements	Heart Volume ml	Maximal Oxygen Uptake liters/min	Maximal Oxygen Uptake ml/kg per min	Heart Rate*	Cardiac Output liters/min	Stroke Volume ml	a-v O_2-diff. ml
LM							
Before	960	3 58	47 7	206	25.5	124	140
16 weeks	950	4.38	58.9	210	28 1	143	142
18 months	950	4 53	61.2	205	30.5	149	149
IS							
Before	920	3.07	45 8	205	23 9	122	126
16 weeks	940	3 87	55.4	205	26.2	134	131
32 months		4 36	63 6	185	27.6	151	158
51 months	1060	4.41	63.0	186	26.6	146	166

*Obtained in experiments without catheters .

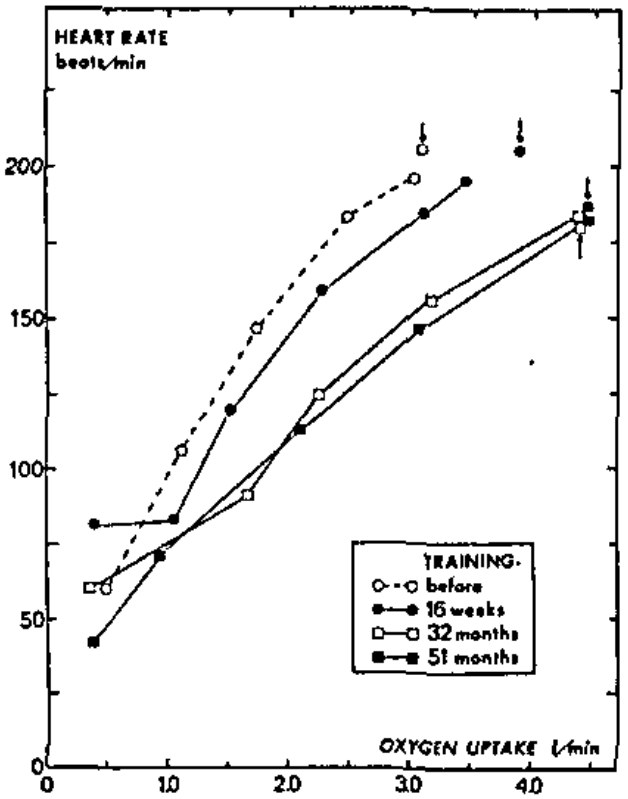


Fig. 6. Heart rate in relation to oxygen uptake in subject *IS* before training and after 16 weeks, 32 months and 51 months of training. Symbols with arrows refer to experiments without catheters inserted.

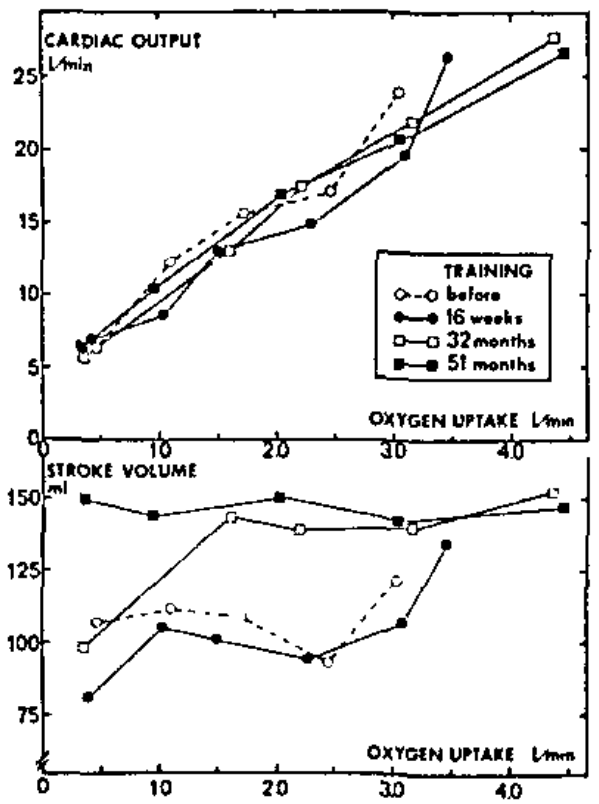


Fig. 7. Cardiac output and stroke volume in relation to oxygen uptake in subject *IS*. Symbols as in Fig. 6.

subjects, *LM* and *IS*, had continued with intensive training, and at certain periods they have trained up to 7 days a week. Some circulatory data obtained during submaximal and maximal work are given in TABLE 2 and Figs. 6, 7 and 8.

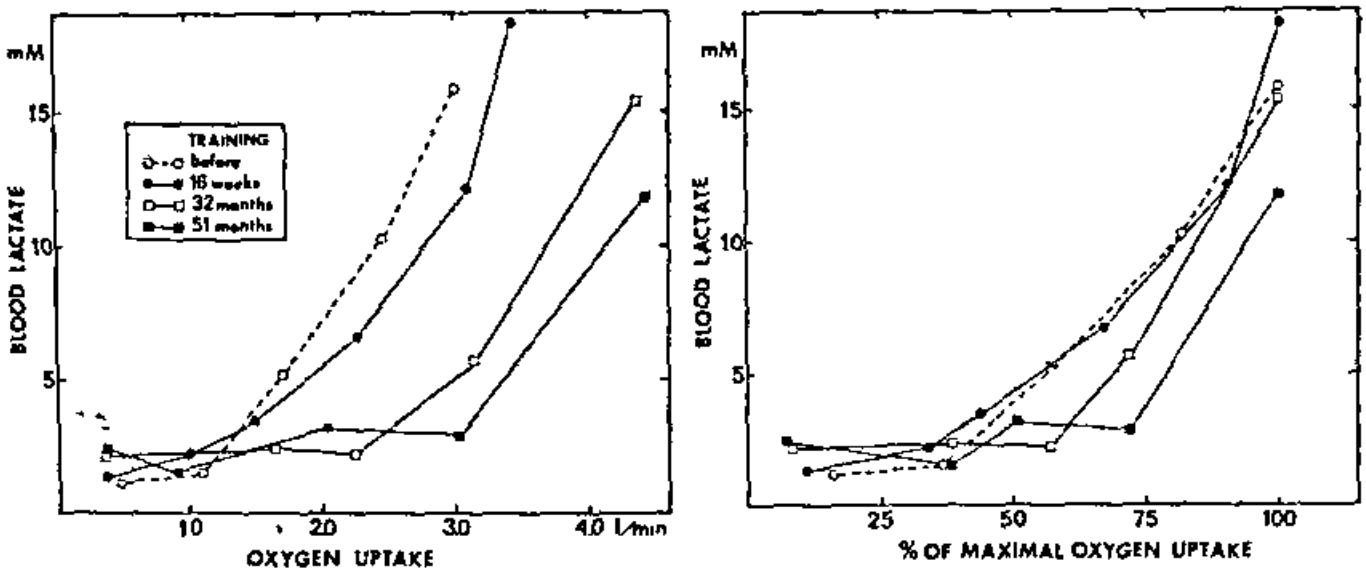


Fig. 8. Blood lactate concentration in relation to absolute values of oxygen uptake (left) and to oxygen uptake in per cent of maximal oxygen uptake (right) in subject *IS*. Symbols as in Fig. 6.

For subject IS the maximal oxygen uptake increased from 3.07 liters/min at the onset of the training to 4.41 liters/min after 51 months of training (total increase 44 per cent). This improvement was ascribable partly to an increase in the cardiac output from 23.9 liters/min to 26.6 liters/min (11 per cent) and partly to an increased extraction of oxygen from the blood (from 126 ml/liter to 166 ml/liter = 32 per cent). The maximal heart rate fell as training continued from 205 beats/min before training to 186 beats/min after 51 months of training. The greater maximal cardiac output was therefore accomplished by a rise in the stroke volume from 122 ml to 146 ml.

The heart rate at a given submaximal work load decreased for subject IS in accordance to the length of training (Fig. 6). As already mentioned the cardiac output for a given submaximal oxygen uptake was lower after 16 weeks of training. However, in the case of the following determinations, recorded after 32 and 51 months of training, respectively, the cardiac output at a given submaximal oxygen uptake was the same as it was before the training started. With an oxygen uptake of 2 liters/min the heart rate fell c. 40 beats/min, and thus the stroke volume correspondingly increased. The highest stroke volume measured during exercise rose from 122 ml before training to 150 ml after 51 months of training. At the same time the heart volume at rest rose from 920 ml prior to training to 1060 ml after 51 months of training.

The concentration of blood lactate when this person exercised at increasing loads is presented in Fig. 8. Both at an absolute and at a relative submaximal oxygen uptake level the concentration of blood lactate was lower after 51 months of training than it was before training commenced.

The mean blood pressure for a given submaximal oxygen uptake was higher after 16 weeks of training than before training started, but after 51 months of training the lower pre-training values were again obtained. However, at maximal exercise the mean blood pressure was higher, but also the cardiac output was as mentioned above higher.

Discussion

The increased capacity for oxygen transportation after 16 weeks of training was partly due to an elevated stroke volume (from 112 ml to 127 ml—or 13 per cent), giving a higher cardiac output during maximal exercise, and partly to a more complete extraction of oxygen from the blood. The reduced blood lactate at a given submaximal load may be taken as a criterion of a more effective regulation of the muscle blood flow. More pronounced vasoconstriction in non-active tissues may explain the rise noted in blood pressure.

As for the long-term physical training, (more than ½ year) which brought to light an asymptotic elevation of the maximal oxygen uptake, the mechanism for the increased capacity for oxygen transportation was the same as for the short-term training. *In both subjects (LM and IS) there was a further increase in stroke* volume, giving a higher maximal cardiac output, and a further widening of the arterio-venous oxygen difference.

The increase in blood lactate concentration for a given submaximal oxygen uptake became less pronounced throughout the training period. A similar change at a relative load was obviously manifested somewhat later. In other words the reduction in blood lactate concentration at a relative oxygen uptake (e.g. 75 per cent of maximal) did not manifest itself after 16 weeks of training, in spite of the fact that maximal oxygen uptake rose about 25 per cent (subject *LM* and *IS*).

EFFECT OF PHYSICAL TRAINING ON CIRCULATION DURING PROLONGED SEVERE EXERCISE IN PREVIOUSLY UNTRAINED MALE ADULTS

a) Previous investigations

Little knowledge is at hand on the effect of physical training on circulation during prolonged hard muscular exercise for more than 30 min. It is a recognized fact (Christensen 1931, Astrand 1960, Saltin and Stenberg 1964, Ekelund and Holmgren 1964) that the heart rate progressively increases during prolonged hard muscular exercise in sedentary as well as in well-trained individuals. Concomitantly stroke volume declines, giving an unchanged cardiac output throughout the work period. This decrease in stroke volume which may be interpreted as a consequence of impaired cardiac function was noticed even during supine work (Saltin and Stenberg 1964, Ekelund 1966).

b) Present investigation (study V)

Seven sedentary subjects were studied before and after 22 weeks of physical training. They cycled for one hour with a load demanding an oxygen uptake of 75 per cent of the individual maximal oxygen uptake. This standard test was performed both before and after the training. The heart rate, oxygen uptake and cardiac output were measured. For details see study V.

In experiments without catheters indwelled the maximal oxygen uptake rose from 3.41 liters/min prior to training to 3.64 liters/min subsequent to training. The maximal heart rate fell from 201 beats/min to 194 beats/min ($p < 0.05$) during this period. The heart volume remained unchanged.

The actual work load in the pre-training test was in average 1010 kpm/min, and in the post-training test 1170 kpm/min. The demand was in both cases 75 per cent of the maximal aerobic power. The explanation to the different work outputs was partly the higher maximal oxygen uptake attained after training and partly a gradually improved mechanical efficiency.

During the early phase of exercise (the 5 and 15 min values) the heart rate was the same prior to and following training. The cardiac output after training was 2.6 liters/min ($p < 0.05$) greater, while the oxygen uptake was 0.1 liters/min higher. The arterio-venous oxygen difference was 127 ml/liter prior to training and 123 ml/liter ($p > 0.05$) afterwards. The stroke volume was significantly larger after training than before—namely 130 and 115 ml, respectively.

From the 5th min up to the 60th min the heart rate gradually increased, but this elevation was equally high prior to and after the training. During this period of time the cardiac output remained virtually unchanged both in the pre-training and the post-training experiment, and therefore the stroke volume fell. Before training this drop was from 116 ml to 111 ml and after training from 132 ml to 123 ml. Mean blood pressure measured at the 5th min was higher after training ($p < 0.05$) and it dropped gradually during exercise both prior to training and afterwards.

The blood lactate content was unchanged prior to training from the 5th min onwards through the entire one hour of exercise (c. 6.8 mM), whereas after training it reached at the 5th min the same value as before training but thereafter it fell successively.

Discussion

The effect of the physical training on the maximal oxygen uptake in this group of subjects was fairly low (c. 8 per cent increase). However, considering that the pre-training mean value was rather high (48 ml/kg per min), the improvement is within the "normal" limits for the increase in maximal aerobic power after short-term physical training in previously untrained young male adults (see Fig. 12).

The combined effect of improved mechanical efficiency and increased maximal oxygen uptake resulted in a much higher work output during the one hour test after training. The total amount of kilopondmeters, that could be performed during the 1 hour test at the same relative load was thus increased from 60,100 kpm before to 70,000 kpm after the training (16 per cent difference). In addition, many subjects spontaneously stated that the prolonged exercise was subjectively felt much easier after training than beforehand. This may partly be explained by an improved local circulation, since the blood lactate content, which prior to training was unchanged throughout the period of exercise after training fell successively during the course of exercise after the initial elevation.

The oxygen pulse was higher after as compared with before the training, depending only on an increased stroke volume. This did not, however, change the circulatory pattern during the prolonged severe exercise, since heart rate rose and stroke volume decreased equally much in the two tests. Thus, whatever the causes may be for the gradual elevation of heart rate and reduction in stroke volume during prolonged exercise, these causes were not affected by training for 22 weeks.

CHAPTER 4

EFFECT OF PHYSICAL TRAINING ON OXYGEN TRANSPORT SYSTEM OF ADOLESCENTS

a) Previous investigations

Relatively little information is available concerning the effect of physical training in individuals in puberty. In the cross-sectional study by Åstrand et al. (1963) on Swedish girl swimmers in the age groups 10—17 years it was definitely shown that various dynamic functions (e.g. maximal oxygen uptake) and static dimensions (e.g. heart volume, vital capacity) in the oxygen transport chain were greater among girl swimmers than among moderately active girls of a corresponding age. Nevertheless, it is impossible on the basis of these results to isolate the effect of training from the effect of constitutional factors.

Few longitudinal studies exist which deal with the effect of training on the organism in adolescence. One complication in this type of studies is to establish a control group of untrained subjects. In most schools a certain degree of physical activity is compulsory and furthermore, young boys and girls are spontaneously very active in leisure hours.

Sprynarova (personal communication) measured maximal oxygen uptake in 90 Czech boys who were 11 years of age, and studied them at intervals for 4—5 years. She noted that the increase in maximal oxygen uptake became more pronounced in the most active boys, when comparing with the ones who were least active. However, no difference in the development of the body dimensions were revealed between the most active and least active of the boys (Pariskova 1967). On the other hand Schwartz et al. (1928) as reviewed by Schneider (1939) studied 68 boys in puberty who were undergoing 4 months of special training. These boys demonstrated a greater increase in body height (significant) and vital capacity (not significant) than a control group of 50 boys of the same age, who were not in special training. For other physiological changes during adolescent growth the reader is referred to the fundamental works of Tanner (1955).

b) Present investigation (study IV)

The present investigation was carried out in order to study the effect of prolonged strenuous physical training during puberty. The maximal oxygen uptake, heart volume and vital capacity of 6 boys—all of them 11 years of age at the start of the investigation—were measured at the onset (T1) and after 6 months of training (T2) and in 5 of these boys again after a further 26 months of

training (T3). A parallel study was made of a "control group" which did not participate in special training. After 6 months of training maximal oxygen uptake had risen from 2.15 liters/min to 2.48 liters/min (15 per cent) for the group in training. The values of the control group were unchanged. The increase in body weight, heart volume and vital capacity during this time was the same in the two groups.

After 32 months of training the maximal oxygen uptake of the training group (5 subjects) rose from 2.22 liters/min to 3.45 liters/min (55 per cent) which corresponded to an increase from 54.3 ml/kg per min to 58.1 ml/kg per min. Moreover the heart volume rose by 43 per cent and vital capacity by 54 per cent. In the control group (4 subjects) the maximal oxygen uptake increased over the

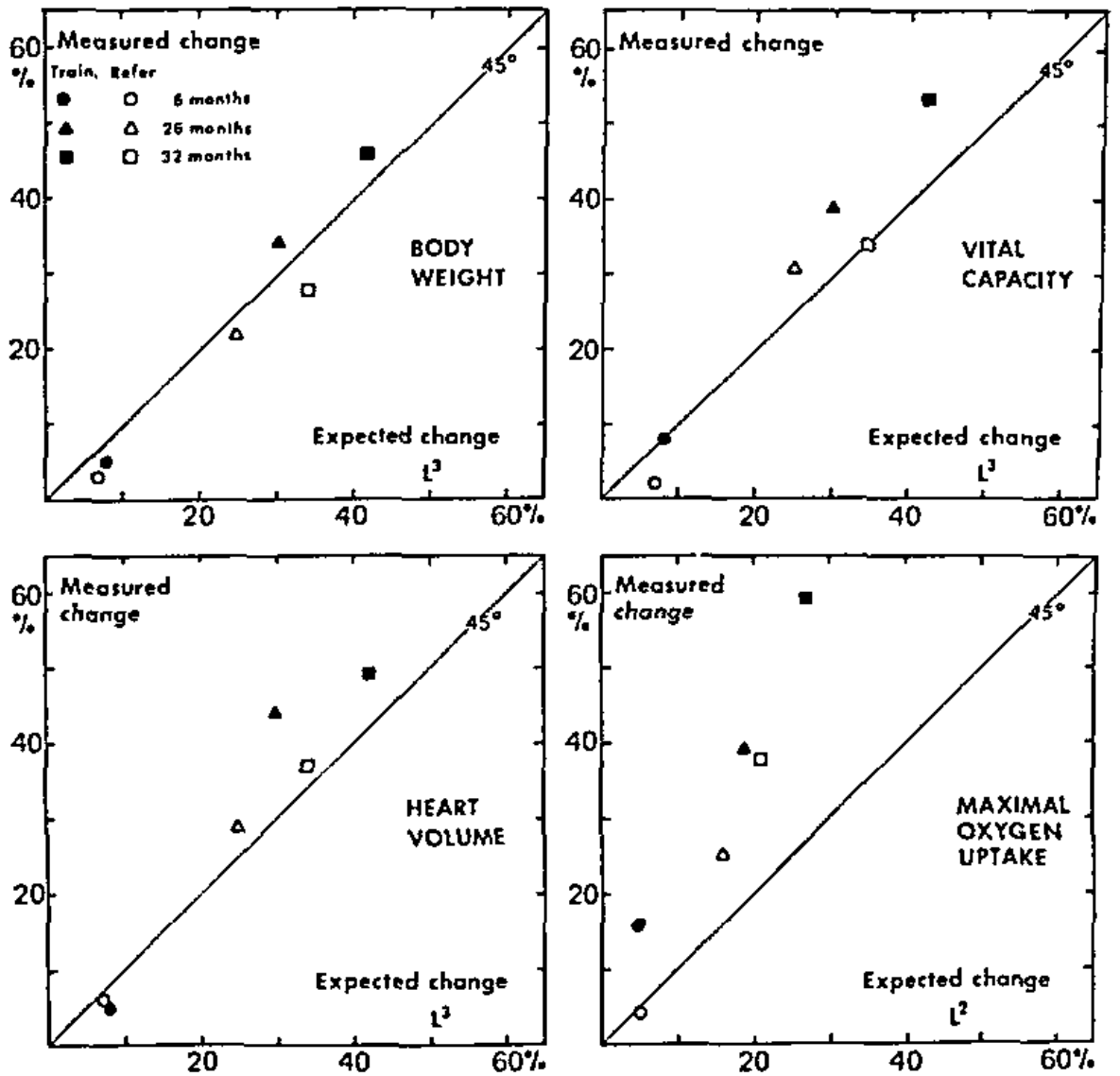


Fig. 9. Group means of measured change in body weight, vital capacity, heart volume and maximal oxygen uptake after 6, 26 and 32 months observation compared with corresponding expected change, as predicted from the relation body height after to body height before, raised to 2.0 (L^2) and 3.0 (L^3).

corresponding period from 1.85 liter/min to 2.54 liter/min (37 per cent), corresponding to 50.3 ml/kg per min and 53.9 ml/kg per min, respectively, while the heart volume rose by 36 per cent and vital capacity by 34 per cent. For four of the training subjects (*AH, HN, JA, LAT*) an increase in growth rate was noted, whereas in the control group none of the subjects exhibited such an accelerated rate of growth (Fig. 11).

Discussion

In order to evaluate the effect of physical training on the oxygen transport system of adults data obtained prior to training can be directly compared with similar data taken after a period of training. With adolescent subjects it is not possible to use this direct method of comparison, since in the case of these individuals—apart from physical training—an age-dependent growth of different static and dynamic functions must be taken into consideration. Neither—in view of individual variations etc.—can one merely make a comparison between the training group and the control group as regards differences in the increase of the parameters which have been selected.

However, theoretically it can be expected that with a specific development in body height certain volumes, such as heart volume and vital capacity should increase in line with the third power of this growth (Asmussen and Heeböll-Nielsen 1955). In this way it is possible to establish a predictable growth for various static and functional dimensions, such as heart volume, vital capacity etc. If there is a descrepancy between the predicted and measured increase, other aspects than purely geometric factors are involved. It will be seen from Fig. 9 that the predicted and measured increase for heart volume and vital capacity agrees in respect of the control group and as regards body weight for both groups. In other measurements the increases noted are greater than expected. As for the heart volume and vital capacity of the training group, the physical training is the probable reason for this difference.

In regard to the maximal oxygen uptake a sharper increase than foreseen occurred in both groups. Von Döbeln (1956) has earlier demonstrated that the theoretically expected difference in maximal oxygen uptake between individuals of different body height should be related to the ratio between the body heights raised to 2.0. However, if this theory is applied on Astrand's investigation on Swedish school children (1952) it will be found that the difference between the divergent ages as regards maximal oxygen uptake corresponded to the difference in body height, raised to 3.3. Thus, other factors than geometrical aspects, such as the effect of hormones, for instance, have played a part in the development of the maximal oxygen uptake. This observation has been confirmed in this longitudinal study. Training has further accentuated the difference between the predictable and attained value.

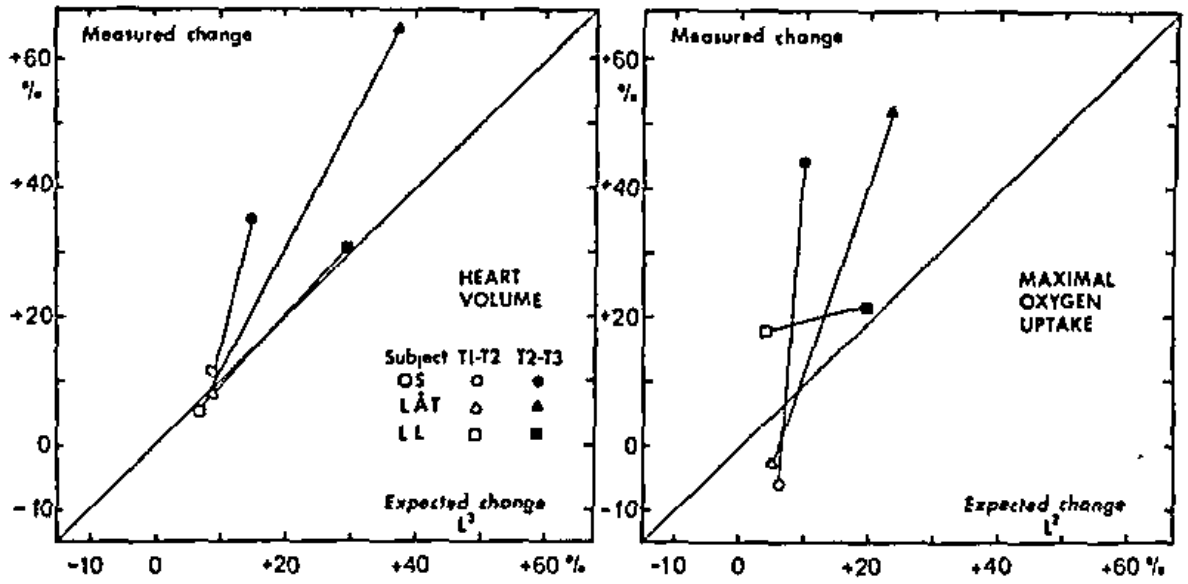


Fig. 10. Measured change in heart volume and maximal oxygen uptake in relation to corresponding expected change, predicted as in Fig. 9, between T1 and T2 (6 months) and T2 and T3 (26 months). Subject *LL* stopped and subject *OS* and *LÅT* started training after T2.

Subject LL from the original training group withdraw from the training after 6 months (at T2). Subject *OS* and *LÅT* from the original reference group started training at T2. *Fig. 10* presents the measured and expected increase in maximal oxygen uptake and heart volume at T2 and T3 for these 3 subjects. During the first 6 months of this study the maximal oxygen uptake in subject *LL* improved about 18 per cent. The heart volume remained on the whole unchanged. During the period between T2 and T3, when he was not in special training, the maximal oxygen uptake increased far less than expected. So when comparing the T1 and T3 values in this subject the measured increase in maximal oxygen uptake (and also heart volume) was identical with an expected value. On the other hand in subject *OS* and *LÅT* maximal oxygen uptake and heart *volume were on the whole unchanged during the first 6 months* (T1 and T2), when they were not in special training, whereas during the hard physical training *during the following 26 months (T2 to T3) their dimensions increased far more* than expected.

For the explanation of all these findings, some recent studies might be of interest. Goldberg and Goodman (1969) have shown that both growth hormone and physical work can induce growth of muscular mass. Concomitently, Sutton (1969) found an elevated concentration of growth hormone in blood, following severe physical exercise. It is likely, therefore, that the rate of growth can be accelerated by physical training during puberty—a period when the individual is evidently sensitive to the action of the growth hormone. This would mean that the *individual would grow more rapidly during a period of physical training than*

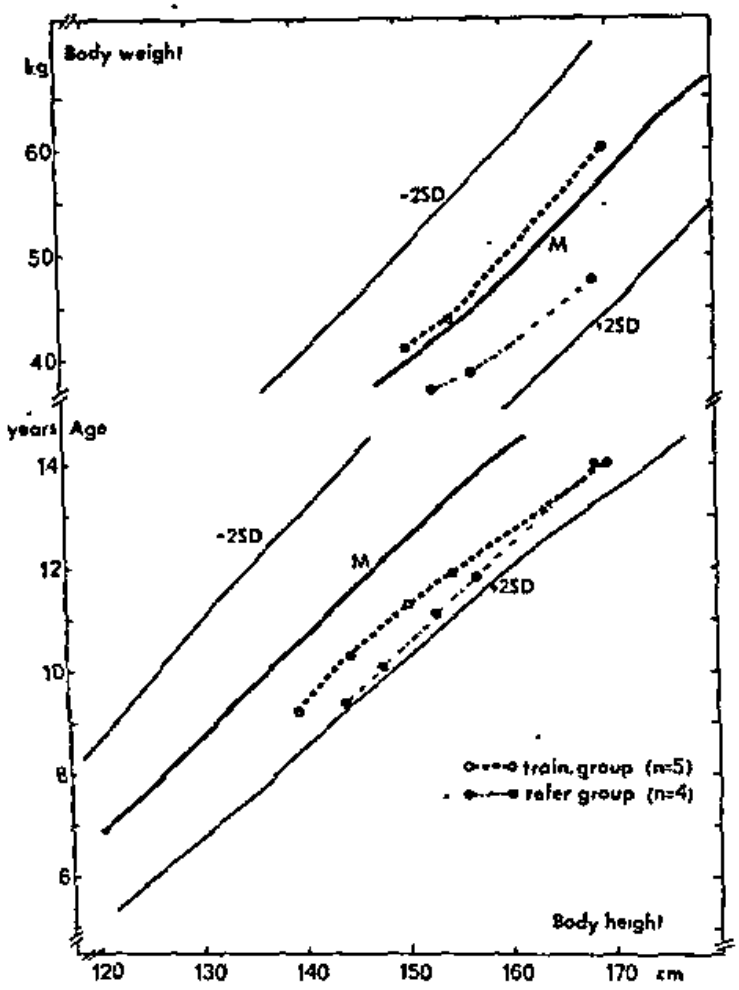


Fig. 11. Group means for body weight and body height for the 5 subjects, who have trained for 32 months (from 11 years) and corresponding non-training reference group of 4 subjects in a Karlberg-Iggbom growth chart (1959).

could be predicted from the body height development as estimated from growth charts. In other words, the individual would deviate from his pre-training age/height percentile in the growth chart, and would instead come into a "higher" percentile grade. This was the case in the present study, as seen in Fig. 11. At 11 years (T1) the body height of the "2 months" training group differed in average + 1.1 SD from the mean value of the diagram but at 14 years (T3) it differed by + 1.6 SD. These data also confirm the earlier study by Schwartz et al. (1928) in which physically active boys increased more in body height than controls.

General discussion

The effect of training on the oxygen transport system of the previously untrained adult can probably be enhanced in different ways at different phases of training. The results of the present as well as of earlier reported studies on training provide the background for the following hypothesis. 1) During the first phase of training a rapid increase in maximal oxygen uptake occurs. This is presumably the outcome of a changed circulatory regulation without any noticable difference in static dimensions. The individual can make better use of existing dimensions. During this first phase an improvement in technique or in mechanical efficiency may also play an important role for the higher work capacity after training; the individual makes better use of the available oxygen. 2) Thereafter, when training has progressed for half a year or so changes in static dimensions, for instance the heart and red cell volume, can occur, but experience points to the fact that these increases in static dimensions are—if they occur at all—relatively moderate. 3) Continued long-term hard training for years does not seem to increase the maximal oxygen uptake any further, whereas on the other hand the heart rate at fixed submaximal loads can continue to decline—indicating an additional improvement in the effectiveness of the oxygen transport system.

The greater maximal oxygen uptake after a short period of training (studies II and V) was brought about by an increase of the maximal cardiac output and a widened maximal arterio-venous oxygen difference. Since the maximal heart rate after training was unchanged (study II) or lower (study V) the entire increase in cardiac output was accounted for by a greater stroke volume. Hence the higher maximal oxygen pulse depended on an increase of both the stroke volume and of the arterio-venous oxygen difference.

In studies II and V the stroke volume increased without any observed change in the heart volume. This may indicate that after training the heart functions with less systolic residual blood volume perhaps because of a greater contractive power. Furthermore, the higher maximal arterio-venous oxygen difference after training indicates a more efficient distribution of the oxygen available in the arterial blood. In connection with long-term training ($> \frac{1}{2}$ year), it clearly emerges that the further increase in maximal oxygen uptake is accomplished by a further elevation of both the stroke volume and the arterio-venous oxygen difference.

It is an interesting observation that in subject *IS* from study II the heart volume increased, and at the same time the maximal heart rate decreased after long-term training. The question arises whether his reduction of the maximal heart rate

from 205 beats/min at the start of training to 186 beats/min after 51 months of training, can be explained by the change in heart volume.

In geometrically similar and qualitatively identical individuals all frequences (like maximal heart rate) should be expected to be inversely proportional to the length scale (von Döbeln 1956) and alla areas and volumes to be proportional to the second third power, respectively, of the length scale (Asmussen and Heeböll-Nielsen 1955). An increased heart volume can be regarded either as A) uniform, i.e. muscle cross-section area and vessel widths increases in proportion to the second and heart volume to the third power of the length scale, or as B) not uniform i.e. caused only by an increased heart blood quantity without changes in the muscle cross-section area and vessel widhts. Regarding these two marginal explanations to an increased heart volume, the maximal heart rate after 51 months training should be expected to be, according to A) $205 \times \sqrt[3]{\frac{920}{1060}} = 196$ beats/min and according to B) $205 \times \frac{920}{1060} = 178$ beats/min. This would signify that the observed change in the maximal heart rate in connection with subject *IS*'s long-term training does not require any other explanation than the change in heart volume.

In a cross-sectional investigation Holmgren and Åstrand (1966) found that the correlation between maximal heart rate and heart volume was low ($r = -0.488$) but it was significant. On the other hand, the maximal heart rate as noted for the athletes in study III, averaged about 190 beats/min, which is not significantly different from the maximal heart rate of untrained persons of the same age, although the heart volume is far greater.

Subject *IS* is still training regularly, but it is not likely that he can increase his maximal oxygen uptake up to the values which are reported for top athletes. In the case of individuals who have trained for many years, hard and intensive training does not seem to increase the maximal oxygen uptake any further. For exemple, the well-trained athletes in group 2 in study III, have not attained maximal oxygen uptake levels comparable with those manifested by top athletes. Saltin and Astrand (1967) also reported that LL, a skier who from 1955 to 1963 trained intensively and participated in World Championships, Olympic Games etc. did only raise his maximal oxygen uptake from 5.49 liters/min to 5.60 liters/min. The present writer has trained intensively for "orienteering" since 1955 (Swedish champion 1964). In 1960 the value noted for maximal oxygen uptake was 4.91 liters/min and for maximal heart rate 191 beats/min. Although severe "athletic" training (see METHODS) has been carried out 5 times a week since then, the maximal oxygen uptake and maximal heart rate have remained unchanged up to 1968. Regular measurements of the heart volume during this time have

shown variations from the mean value on the order of ±5 per cent. However, the heart rate noted at submaximal exercise has fallen from c. 155 beats/min at 1500 kpm/min (c. 250 watts) in 1960 to 130 beats/min at the same load in 1968. Corresponding observations were made on subject *AH*, see below. This decreasee in heart rate at a given submaximal work load, without a corresponding increase in maximal oxygen uptake or heart volume might be caused by an increased stroke volume.

Subject *AH* mentioned in study III, who was a top athlete in cross-country skiing and long distance running, is an interesting case in respect of the effect of prolonged hard training. Cardiac output was measured twice with a 5-year interval. Although he continued to train very hard during this time, the maximal oxygen uptake did not change, whereas the maximal cardiac output had risen from 28.8 liters/min (1962) to 34.4 liters/min (1967). Concurrently the arterial oxygen content during maximal exercise was—because of a low Hb-concentration —lower in 1967 than in 1962, a circumstance which provides an explanation for the lower maximal arterio-venous oxygen difference in 1967 and for an unchanged maximal oxygen uptake. The maximal heart rate was 8 beats/min lower in 1967 than in 1962, which means that the stroke volume during maximal work was about 50 ml higher in 1967 than in 1962. It is remarkable that at the same time the heart volume was unchanged. It can not be excluded that part of the explanation for the higher stroke volume in the 1967-study was the lower hemoglobin concentration and thus lower blood viscosity. This was also the case for the majority of the well-trained athletes, who have maximal stroke volumes greater than would be anticipated on the basis of the heart volume, as judged from data on sedentary and moderately active individuals.

Many extremely well-trained athletes have a low Hb-concentration (Grimby and Saltin 1966, study III). The cause for this is not known. Is it due to a physiological adjustment to hard physical training, or does it arise from an increased breaking down of red blood cells during training? Anyhow it should be observed that a reduction of the Hb-concentration in connection with physical training may not be an indication of iron deficiency.

The circumstance that there are possibly different phases in the changes of maximal oxygen uptake by training can be the explanation for the conflicting results which are reported in various studies on training.

When evaluating the physiological effects of training one must concider differences in the duration and intensity of the training, the subject's physical condition before training started, etc. and also the possible existence of considerable individual disparities in the response to a given amount of physical training, (see for example subject *JA* compared with the other subjects in study IV). Some studies of training may have recorded changes during the regulatory stage i.e. phase 1 (Holmgren et al. 1960, study II and V) and others with changes between

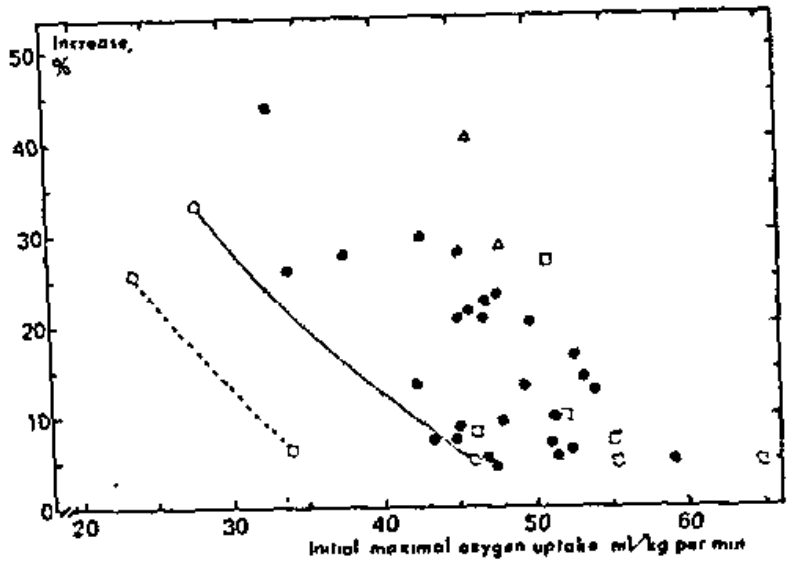


Fig. 12. The per cent increase in maximal oxygen uptake after short-term training (except triangles, see below) in relation to the pre-training maximal oxygen uptake in previously untrained individuals. Unfilled squares refer to adoloescent boys (study IV) and filled circles to young males (Rowell 1962, Saltin et. el. 1968, study II and V). Solid and dotted line refer to the mean value for middle-aged (35 to 50 years) and old (50 to 63 years) men, respectively, (Saltin et al. 1969). Triangles refer to the increase in maximal oxygen uptake in subject *LM* and *IS* from study II after 18 and 51 months of training, respectively.

phase 1 and phase 2 (Saltin et al. 1968). Douglas and Bachlake (1968) have studied the effect of training on individuals who had already undergone thorough training and who had probably reached the most advanced stage (phase 3) when the observations were conducted.

The magnitude of the increase in maximal oxygen uptake depends upon many factors, and some of interest are illustrated in Fig. 12. This diagram clearly illustrates that the expectation of an increase in maximal oxygen uptake after a short period of training (less than half a year) is greatest for subjects with the lowest level of pretraining maximal oxygen uptake. At a given level of pretraining maximal oxygen uptake the improvement seems less pronounced for individuals of 35 to 50 years and 50—63 years of age than for men of 20 to 30 years of age. Where adolescents are involved their improvements lie at the upper limit for adult men of 20 to 30 years of age. The importance of the duration of training is revealed by the data on subjects *LM* and *IS* from study II, whose maximal oxygen uptake increased far more than that of any person with approximately the same pretraining maximal aerobic power, who participated in short-term training.

Athletes represent a selected group of individuals with very special preconditions for outstanding physical performances. In addition they have trained hard for many years. These athletes provide insight on the upper performance limit for the human circulation. The differences in the maximal oxygen uptake between athletes and habitually sedentaries are connected with disparities partly in the stroke volume and partly in the arterio-venous oxygen difference. Differences between athletes in endurence events with different maximal aerobic power depend mainly in the magnitude of the stroke volume, since during maximal work

the oxygen content in the mixed venous blood for almost all had the lowest conceivable value taking into account the necessary circulation in the inactive organs.

After puberty the possibility to increase the maximal oxygen uptake by means of developing the static dimensions seems to be limited. The training of the adolescent boys gave data which tends to support the assumption that long-term—as opposed to short-term—training results in a more pronounced development of *certain dimensions, such as heart volume and vital capacity, than would have been* expected from the standpoint of age-dependent increase. On the basis of these observations it may be concluded that rational training during adolescent growth would yield the best results. Thereafter a previously sedentary person may well have forfeited his chance of developing his dimensions to a higher degree.

In all the longitudinal studies the common pattern for circulatory response to submaximal work levels has been that the heart rate and the blood lactate concentration *at a given submaximal load has been lower after training than before* In some cases the lower heart rate has been brought about by the improved *mechanical efficiency and consequently a lower oxygen uptake (studies II, IV* and V) but for almost all subjects a higher oxygen pulse has been a common finding. This might be wholly or partly attributable to a higher stroke volume (studies II and V) since the arterio-venous oxygen difference did not change regularly. However, after the long-term training (see Chapter 2) where a reduction in heart rate of 30—40 beats/min was recorded for a given submaximal oxygen uptake the higher oxygen pulse depended solely on the raised stroke *volume, whereas the cardiac output was at the same level as at the pretraining* stage. When comparing athletes (study III) with sedentaries (study II) the stroke volume for a given submaximal oxygen uptake was definitely higher for athletes, whereas the arterio-venous oxygen difference was on a whole similar for both groups.

The above mentioned changes in heart function following training raises the question, whether the reduction of heart rate or the increase in stroke volume is *the primary factor. This is not known. However, if the heart rate at a given* oxygen uptake is increased by using a pacemaker (Bevegård 1962) or at atrial flutter (Åstrand et al 1963), the stroke volume is correspondingly decreased so that the cardiac output is unchanged. Thus, in such cases stroke volume is secondary to heart rate. Whether the low heart rate and thus high stroke volume, characteristic for the trained state mainly depend on in improved myocardial function or on a reduced sympathetic and/or increased parasympathetic effect on *the myocardium, is still unsettled.*

The above mentioned changes in the circulatory adaptation to submaximal exercise indicate a better adjustment after training, i.e. the changes in the body's homeostasis between rest and work are less pronounced. Theses changes are

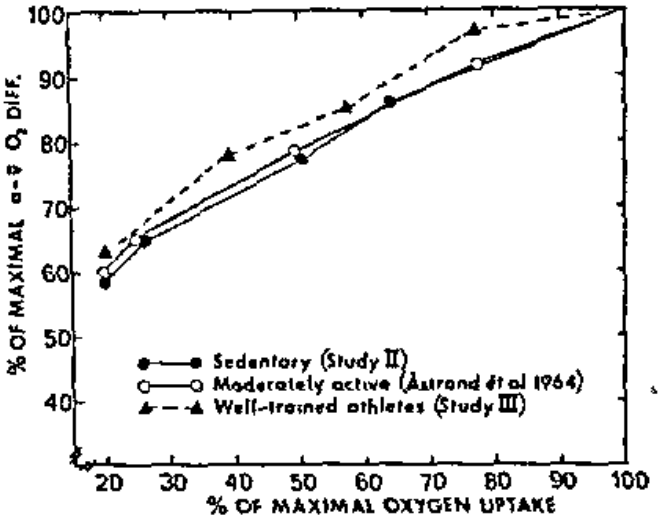


Fig. 13. Mean values of arterio-venous oxygen difference in per cent of maximal in relation to oxygen uptake in per cent of maximal in three groups of subjects.

possible to bring about by an improved peripheral blood distribution within a relatively short span of time. The developments which would occur over a somewhat longer time might be increased muscular capillary density (Petrén et al. 1936), greater concentration in the muscular cells of ATP and creatine phosphate (Yakovlev 1958), myoglobin (Wipple 1926) and mitochondria activtivy (Holloszy 1967).

From the neurophysiological standpoint it has been postulated that with a gradually increasing muscular contraction emanating from a certain motor pool, first the "red" ("aerobic") muscle fibres are brought into action and then, as the load becomes heavier, also the "white" ("anaerobic") fibres (Romanul 1964, Dawson and Romanul 1964, Kugelberg and Edström 1968). Hence it could be assumed that after training the muscle involved in order to perform a given submaximal load could be distributed on a larger motor pool, and that the relative share of the exertion borne by the "red" muscle fibers is greater after training than before.

An assumption along these lines does also provide part of the explanation for the fact that during extended (long-term) hard physical training the blood lactate concentration declines not only for a given absolute but also for a given relative oxygen uptake (see Fig. 8). Furthermore, if sedentary (study II) and moderately active (Åstrand et al. 1964) individuals are compared with the well-trained athletes (study III) it will be found, that athletes have a higher relative arteriovenous oxygen difference at a given relative oxygen uptake (see Fig. 13). .However, since the arterial oxygen content may vary highly (see study III), an even better variable, reflecting the efficiency of the peripheral circulation may be the oxygen content of the mixed venous blood. As seen in Fig. 14, well trained athletes have a definitely lower oxygen content in the mixed venous blood at a given load that non-athletes. It should be observed that a well-trained athlete and a cardiac patient may have the same low oxygen content in the mixed venous

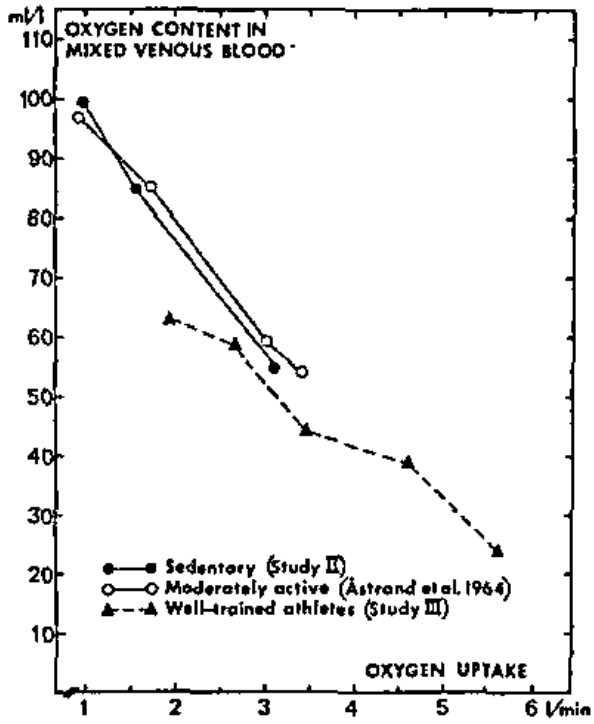


Fig. 14. Mean values of calculated oxygen content in mixed venous blood in relation to oxygen uptake during subamaximal and maximal exercise. Same subjects as in Fig. 13.

blood at the same submaximal oxygen uptake, but the causes for this are different. As for the athlete it depends on an effective distribution of offered oxygen from the heart, while the circulation in the cardiac patient may be hypokinetic due to some myocardial or other reason.

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Stockholm in March 1969.

BJÖRN EKBLOM

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ACTA PHYSIOLOGICA SCANDINAVICA
SUPPLEMENTUM 329

ELECTRON MICROSCOPIC AND HISTOCHEMICAL OBSERVATIONS ON THE SUPRAOPTIC NUCLEUS OF NORMAL AND DEHYDRATED RATS

BY
LEENA RECHARDT

HELSINKI 1969

ACTA PHYSIOLOGICA SCANDINAVICA
SUPPLEMENTUM 329

FROM THE DEPARTMENT OF ANATOMY, UNIVERSITY OF HELSINKI
SILTAVUORENPENGER, HELSINKI, FINLAND

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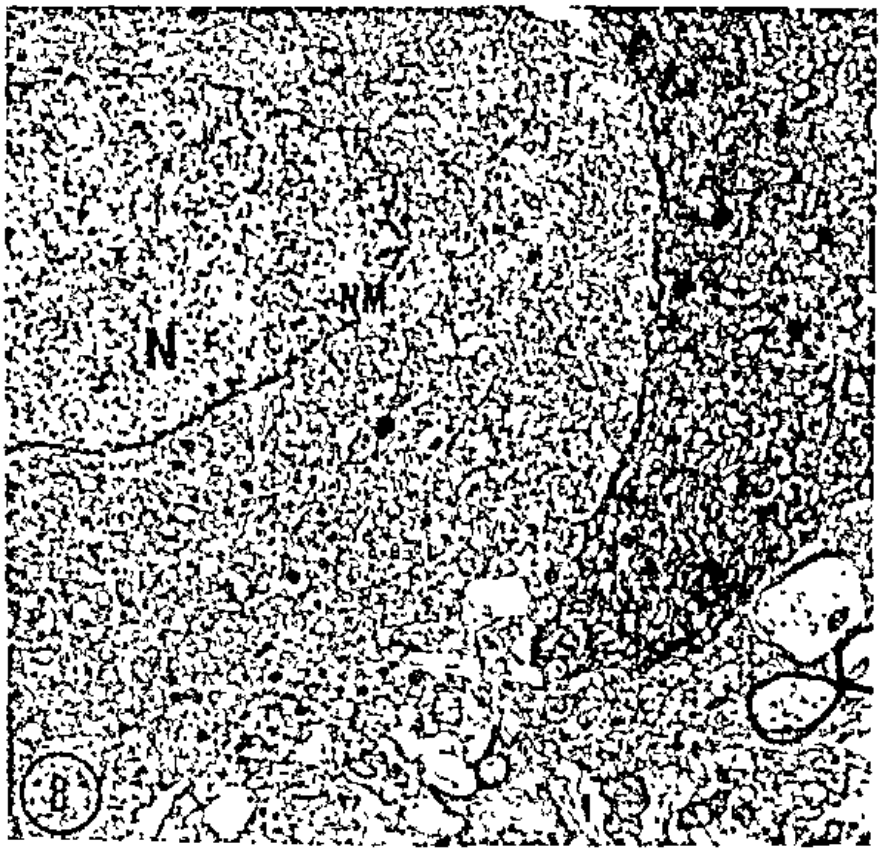
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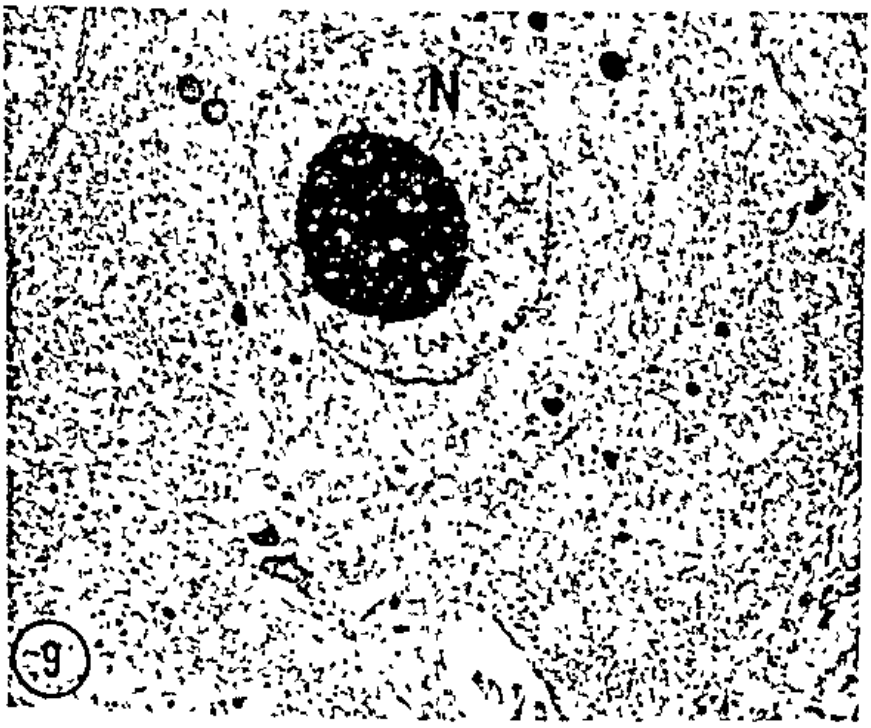
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Figs. 8, 9, 16, 17, 43 and 44 are reprinted because of differing reproduction from the original pictures due to technical reasons.

ERRATA

Page 10: Material and "metdods", read methods
Page 26: Line 8, "mitochodria", read mitochondria
Page 26: Line 37, "neuroscecretory", read neurosecretory
Page 30: in title "neurosectory", read neurosecretory
Page 32: Line 1, "elecetron", read electron
Page 37: Line 3, "proced res", read procedures
Page 51: in title "dehyrated", read dehydrated
Page 51: Line 16: "secretery", read secretory
Page 58: Line 24: "sectios", read sections
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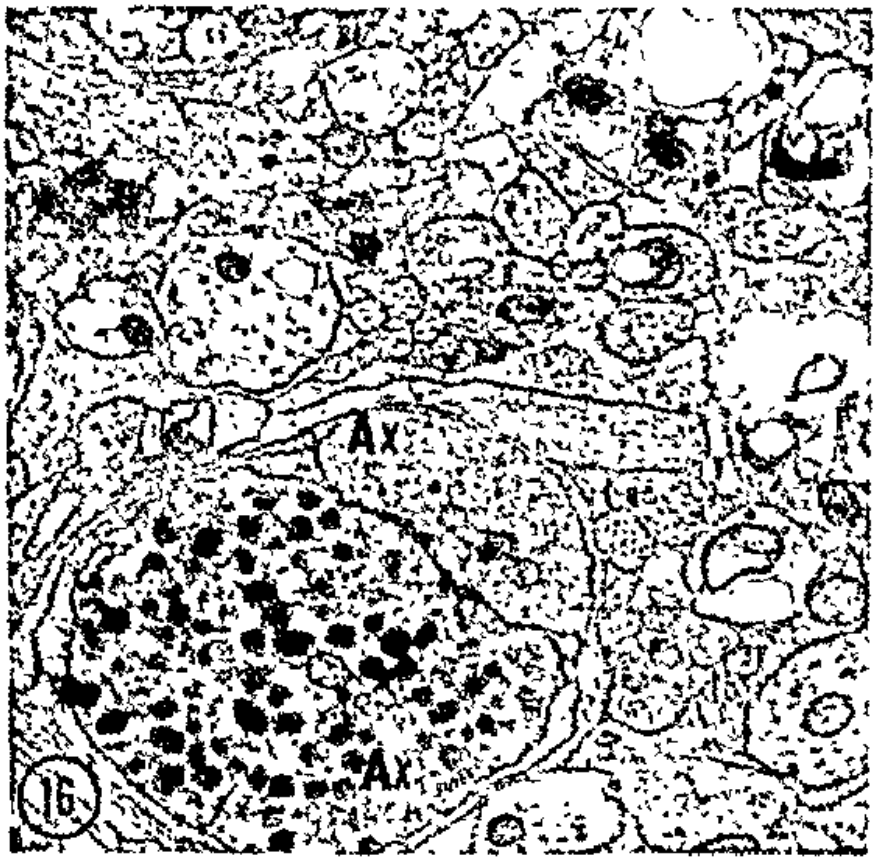


Fig. 16
Transverse section of an axon with synapsis.
An axon terminal with two large granular vesicles is in contact with an axon. The latter is crowded with granules of varying electron opacity. The synaptic nerve fibres are encircled by a glial element. Specimen processed as in Fig. 3. ×25 500



Fig. 17
Part of a capillary wall and axonal swellings in control rat SON.
Two axonal swellings are seen in the vicinity of the capillary wall. There are thin glial sheets between them and the basal lamina. Specimen processed as in Fig. 3. ×25 500

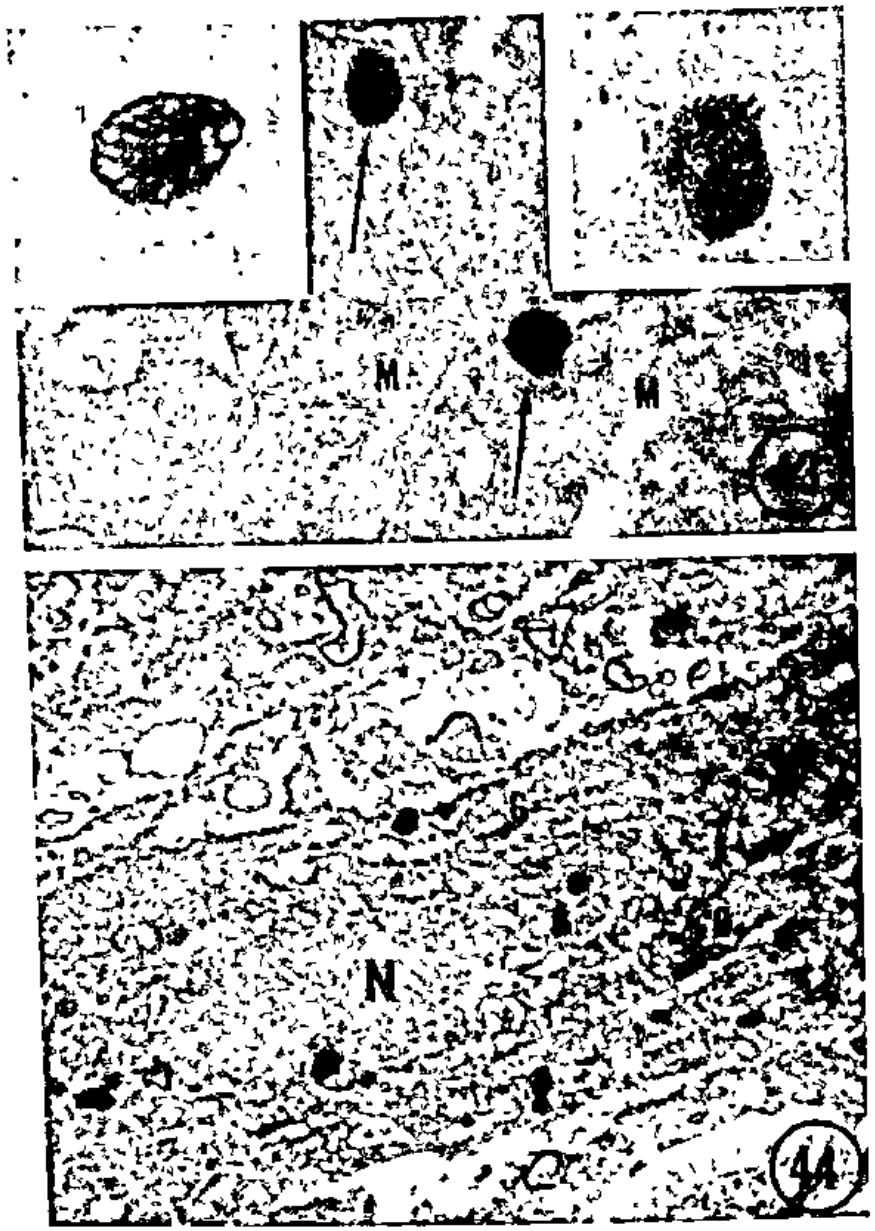


Fig. 43 AcPh activity in the large inclusion bodies of a light neurosecretory cell in control rat SON. Two large inclusion bodies (arrows), which are seen vary in the amount of reaction product. The lead salt is very finely granular as seen in the inset pictures with higher magnification. Procedures as in Fig. 41. × 25 500 × 39 000

Fig. 44 AcPh activity in the dark neurosecretory cell of rat SON after 7 days' dehydration. The large inclusion bodies are heavily covered with lead precipitate. The Golgi lamellae (G) are also reactive. Specimen processed as in Fig. 41. × 6 600

PREFACE

The present work was carried out in 1967—1969 in the Department of Anatomy, University of Helsinki, under the supervision of the Head of the Department, Professor Olavi Eränkö, M. D., Ph. D. He suggested to me the subject of this investigation, which forms a part of the neurohistochemical project now in progress in this Department. I am deeply indebted to him for his valuable advice and for his unsparing help and guidance.

I also wish to thank Docent Arto Palkama, M. D., for reading the manuscript and for his advice and criticism.

My sincere thanks are due to numerous colleagues in the Department of Anatomy: especially to Dr. Aulikki Kokko, M. D., with whom I had the advantage and pleasure to co-operate in the field of histochemistry; to Mrs. Liisa Eränkö, Phil. Mag., Docent Martti Kormano, M. D., Dr. Antti Penttilä, M. D., Phil. Cand., Dr. Heikki Terävainen, M. D. for many stimulating discussions.

I should like to thank the members of the staff of the Department of Anatomy for their friendly and always helpful attitude; especially Mrs. Tuula Stjernvall, Miss Laila Hänninen and Mrs. Liisa Kangasvuo for preparing the specimens; Miss Helmi Palomäki, Librarian, for collecting the literature, and Mrs. Helmi Savolainen for the typing of manuscript.

The English text was checked by Miss Aino Wuolle, Phil. Mag., which I wish to acknowledge here.

The Department of Electron Microscopy, University of Helsinki, placed at my disposal the facilities for electron microscopy. I am very grateful to Professor Antti Telkkä, M. D., Head of that Department, and to Mr. Mauri Nyholm, Phil. Mag., for much help on practical matters. The Institute of Photography, University of Helsinki, prepared part of the prints, and I take this opportunity to thank its staff for their helpful co-operation.

Last but not least, I owe thanks to my family, especially my husband Tapani, for their constant encouragement during this study.

Helsinki, April 1969

Leena Rechardt

CONTENTS

PREFACE 3
CONTENTS 4
ABBREVIATIONS 6
INTRODUCTION 7
I. ULTRASTRUCTURE OF THE SUPRAOPTIC NUCLEUS OF NORMAL AND DEHYDRATED RATS 9
Review of the literature 9
Light microscopic observations 9
Electron microscopic observations 10
Material and methods 10
Results 12
Electron microscopic observations 12
Techniques 12
The neurosecretory cells 14
Endoplasmic reticulum 14
The Golgi apparatus 16
Mitochondria 16
Inclusion bodies 16
a) Large bodies 16
b) Small bodies 16
The nerve fibres 18
Synapses 18
Synaptic vesicles 18
Axo-somatic synapses 22
Axo-dendritic synapses 22
Axo-axonal synapses 22
Capillaries 22
Glial cells 22
Discussion 24
II. EFFECT OF SYMPATHECTOMY, RESERPINE AND OXYPERTIN ON THE NEUROSECRETORY CELLS AND NERVE ENDINGS IN THE RAT *SUPRAOPTIC NUCLEUS* 30
Review of the literature 30
Sympathectomy 30
Effect of reserpine 30
Effect of Oxypertin 31
Material and methods 31
Results 32
Fluorescence microscopy 32

Electron microscopic observations 32
Neurosecretory cells 32
Synapses 34
Discussion 34
III. CHOLINESTERASE ACTIVITY IN THE SUPRAOPTIC NUCLEUS OF NORMAL AND DEHYDRATED RATS 40
Review of the literature 40
Light microscopic observations 40
Electron microscopic observations 41
Material and methods 41
Results 43
A. AChE activity 43
Light microscopic observations 43
Electron microscopic observations 44
B. Ns.ChE activity 46
Light microscopic observations 46
Electron microscopic observations 47
Discussion 47
Techniques 47
A. AChE activity 47
B. Ns.ChE activity 50
IV. ACID PHOSPHATASE ACTIVITY IN THE SUPRAOPTIC NUCLEUS OF NORMAL AND DEHYDRATED RATS 51
Review of the literature 51
Light microscopic observations 51
Electron microscopic observations 51
Material and methods 51
Results 52
Techniques 52
Light microscopic observations 52
Electron microscopic observations 54
Discussion 54
V. NUCLEOSIDE PHOSPHATASE ACTIVITY IN THE SUPRAOPTIC NUCLEUS OF NORMAL AND DEHYDRATED RATS 57
Review of the literature 57
Light microscopic observations 57
Electron microscopic observations 57
Material and methods 58
Results 58
Light microscopic observations 58
Electron microscopic observations 59
Neurosecretory cells 59
The nerve fibres 59
Capillaries 62
Control studies 62
Discussion 65
SUMMARY AND CONCLUSIONS 67
REFERENCES 71

ABBREVIATIONS

AChE	acetylcholinesterase
AcPh	acid phosphatase
ADP	adenosine diphosphate
ATP	adenosine triphosphate
ATPase	adenosine triphosphatase
ChE	cholinesterase
IDP	inosine diphosphate
ITP	inosine triphosphate
NSM	neurosecretory material
ns.ChE	non-specific cholinesterase
SON	supraoptic nucleus
TPP	thiamine pyrophosphate
UDP	uridine diphosphate
iso-OMPA	tetra-isopropylpyrophosphoramide
284C51	(1.5-bis(4-allyldimethylammoniumphenyl)pentan-3-one dibromide
5-HT	5-hydroxytryptamine

ABBREVIATIONS USED IN FIGURES

Ax	axon
Bl	basal lamina
Ch	chiasma opticorum
D	dendrite
ER	endoplasmic reticulum
G	the Golgi apparatus
Gl	glia
M	mitochondrion
N	nucleus
NM	nuclear membrane
VLb	vacuolated large body

INTRODUCTION

A century ago Meynert (1869) described a paired hypothalamic group of nerve cells, which he called »basale Opticus-ganglion». Lenhossék (1887) introduced the term nucleus supraopticus (SON), and this term is now generally accepted.

In the mammals, the SON is formed by closely packed, large nerve cells laterocaudally to the optic chiasma. In the rat, some diffusely scattered large nerve cells form an accessory SON above the main SON (Scharrer and Scharrer 1954).

These nerve cells show several peculiar features which suggest functions other than impulse conduction. On the basis of the pioneer work of Scharrer and Scharrer (1945, 1954) and further studies by Bargmann (1949) and Bargmann *et al.* (1950), it was established that the SON nerve cells were secretory. With various basic stains, *e.g.* chrom-alum haematoxylin (Gomori 1941a), aldehyde fuchsin (Gomori 1950), Alcian blue (Adams and Sloper 1955), and paraldehyde thionin (Huber 1963), it was possible to reveal typical material in the supraoptic nerve cells. This was subsequently called »Gomori-positive material» or neurosecretory material (NSM). With these staining techniques it was possible to demonstrate a nervous pathway from the SON to the neurohypophysis. The nerve terminals, which also showed NSM in their interior, ended in the pericapillary spaces of the neurohypophysis. Some axons were reported to terminate in the capillary loops of the portal system (Arko and Kivalo 1958).

In 1924 Abel reported the existence of two hormone-like substances in the mammalian hypothalamus and in the neurohypophysis, and the production of vasopressin and oxytocin has since been related to these neurons. An extensive literature is available on the morphology, bio- and histochemistry of the SON (*e.g.* Heller and Clark 1962, Stutinsky 1967).

Comprehensive light and electron microscopic studies by Eneström (1967) and Streefkerk (1967) dealing with the rat SON contributed to elucidate mainly the morphological correlations of the neurosecretory cells in normal and experimental conditions. Nerve terminals, capillaries and glial elements of the SON area have aroused minor interest (Zambrano 1968). In the present study, some aspects dealing with those structures and especially the innervation of the neurosecretory cells and adjacent nerve fibres will be examined.

Although the enzyme histochemistry of the SON has been widely studied by light microscopy in normal rats and in rats subjected to various experimental conditions (Eränkö 1951a, Pepler and Pearse 1957, Kivalo *et al.* 1958, Uemura 1965, Pilgrim 1967), no ultrastructural histochemical studies on rat SON are available, except for one dealing with acid phosphatase activity in normal rat SON (Osinchak 1964a). There are, however, biochemical observations on several enzymes in the SONs of osmotically stressed rats (Kobayashi *et al.* 1962, Jongkind

1967, 1969, Ferreyra Moyano 1967). Light microscopic and biochemical findings on the enzyme activities have been controversial to some extent.

The development of new perfusion fixation methods for electron microscopy of brain tissue (Palay *et al.* 1962, González Aguilar and De Robertis 1963) and advances in histochemistry (*e.g.* Wood and Barrnett 1964, Adams 1965) have made possible the demonstration of several enzymes or enzyme groups in nervous tissue. Therefore it appeared likely that useful information might be obtained by studying the electron microscopic histochemistry of the neurosecretory cells under normal and experimental conditions. The present study was undertaken to find such information on some of those enzymes which can be demonstrated electron microscopically with the methods currently available.

I. ULTRASTRUCTURE OF THE SUPRAOPTIC NUCLEUS OF NORMAL AND DEHYDRATED RATS

REVIEW OF THE LITERATURE

LIGHT MICROSCOPIC OBSERVATIONS

Structural peculiarities of the cells in the SON suggesting that these cells are not ordinary nerve cells were first observed by Collin (1931), Scharrer and Gaupp (1933), Scharrer and Scharrer (1940), Scharrer *et al.* (1945). They assumed the multipolar nerve cells of the SON to be secretory and introduced the term neurosecretion, previously used by some other authors (see Scharrer and Scharrer 1940) to explain the presence of certain granules in other nerve cells.

Early efforts to find signs of neurosecretion in the SON of the rat often failed until methods were developed by which the secretory granules could be more selectively demonstrated. The cells of the rat SON were studied with such methods by Ortmann (1951), Eränkö (1951b), Stutinsky (1952) and later by many others (see *e.g.* Diepen 1962 a, Enestrom 1967, Streefkerk 1967). Eränkö (1951b) found two different types of nerve cells: light and dark cells. The dark cells were smaller and stained more intensely with basic dyes. Later Zambrano and Mordoh (1966) confirmed this finding and related it to the secretory rhythm of the cells. Enestrom (1967) described A-cells and B-cells on the basis of their staining qualities and electron microscopic appearance.

The neuronal character of the cells of the SON was determined at the light microscopic level by the existence of neurofilaments, Nissl substance and axon cylinders leading to the neurohypophysis (*e.g.* Scharrer and Scharrer 1954, Diepen 1962b).

Release of antidiuretic hormone can be induced by depriving the animals of water or by injecting hypertonic solutions. Several reports deal with the structural changes thus caused in the SON: increase in the cellular, nuclear and nucleolar volumes, increase in the ribosomal content and loss of neurosecretory material was demonstrated light microscopically by Hillarp (1949), Ortmann (1951), Eichner (1952), Edström and Eichner (1958), Ifft and Berkowitz (1965), whereas a loss of Nissl substance was reported by Bargmann (1954), Scharrer and Scharrer (1954) and Bachrach (1957).

ELECTRON MICROSCOPIC OBSERVATIONS

Ultrastructural studies of the SON were made possible by the development of suitable methods for the fixation of brain tissue. In 1960 Palay described in detail the ultrastructure of the preoptic nucleus of the gold fish. He used an OsO_4 perfusion technique for fixing the tissue. Since then the homologous nuclei have been studied in amphibia (Murakami 1964), in reptiles (Murakami 1961), and in birds (Nishioka 1967). The SON was described in the mouse by Murakami (1962), in the dog by Sloper and Bateson (1965) and Zambrano and De Robertis (1967), and in the rat by Duncan and Alexander (1961), Osinchak (1964b), Nemetschek-Gansler (1965), Sloper and Bateson (1965), Zambrano and De Robertis (1966), Enestrom (1967) and Streefkerk (1967). In these studies, the various cytoplasmic organelles were the targets of main interest, above all the so-called »neurosecretory elementary granules» and their possible site of formation.

Peterson (1965) called attention to the synapses between the neurosecretory cells and nerve terminals in the rat SON and found relatively few terminals. Polenov and Senchik (1966) were the first to report the presence of agranular and granular synaptic vesicles in the axo-somatic synapses of the mouse SON (Polenov and Senchik 1966). Enestrom and Svalander (1967) and Zambrano (1968) recently reported observations on synapses in the rat SON.

Nemetschek-Gansler (1965), Zambrano and De Robertis (1966), Enestrom (1967) and Streefkerk (1967) described ultrastructural changes in neurosecretory cells after dehydration. Proliferation and vacuolation of the endoplasmic reticulum, together with an increase in the amount of ribosomes, was found in all these studies. Observations concerning the disappearance and reappearance of neurosecretory elementary granules during and after dehydration were controversial to some extent (see Zambrano and De Robertis 1966, Streefkerk 1967).

MATERIAL AND METDODS

For this part of the present study, 55 adult male rats of the albino Sprague-Dawley strain, weighing about 300 g, were used. The studies were carried out between September and June. The rats were kept in the daytime under artificial illumination. The control rats received dried food and water *ad libitum*. The rats to be dehydrated for 3, 5 or 7 days were given only dry food. The rats were weighed before dehydration and before being sacrificed.

Perfusion-fixation was carried out under Nembutal (50 mg/kg body weight i.p.) anaesthesia. Several fixation fluids at room temperature were used: (a) 3.5 % formaldehyde *pro analysi* grade (E. Merck AG., Darmstadt) in 0.075 M phosphate-buffered 0.3 M sucrose, (b) 2.5 % glutaraldehyde (Taab Laboratories, Emmer Green. Reading. England) in 0.1 M phosphate buffer, (c) 2.5 % glutaraldehyde and 1 % formaldehyde in 0.1 M phosphate buffer, a modification of Karnovsky's (1965) fixative and (d) 2 % potassium permanganate in 0.1 M phosphate buffer, a modification of Richardson's (1964) fixative. The pH of all these fixatives was adjusted to 6.9, and the respective approximate osmolalities were (a) 1300, (b) 470, (c) 700 and (d) 350 mOsM, determined by osmometer.

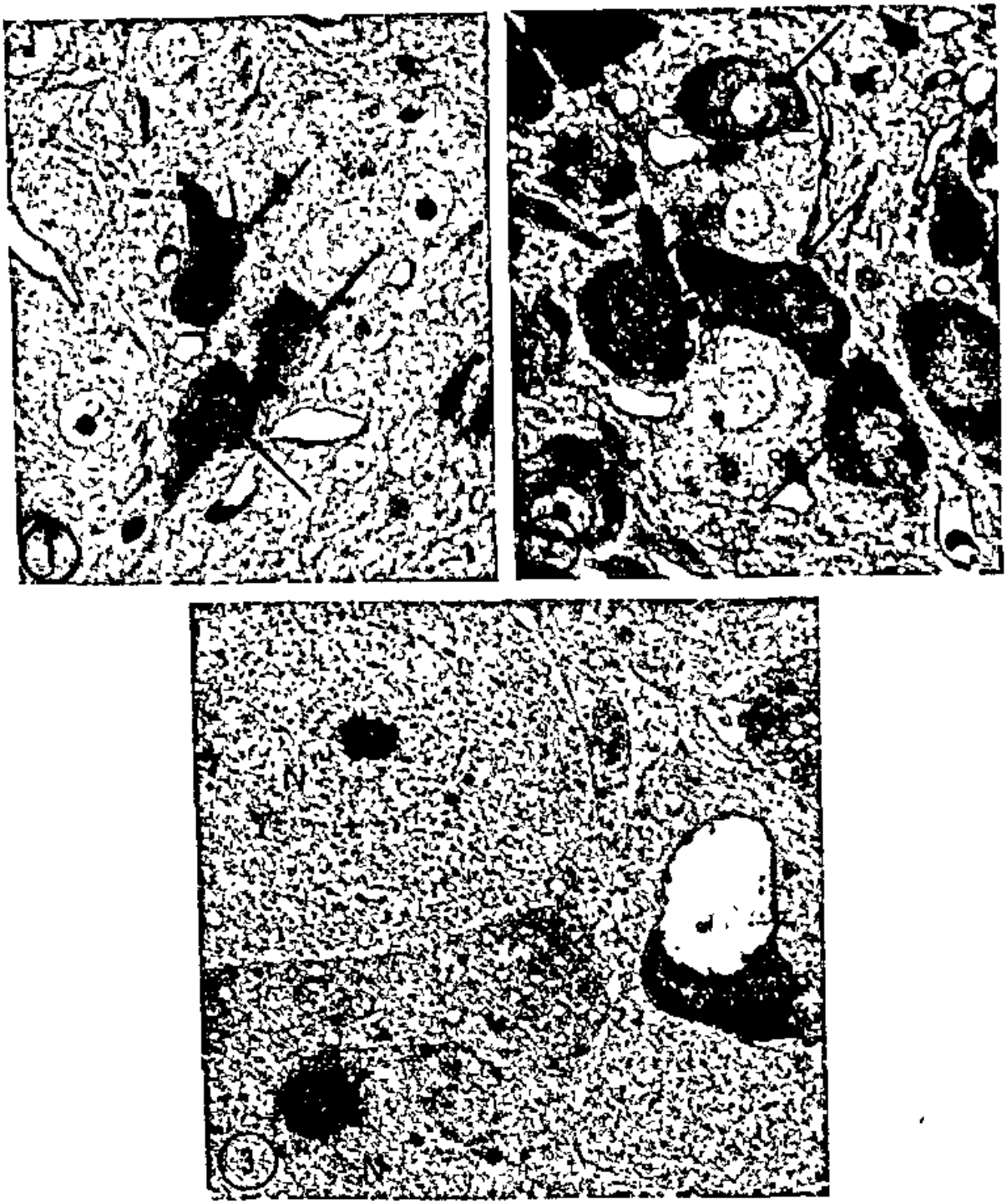


Fig. 1 Light microscopic view of the neurosecretory cells in control rat SON. Three fusiform dark neurosecretory cells, pointed by arrows, are seen among the light neurosecretory cells. Specimen perfused with Karnovsky's fixative and refixed with OsO_4 Toluidine blue stained Epon section × 750

Fig. 2 Light microscopic view of the neurosecretory cells in rat SON after 7 days' dehydration. Several dark neurons, pointed by arrows, surround two light nerve cell bodies. Note enlarged size of the cells. Specimen processed as in Fig. 1. × 750

Fig 3 Two types of neurosecretory cells in control rat SON. The neurosecretory cells are interconnected with a thin glial sheet. Note the different shade of the nucleus in the dark cell compared to the nucleus in the light cell above. Specimen glutaraldehyde perfusion fixed and refixed with OsO_4. Poststained with uranyl acetate and lead citrate × 3000

A simple blood transfusion apparatus consisting of a bottle, drop-enlargement and clamped tube was used in the perfusion procedure. The bottle was raised to obtain a hydrostatic pressure of 115 cm of the perfusion fluid, and perfusion speed was about 20 drops per minute. The perfusion was performed either *via* the abdominal aorta or intracardially *via* the left ventricle without any artificial respiration. Both external jugular veins were opened just before the perfusion was started. The abdominal aorta was clamped if intracardial perfusion was used. When glutaraldehyde or potassium permanganate was used, a prewash perfusion with 20 cc physiological saline at 39°C was administered. Perfusion time was 15 minutes. The brain was dissected out immediately after perfusion.

Thin slices of the hypothalamus containing both supraoptic nuclei were cut with a razor blade. Fixation was further prolonged by immersion of these slices for 3 hours at 4°C in the same fixative as used for perfusion. Pieces were then dissected out under a stereo-microscope containing only the SON and the optic chiasma, and these pieces were further fixed for one hour in ice-cold OsO_4 fixative as described by Rhodin and Zetterquist (Rhodin 1954). This postfixation step was omitted in potassium permanganate-fixed specimens.

After a short dehydration procedure, described in detail by Kokko and Rechardt (1968), the pieces were embedded in Epon epoxy resin (Luft 1961) and sectioned with an LKB Ultrotome. The SONs of two rats were cut serially into 1 μ sections, stained with toluidine blue, and studied under a light microscope. The resin block was further trimmed so that a small part of the optic chiasma was left in every block. Silver or grey sections were cut with an LKB Ultrotome and stained on grids with uranyl acetate and lead citrate (Watson 1958), or with lead citrate alone (Reynolds 1963). The sections were viewed and photographed with Siemens Elmiskop I and Philips EM 200 operated at 80 kV.

RESULTS

The mean weights of the rats dehydrated for 3, 5 or 7 days were reduced to 85, 81 or 68 per cent of the original, respectively. After 7 days' dehydration the rats were ataetic, soporific and cachectic.

ELECTRON MICROSCOPIC OBSERVATIONS

Techniques

Perfusion fixation proved to be essential for good structural preservation of the SON. In preliminary studies, in which only immersion fixation was used, an enlarged extracellular space, swollen mitochondria and loose myelin layers were observed. A comparison of aortic and intracardial perfusion techniques showed no differences in the fine structure of the SON, using the neurosecretory elementary granules and the granular synaptic vesicles as criteria. Preperfusion with physiological saline was necessary before glutaraldehyde or potassium permanganate. If omitted, red blood cells were found in the capillaries, and the ultrastructure was inferior.

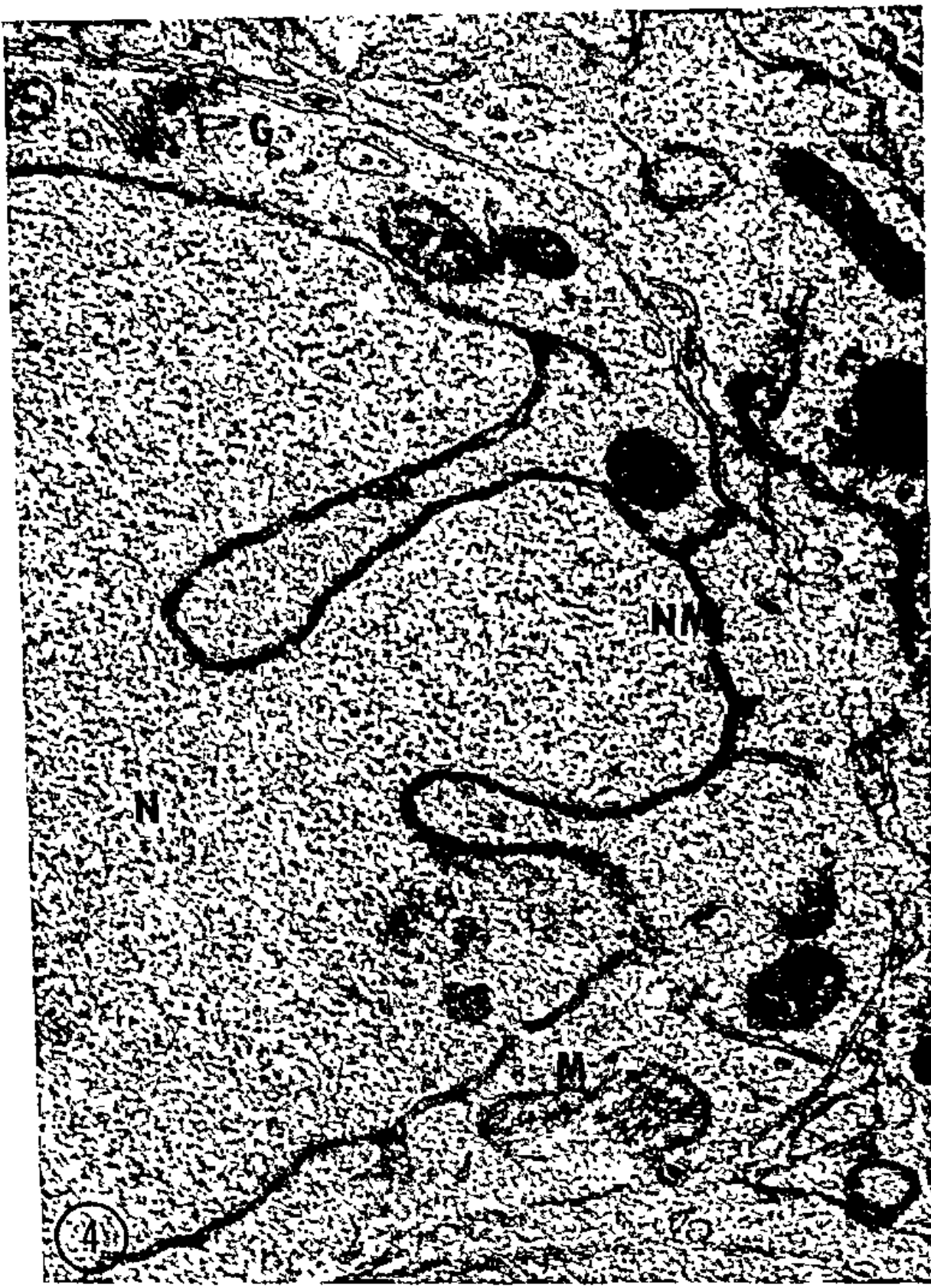


Fig. 4 A view of the nuclear membrane of neurosecretory cell in control rat SON. Nuclear indentations, pores and budding of the endoplasmic reticulum from the nuclear membrane are demonstrated in this potassium permanganate-fixed specimen. Poststained with lead citrate. × 28 000

The neurosecretory cells

When formaldehyde- and glutaraldehyde-containing primary fixatives were employed, two types of neurosecretory cells were found, light and dark cells (Figs. 1 and 3). (a) The light cells were larger and circular or oval in shape. The nuclei of these cells were large, pale and eccentrically placed.

(b) Only a few dark cells were found. These were randomly scattered amongst the light cells. The dark cells were smaller and fusiform. The nuclei were smaller than those of the light cells and their electron density was more homogeneous.

The main criterion between light and dark cells was the relative number of free ribosomes. When potassium permanganate was used as a fixative, no difference was observed in the density of the cell cytoplasm.

After dehydration the size of both light and dark cells increased considerably (Fig. 2). This seemed to be due to the proliferation of the endoplasmic reticulum, which showed dilatation of cisternae and increase in the number of free ribosomes (Fig. 8). The increase of cell size was already observed after 3 days' dehydration, and it appeared about equal to that noticed after 7 days. Numerous intercellular contacts were seen. Disappearance of glial elements between the cells was noticeable. The space between the neurosecretory cells and the capillaries was, however, wider, and the glial elements in this space were more voluminous than in the control sections, as seen in Figs. 10 and 19.

The nuclei of normal control cells often showed several infoldings and protrusions, especially in potassium permanganate-fixed specimens. Nuclear pores and the budding of endoplasmic reticulum of the nuclear membrane is clearly seen in Fig. 4.

After dehydration, the relative size of nuclei appeared to increase. The nuclei became rounded and lost their indentations (Figs. 8 and 9). These changes were observed after 3, 5 or 7 days' dehydration. The nucleolus became prominent and its size increased (Fig. 9).

Endoplasmic reticulum

The endoplasmic reticulum was mainly granular and it was situated at the periphery of the cells, forming parallel cisternae. In the dark cells, free ribosomes were especially abundant and the cisternae appeared more dilated than those in the light cells (Fig. 5). In potassium permanganate-fixed tissues a parallel arrangement of the cisternae was seldom seen. Instead, the cisternae were dilated in all cells, ribosomes were not visible using this fixative (Fig. 12).

After 3 days' dehydration the endoplasmic cisternae of the light cells appeared vacuolated and the number of free ribosomes appeared increased (Fig. 11). In the dark cells the endoplasmic reticulum was essentially unchanged even after 5 days' dehydration. After 7 days' dehydration there was an abundance of free ribosomes between the cisternae and the electron-dense material inside them, and this made the dark cells appear even darker than in the control rats (Figs. 8 and 3).

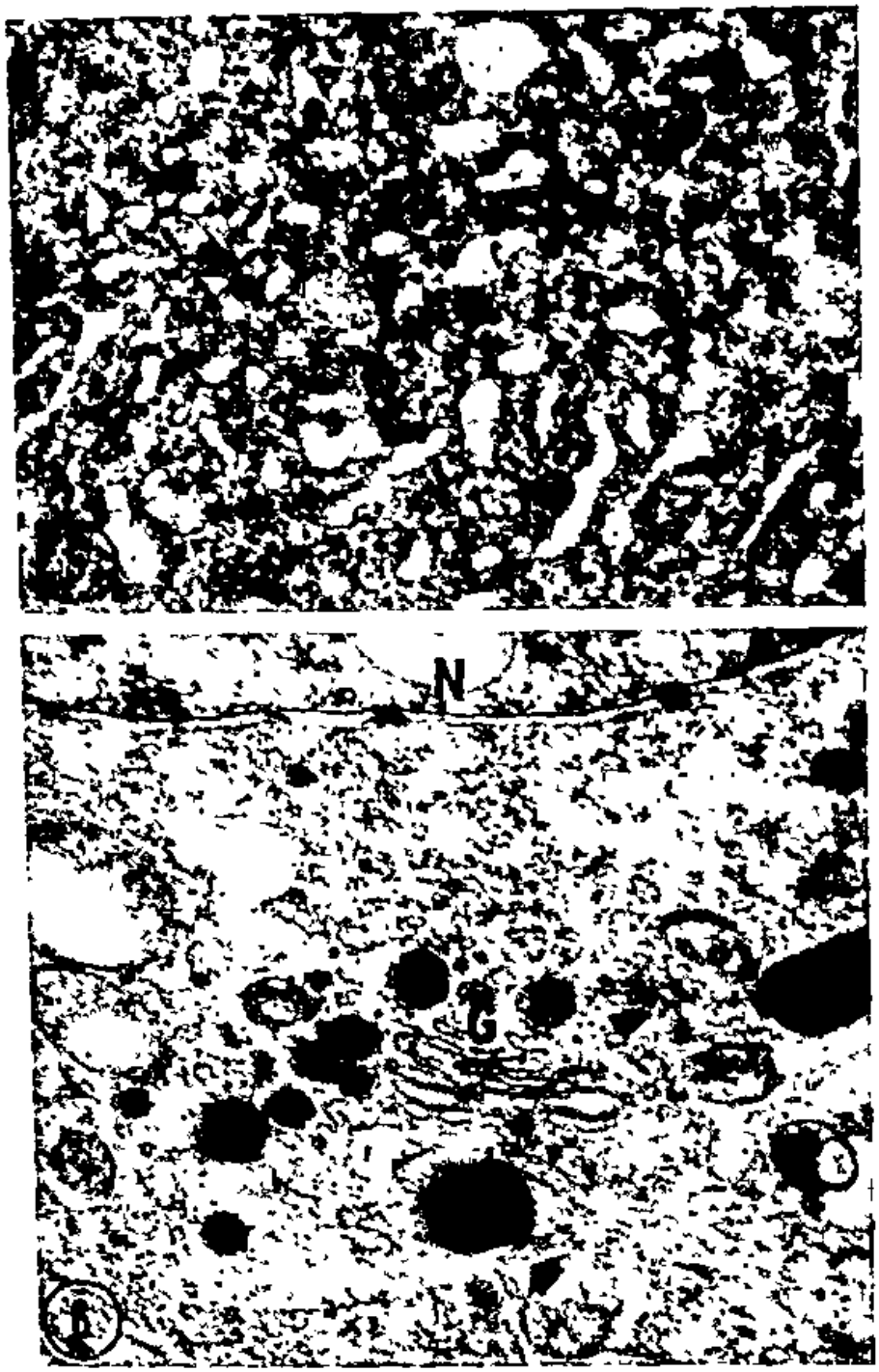


Fig. 5 A higher magnification from the periphery of a dark neurosecretory cell in control rat SON.
The endoplasmic cisternae are dilated and between them there are a great number of numerous rosettes formed by free ribosomes. Specimen processed as in Fig. 3. × 25 500

Fig. 6 Small inclusion bodies in proximity to the Golgi apparatus in a light neurosecretory cell of control rat SON.
The small inclusion bodies, about 1100 Å in diameter, have central cores which show different osmiophility. The arrow indicates a large inclusion body of type I. Specimen processed as in Fig. 3. × 39 900

The Golgi apparatus

The Golgi apparatus consisted of flattened cisternae with associated small vesicles. There were only a few large vesicles in association with the Golgi apparatus (Fig. 6). In potassium permanganate-fixed tissues, the Golgi apparatus was very prominent.

Compared with the controls, flattened vesicles were more numerous even after three days' dehydration in both types of neurosecretory cells. Empty vesicles were observed in the vicinity of the Golgi cisternae, as seen in Fig. 11.

Mitochondria

Most mitochondria were oval and varied in length from 0.3 μ to 1.0 μ. The light and dark cells were not found to differ in number or appearance of the mitochondria. The cristae were generally arranged transversally (Fig. 7); only occasionally there were longitudinally arranged cristae. Preservation of mitochondria was equally good using both formaldehyde and potassium permanganate fixatives, as seen in Figs. 7 and 4. In glutaraldehyde-fixed tissue it was usual to find swollen mitochondria (Fig. 6) with reduced number of cristae.

The shape,size and number of mitochondria did not change after dehydration.

Inclusion bodies

a) *Large bodies.* — Two types of large inclusion bodies were demonstrated. *Type I* bodies were very electron-dense and oval bodies with homogeneous contents and surrounded by a single unit membrane (Fig. 7). The size of these bodies varied from 0.5 μ to 1.0 μ.

Type II bodies were round, approximately 1.0 μ in diameter and vacuolated. They were surrounded by a single membrane and contained 1—5 vacuoles (Fig. 7). This type was rare.

The number of large inclusion bodies appeared to be unchanged in both light and dark cells after 3, 5 and 7 days' dehydration.

b) *Small bodies.* — Round or slightly oval small inclusion bodies were observed in the light and dark cells. They were mostly scattered round the nucleus or in the neighbourhood of the Golgi apparatus. These small bodies were approximately 1100 Å in diameter and were surrounded by a unit membrane. Inside this membrane there was a clear zone of 150Å. The central cores were of variable electron density, and small inclusion bodies of different shades were usually seen in the cell, as illustrated in Fig. 6.

In potassium-permanganate-fixed tissue only empty vesicles, all of the same size, about 700 Å, were found in the area of the Golgi apparatus. When formaldehyde- and glutaraldehyde-fixed tissue was compared, no outstanding difference was found in appearance, shade or size of the small inclusion bodies.

After 3 days' dehydration a distinct decrease was observed in the number of small inclusion bodies. Empty vesicles of the same size were found instead at the ends of the flattened Golgi vesicles, as seen in Fig. 11. The disappearance of small bodies was almost complete after 5 or 7 days' dehydration (Figs. 9 and 8).

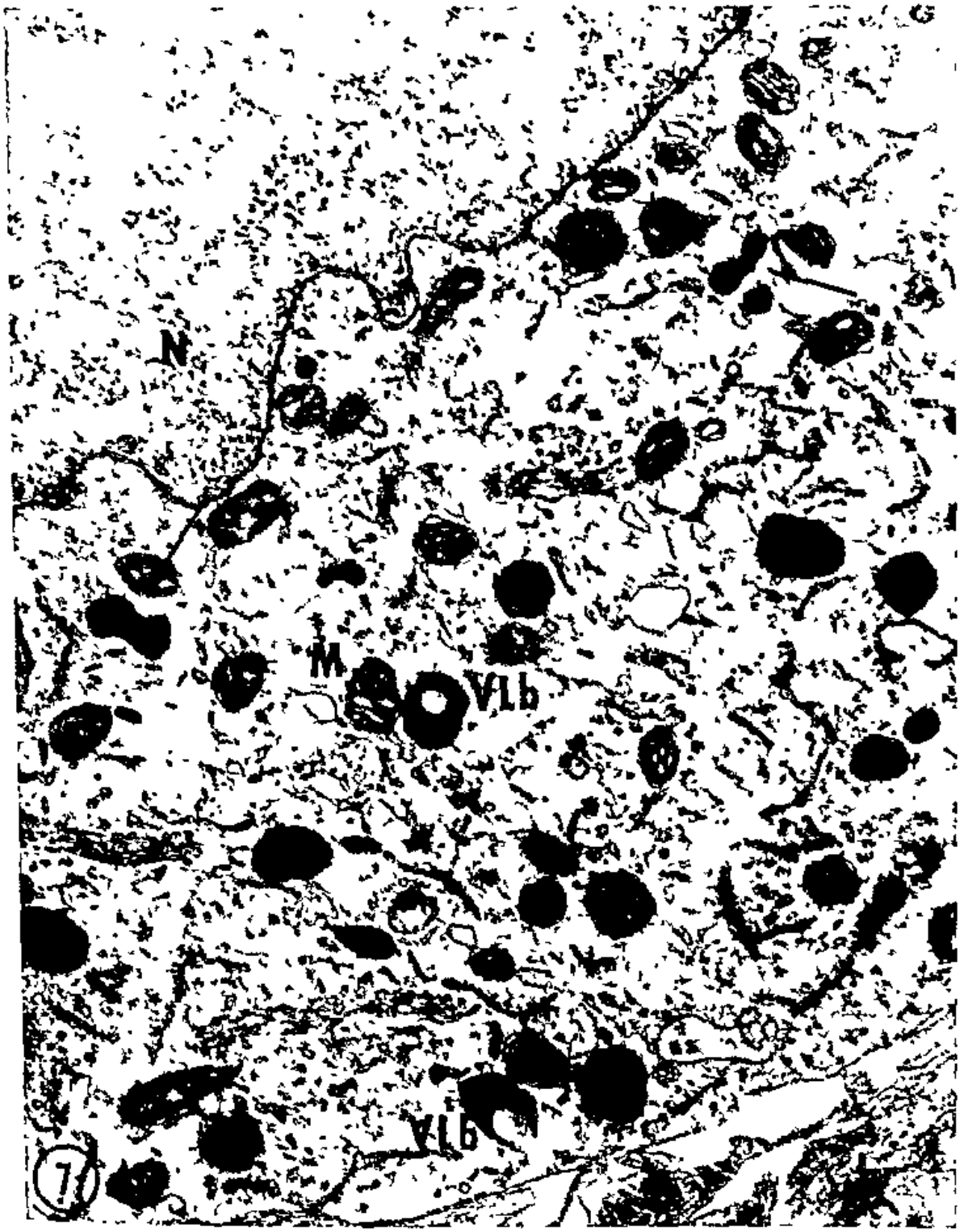


Fig. 7 Various cell organelles in the light neurosecretory cell of control rat SON. Several large inclusion bodies are scattered in the cytoplasm, two large bodies of type II are seen (VLb). The mitochondria have transversal cristae. The arrows point to budding inclusion bodies, probably of larger type. Specimen was formaldehyde perfusion fixed and refixed with OsO_4. Poststained with lead citrate. × 18 800

The nerve fibres

Neurosecretory cells and numerous capillaries occupied the bulk of the SON, the nerve fibres and glial elements being only minor constituents.

Axons and dendrites leaving the cell body were seldom present. However, Fig. 12 shows a neurosecretory cell with an axon branching off from it.

The axons of the SON neurosecretory cells were unmyelinated. Large axonal swellings, so-called Herring bodies, were often seen near capillaries. These contained round granules of different size and electron opacity (Figs. 16 and 17). These granules were larger than the small inclusion bodies in the neurosecretory cells and the large granulated vesicles in the synaptic endings. They were 1000—1750 Å in diameter. Neurotubules of 300 Å diameter, vesicles of various sizes, mitochondria and ribosomes were other components of the axonal swellings.

The dendrites showed a regular inner structure with neurotubules, long and slender mitochondria, vesicular membraneous structures and fragments of the endoplasmic reticulum (Fig. 15). The dendrites were densely covered by synapses.

After 3, 5 or 7 days' dehydration, the large granules in the axonal swellings were larger (about 2000 Å) than in the control sections. Empty vesicles of the same size were abundantly present in the axonal swellings (Fig. 10).

Synapses

Three types of synapses were found in the SON: axo-somatic, axo-dendritic and axo-axonal synapses. In all these types three different kinds of vesicles were observed in axon terminals depending on the fixative employed.

No distinct changes were observed in the synapses after dehydration, when compared to those in the control specimens.

Synaptic vesicles

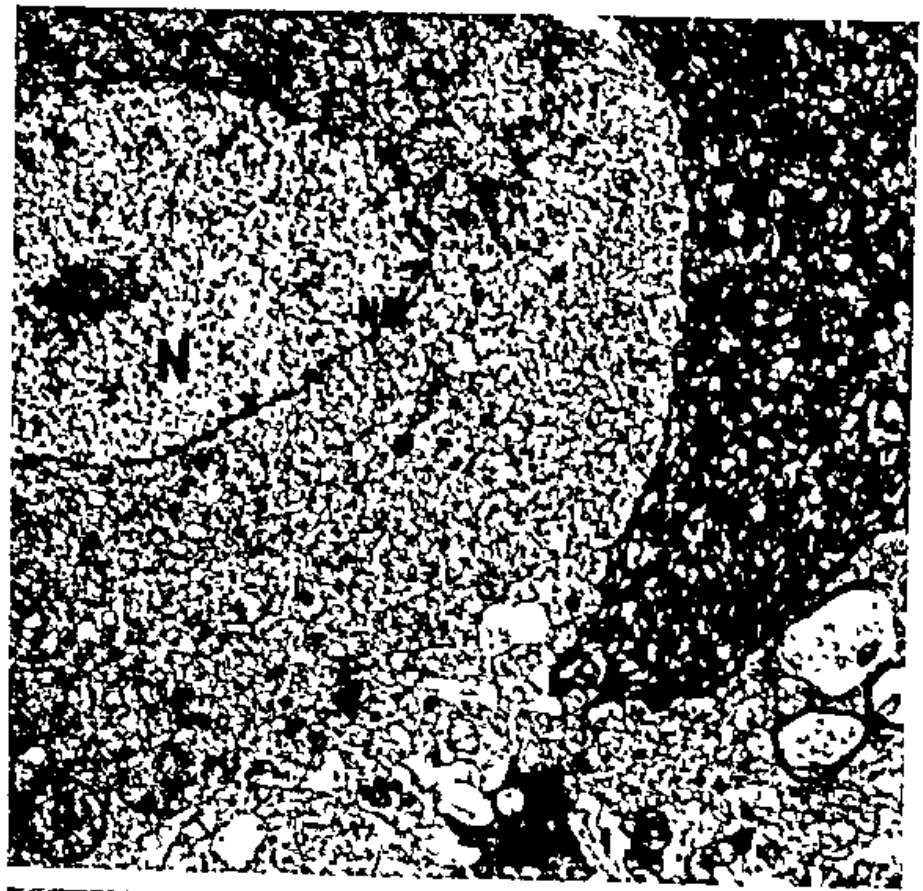
The main part of axon terminals contained only agranular vesicles. In specimens fixed with formaldehyde and glutaraldehyde their size was 300—450 Å. In specimens fixed with potassium permanganate the diameter was 250—280 Å.

Large granular vesicles were demonstrated with all the fixatives used and their size varied between 800 and 1200 Å; when fixed with potassium permanganate it was 600—750 Å. Only two or three such vesicles were seen amongst the agranular vesicles in each axon terminal. The centrally located granule was about 600 Å in diameter (Figs. 14 and 15). The electron opacity of these granules varied greatly even inside one and the same axon.

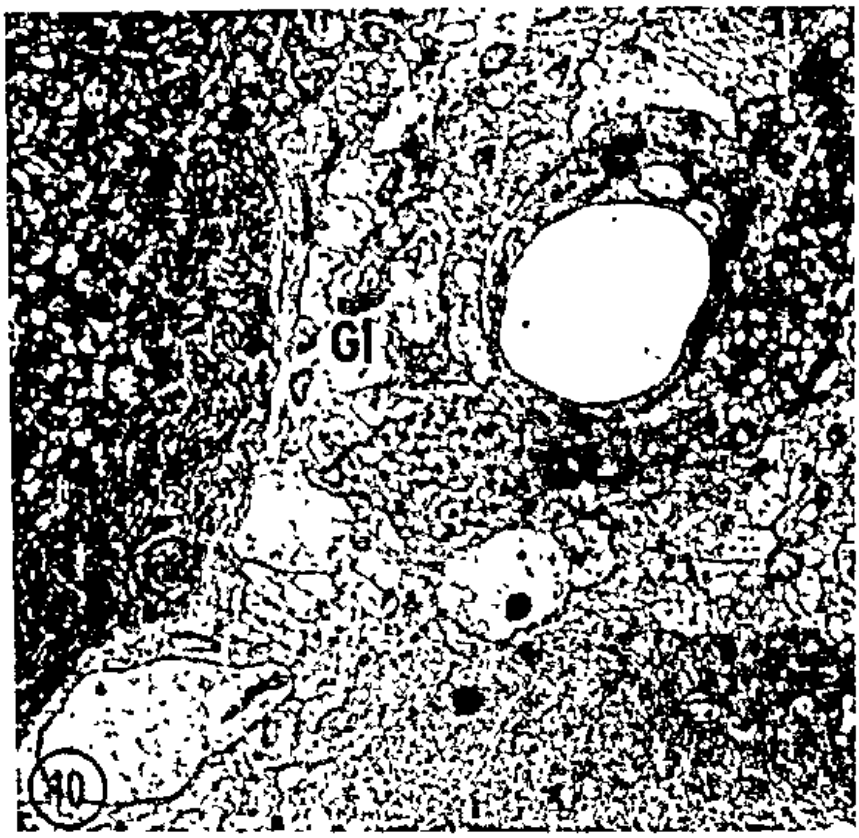
Small granular vesicles were observed only in specimens fixed by potassium permanganate perfusion. They varied in diameter between 250 and 300 Å and occurred amongst the agranular vesicles (Figs. 12 and 13).

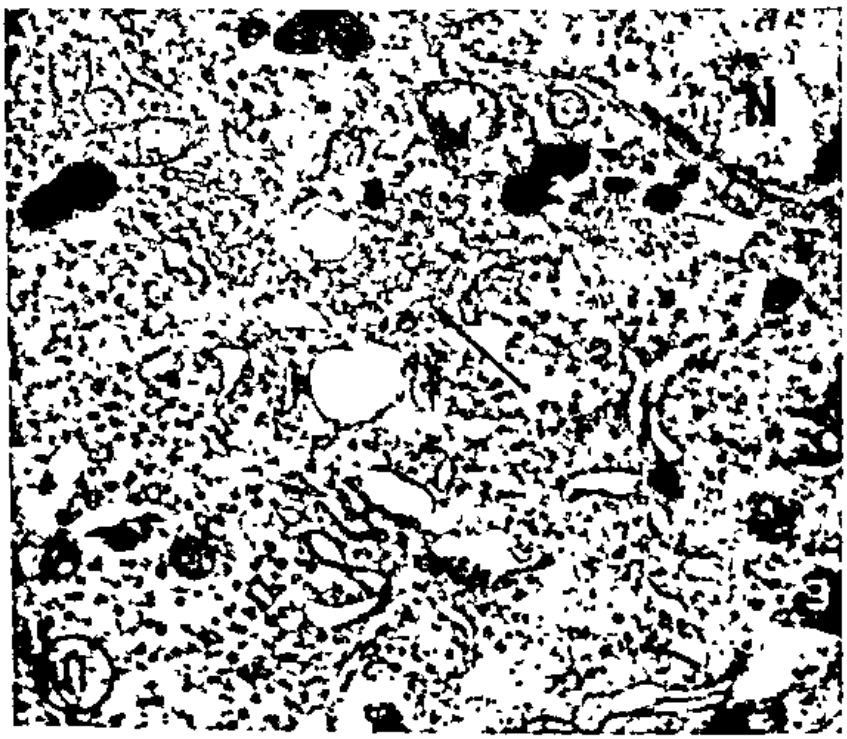
Fig. 8 A light and a dark neurosecretory cell in rat SON after 7 days' dehydration. Glial elements have disappeared between the neurosecretory cells and they lie in close contact with each other. The nuclear membrane has lost its indentations. Large inclusion bodies are seen in both of the cells, but small ones are almost completely abolished. The endoplasmic cisternae in the dark cell in the right side are filled with amorphous material. Specimen processed as in Fig. 3. × 5 100

Fig. 9 Nucleus and nucleolus in rat SON after 5 days' dehydration. The nucleus has become rounded. Note the very prominent nucleolus in this light neurosecretory cell. Only few small inclusion bodies are visible in these two light cells. Specimen processed as in Fig. 3 × 5 100


N


N
9


Gl
10


N
11

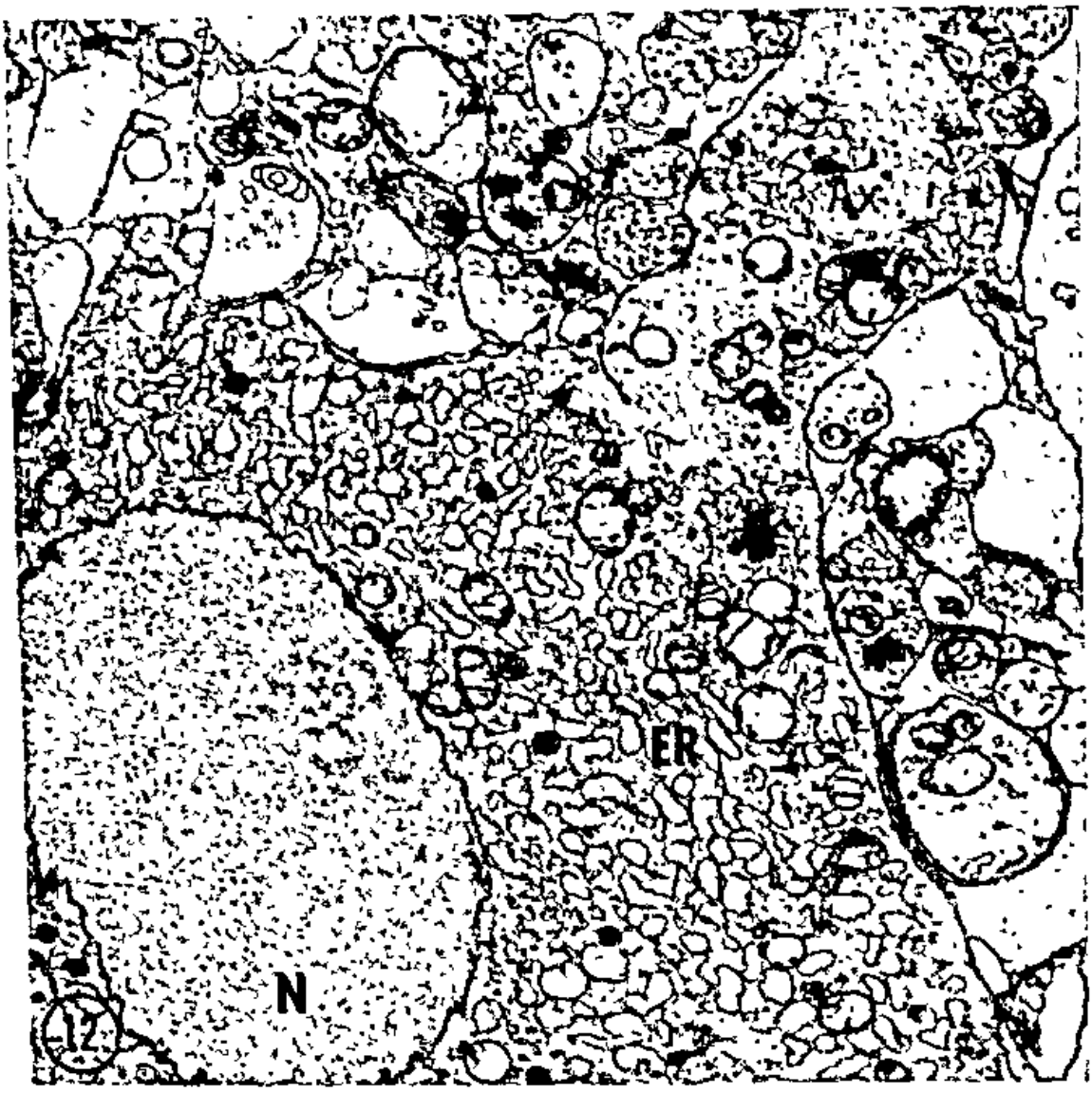


Fig. 10 General view of a capillary, glial tissue and two neurosecretory cells in rat SON after 7 days' dehydration.
Glial processes are increased round the capillary. They look pale and swollen. On the right and below the capillary an axon (Ax) is seen. It is filled with osmiophilic granules and empty vesicles of the same size. Specimen processed as in Fig. 3. × 5 100

Fig. 11 A view of a light neurosecretory cell in rat SON after 3 days' dehydration.
The amount of free ribosomes is increased and the endoplasmic cisternae, which look empty, are dilated. There are some small inclusion bodies, but in the proximity of the Golgi apparatus small empty vesicles are found (arrow). Specimen processed as in Fig. 3. × 25 000

Fig. 12 Neurosecretory cell with a branching nerve fibre. A thick nerve process is leaving the cell body. This is probably an axon with abundant endoplasmic cisternae. Several synaptic terminals are seen. Note the various vesicles inside them. Potassium permanganate perfusion and immersion fixed. No poststaining. × 13 600

Axo-somatic synapses

Only a small area of the neurosecretory cell surfaces was covered by synapses. These axo-somatic synapses contained only agranular vesicles or both small and large granular vesicles, as shown in Figs. 12 and 14. There was no difference in the number of synapses or in the proportion of the different vesicle types in the synapses ending on the light and dark cells.

Axo-dendritic synapses

The dendrites appeared to be covered very densely with synapses. Cross-sectioned dendrites showed several synapses containing all types of vesicles or only agranular ones. Typical axo-dendritic synaptic contacts are seen in Figs. 13 and 15.

Axo-axonal synapses

Synapses of this type were rare. When present, they were always located near a capillary (Fig. 16).

Capillaries

The fine structure of the capillary walls was found to be similar irrespective of the fixative used. However, the endothelial cells were more swollen after perfusion with glutaraldehyde or potassium permanganate than after perfusion with formaldehyde.

No fenestrations could be demonstrated in the endothelial cells, which showed but few pinocytotic vesicles. The basal lamina was about 400 Å thick and sometimes formed fingerlike indentations between the glial elements. When the specimen was adequately perfused, no perivascular space was noticed round the basal lamina. Some direct contacts between the neurosecretory cells and the capillaries were seen, although there were usually some glial end feet. The glial end feet were always situated between synapses and capillary walls. Axonal swellings with various granules were often found in the vicinity of the capillaries. They were not in direct contact with capillary walls (Fig. 17).

A pericyte sometimes surrounded the capillary wall, especially in the larger capillaries. Its nucleus stained intensely in the periphery, when glutaraldehyde fixation was used (Fig. 18). This type of glial cell was probably a protoplasmic astrocyte (Hartmann 1958, Palay 1958).

After 3, 5 or 7 days' dehydration the capillaries were narrowed and the endothelial cells became swollen. The basal lamina varied in thickness between 500 and 600 Å, *i.e.*, it was thicker than that in the controls. The glial elements round the capillaries appeared to be hypertrophied, swollen and pale (Figs. 10 and 19).

Glial cells

The glial cells occurred in perineuronal or pericapillary sites in the SON area. The predominant glial cell type was the protoplasmic astrocyte, as judged by

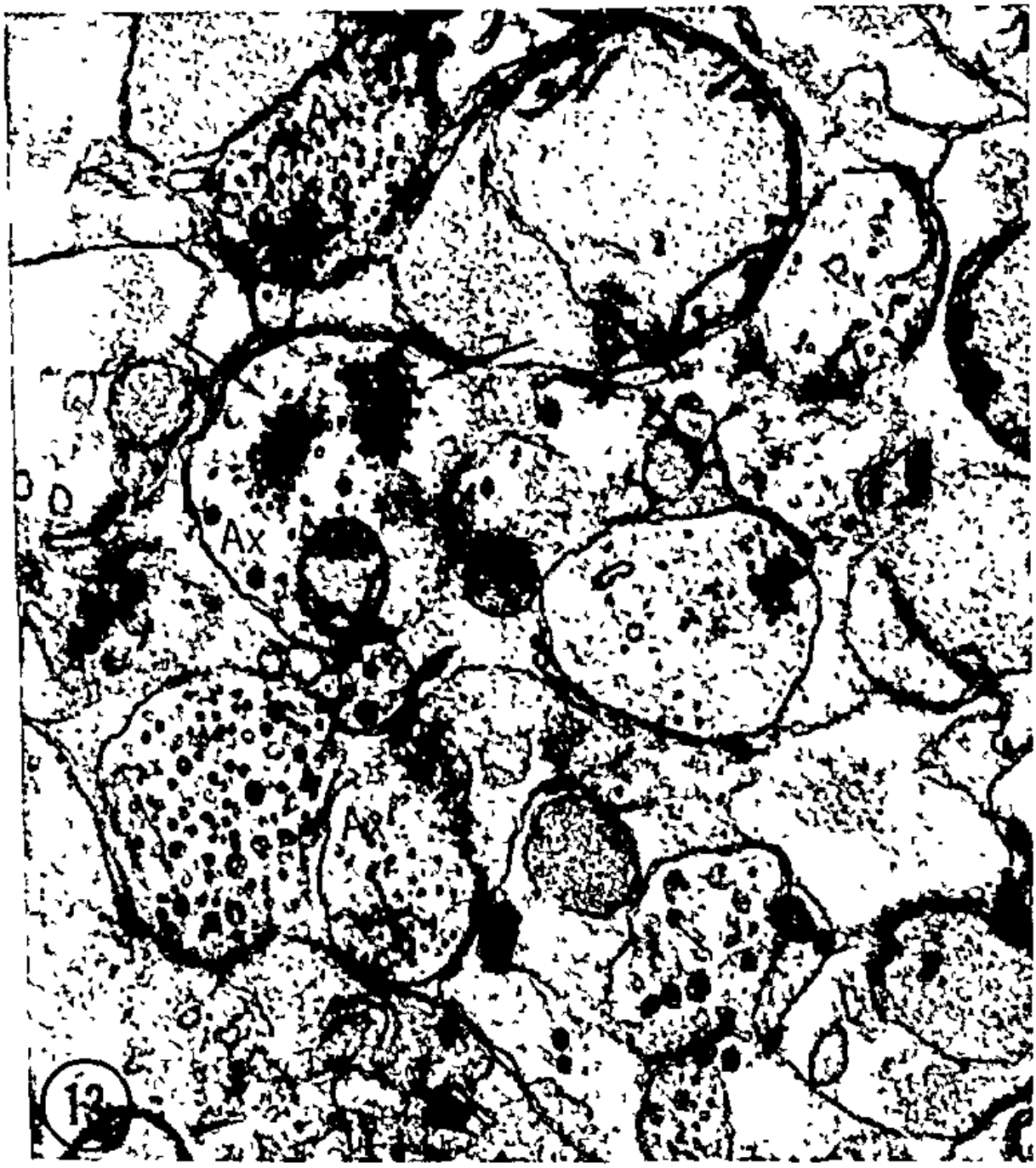


Fig. 13 Axo-dendritic synapses after potassium permanganate fixation. Among the agranular and large granular vesicles there are small electron dense vesicles. Sometimes these latter occur in groups, as indicated by the arrows. Specimen processed as in Fig. 12. × 42 500

electron microscopic criteria, which are not absolutely accurate (Hartmann 1958, Palay 1958). The scanty cytoplasm of these astrocytes consisted of endoplasmic reticulum, Golgi apparatus, a few mitochondria and lysosomes (Fig. 18).

In the vicinity of the optic chiasma, oligodendrocytes were sometimes found. No microglial cells were observed.

After dehydration, the number of glial cells was not found to be increased. The glial processes were, however, more numerous and appeared swollen and pale round the capillaries (Fig. 19). Between the neurosecretory cells the glial processes were narrow and tightly packed between the hypertrophic neurosecretory cells.

DISCUSSION

In the present study, fixation by perfusion was found to be clearly superior to immersion fixation, which was used by Enestrom (1967) and Streefkerk (1967). Indeed, perfusion fixation of nervous tissue has been strongly recommended by several authors (Palay 1960, Gonzáles Aguilar and De Robertis 1963 and Karlsson and Schultz 1965) as giving reliable and reproducible results. The perfusion technique was easy using a blood-transfusion apparatus described in the present study. All the fixatives were hyperosmolar compared to the osmolarity of the blood plasma. Potassium permanganate was used here for the first time as a perfusion fixative. Earlier it has been used exclusively as an immersion fixative (Luft 1956, Richardson 1966 and Hökfelt 1968).

Two types of neurosecretory cells were demonstrated both by light microscopy in toluidine-blue-stained epoxy resin sections and by electron microscopy. The existence of dark cells has been a matter of dispute. The dark cells have been ascribed to poor tissue preservation or to trauma due to tissue manipulation before immersion fixation (Mugnaini 1965, Wood 1967). There have been controversial reports concerning the heterogeneity of the neurosecretory cells in the rat SON. Zambrano and De Robertis (1966) found two different types of cells in formaldehyde perfused rat's SON. On the other hand, Nemetschek-Gansler (1965) did not find any difference between the cells in glutaraldehyde perfused SON. Only osmotically stressed rats' neurosecretory cells showed some variety. Enestrom (1967) described two types of cells in glutaraldehyde immersion fixed material, but Streefkerk (1967) found only one type of neurosecretory cells in SON immersion fixed in glutaraldehyde. Enestrom's (1967) A-cells are probably identical with the type II cells of Zambrano and De Robertis (1966) and with the dark cells described in the present study, in which the cell types were observed with all fixatives with the exception of potassium permanganate, which is known to dissolve proteins (Hake 1965).

A large amount of ribosomes was characteristic of the dark cells. Free ribosomes increased after 3, 5 or 7 days' dehydration. In the light cells a similar response was observed after 3 and 5 days' dehydration, but a decrease of ribosomes was noticed after 7 days' dehydration. This might reflect a failure of these cells in producing protein, or perhaps neurosecretory production occurs at this functional stage at the expence of the Nissl substance or endoplasmic reticulum (Bargmann



Fig. 14
Axo-somatic synapses of a light neurosecretory cell in control rat SON. Two synaptic terminals are in contact with a light neurosecretory cell. There are several large granulated vesicles among agranular vesicles. Specimen processed as in Fig. 3. × 25 500

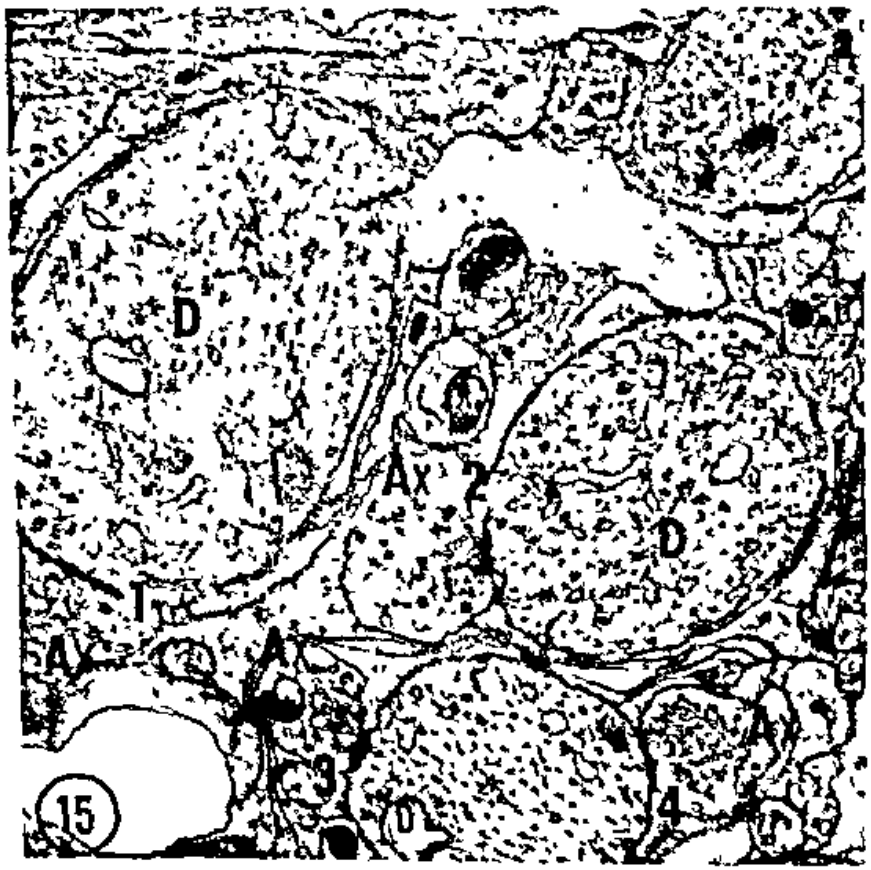


Fig. 15
Transverse section of three large dendrites with synapses. Four axo-dendritic synapses are seen. (1, 2, 3 and 4). Three of them (1, 2 and 3) contain large granular vesicles. Specimen processed as in Fig. 3. × 25 500

1949, 1954, Nemetschek-Gansler 1965, and Zambrano and De Robertis 1966). No decrease of ribosomes was noticed in the dark cells. This is in agreement with the findings of Nemetschek-Gansler (1965).

Swollen mitochondria were sometimes found after primary glutaraldehyde fixation. These are probably attributable to the fixative itself and do not represent any special functional stages in contrast to what was proposed by Streefkerk (1967). With other fixatives the preservation of mitochondria was uniform. The size and shape of mitochodria did not change after dehydration. This is in agreement with the studies of Eneström and Hamberger (1968).

The large inclusion bodies found in the present study in both the light and the dark cells are possibly lysosomes, and the type II large inclusion bodies would fit into the category of multivesicular bodies (Palay 1960, Osinchak 1964a, Zambrano and De Robertis 1966). The function of lysosomes will be further discussed in Part IV.

The small inclusion bodies are interpreted as neurosecretory elementary granules by several authors (Palay 1960, Bern *et al.* 1961; see *e.g.* Streefkerk 1967). In the present study, these granules were often seen budding off from the flattened Golgi cisternae. This observation favours the theory that the neurosecretory granules, at least during the final steps, are processed by the Golgi apparatus, as suggested by Zambrano and De Robertis (1966).

The almost complete disappearance of the elementary granules here obtained after 5 or 7 days' dehydration of the rats is in agreement with the findings of Streefkerk (1967). After 3 days' dehydration of the rats the amount of the elementary granules decreased considerably. At this stage of dehydration, Zambrano and De Robertis (1966) reported an increase and dispersion of the elementary granules in the neurosecretory cells.

All three types of synapses were of Gray's type I or II (Gray 1959). No spine apparatuses were noticed. The existence of both agranular and granular vesicles in the axonal synaptic terminals is suggestive of both cholinergic and adrenergic mechanisms in neuronal transmission of the SON of the rat (De Robertis 1959, Koelle 1962, Pellegrino De Iraldi *et al.* 1963, Hökfelt 1968). Especially the small granulated vesicles, demonstrated only with potassium permanganate fixation, are highly suggestive of catecholamine-containing nerve terminals in the SON (Hökfelt 1968). The possible stimulating and inhibitory roles of these two transmitter types are discussed in detail in chapters II and III.

The axo-axonal synapses were also described by Klein et al. (1968). Although the main branch of the neurosecretory cell is functionally considered an axon, it has an inner structure of a dendrite, as already pointed out by Palay (1960).

The ultrastructure of the capillaries in the rat SON was essentially similar to that of the capillaries of other brain areas (Bloom and Fawcett 1968). No pericapillary space was found, such as was described by Streefkerk (1967) after immersion fixation. Glial processes in most cases separated the neurosecretory cells and axonal swellings from the capillaries. The glial processes obviously play an important role in the osmoreceptory functions between the capillaries, axons and the neurosecretory cells.

After 5 or 7 days' dehydration, when the nerve cell bodies were greatly hypertrophic, the glial elements between the cells appeared narrow. On the other hand, the distance between the capillaries and the neurosecretory cells increased and

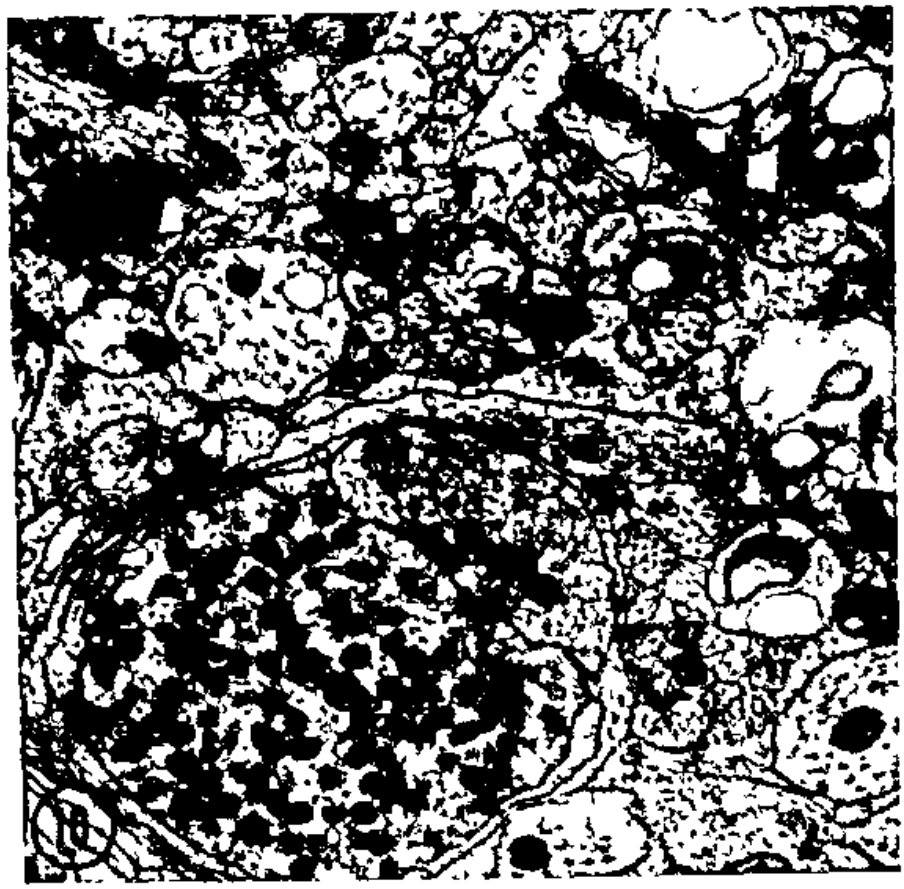


Fig. 16
Transverse section of an axon with synapsis.
An axon terminal with two large granular vesicles is in contact with an axon. The latter is crowded with granules of varying electron opacity. The synaptic nerve fibres are encircled by a glial element. Specimen processed as in Fig. 3. ×25 500



Fig. 17
Part of a capillary wall and axonal swellings in control rat SON.
Two axonal swellings are seen in the vicinity of the capillary wall. There are thin glial sheets between them and the basal lamina. Specimen processed as in Fig. 3. ×25 500

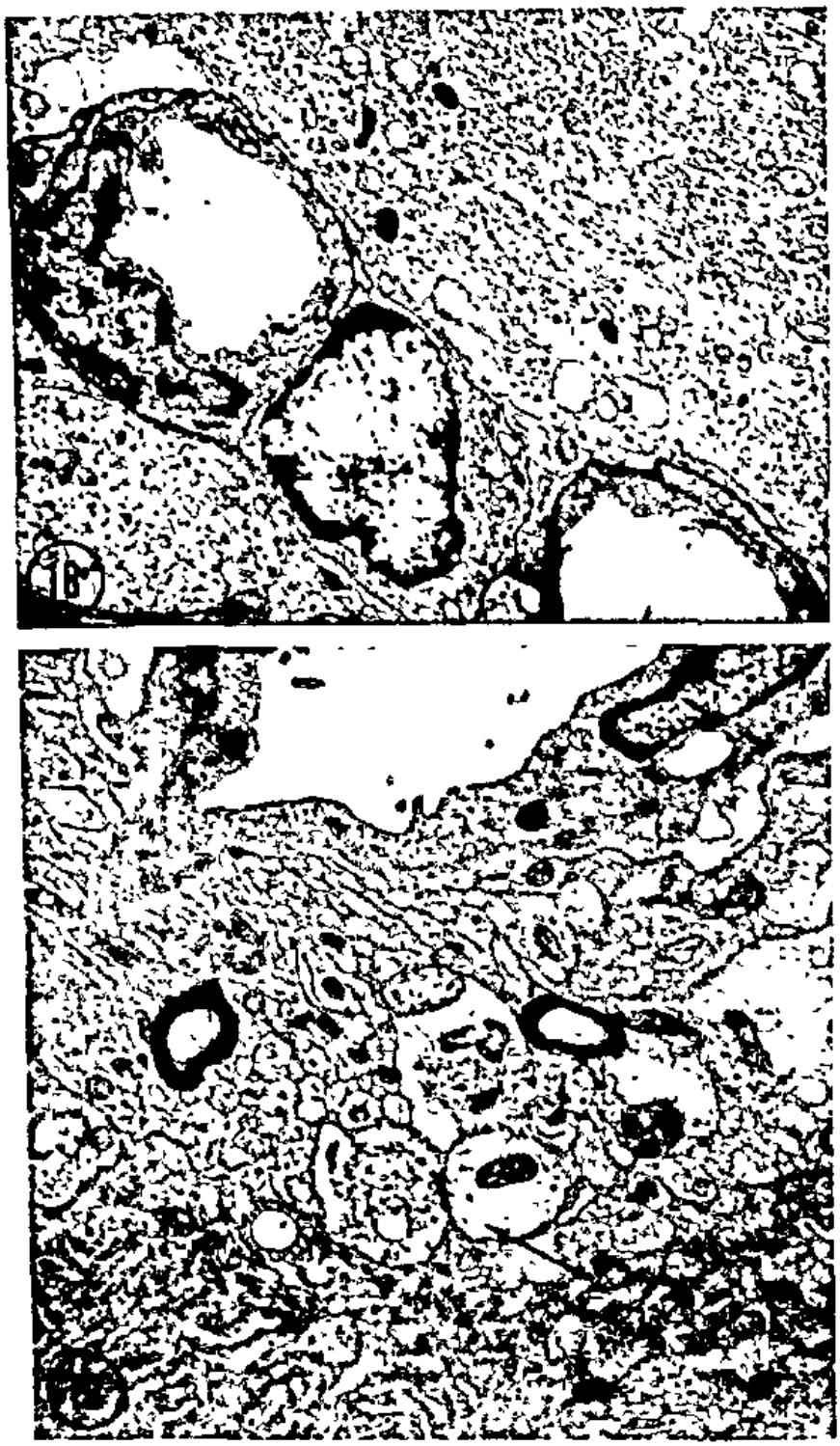


Fig. 18 A glial cell between two capillaries.
A protoplasmic astrocyte is located between two capillaries closely connected with a light neurosecretory cell. Specimen processed as in Fig. 3. × 6 600

Fig. 19 Capillary wall and glial processes after 7 days' dehydration. Note the increased distance between the capillary wall and the dark neurosecretory cell beneath. The basal lamina appears thicker than in the control capillaries. An arrow points to an axo-dendritic synapsis. Specimen processed as in Fig. 3. × 10 200

the glial elements appeared hypertrophied and swollen. This observation is entirely different from that made by Enestrom (1967), who used immersion fixation. According to him, all the glial elements were reduced after dehydration stress. The disparity between our observations might be due to the different fixation procedures. The present writer's observation agrees with that of Murray (1968), who reported a distinct proliferation of neuroglial cells in the rat SON after salt loading. It is of interest in this context that the number of pituicytes is known to increase after dehydration (Ortmann 1951).

II. EFFECT OF SYMPATHECTOMY, RESERPINE AND OXYPERTIN ON THE NEUROSECTORY CELLS AND NERVE ENDINGS IN THE RAT SUPRAOPTIC NUCLEUS

REVIEW OF THE LITERATURE

SYMPATHECTOMY

The localization of brain monoamines was studied with formaldehyde-induced fluorescence by Carlsson *et al.* (1962). The nerve cells in the rat SON were surrounded by fine varicose fibres, which emitted greenish fluorescence. Bilateral cervical sympathectomy had no effect on the fluorescence of these fibres.

So far there have been no reports of electron microscopic observations on the SON after cervical sympathectomy.

EFFECT OF RESERPINE

It is generally agreed that reserpine depletes noradrenaline, dopamine and serotonin from the nervous tissue by blocking the uptake and storage mechanism of the granules (Brodie *et al.* 1957, Shore *et al.* 1957 and Bertler 1961).

The fluorescent varicose fibres round the non-fluorescent nerve cells in the rat SON have been reported to disappear completely after a single dose of reserpine as low as 0.5 mg/kg i.p. (Carlsson *et al.* 1962).

There is division of opinion as regards the fate of the large granular vesicles in brain tissue after reserpine treatment. A marked decrease in the number of these vesicles in the anterior hypothalamus of the rabbit was reported by Shimizu and Ishii (1964). Pellegrino De Iraldi and De Robertis (1961) described a complete disappearance of granulated vesicles from the pineal body of the rat after reserpinization. On the contrary, Fuxe *et al.* (1965, 1966) and Bloom and Aghajanian (1967, 1968) found that reserpine treatment had only little, if any effect on the rat hypothalamus.

The SON of the rat was not examined at all in any of the above-mentioned studies.

EFFECT OF OXYPERTIN

Oxypertin, also called Win 18501-2, or 1- [(5.6-dimethoxy-2-methyl-3-indole)-ethyl]-4-phenylpiperazine has been reported to deplete catecholamines from the adrenergic nerve terminals.

The number of noradrenaline-containing granular vesicles decreased without any notable effect on 5-HT (Spector *et al.* 1962) and dopamine (Matsuoka *et al.* 1965) containing granular vesicles. However, the concentration of biochemically determined dopamine in hypothalamus has been found to be increased (Matsuoka *et al.* 1965). Fuxe *et al.* (1967) reported that after a relatively high dosis of Oxypertin all these amines were affected. However, the noradrenaline content of the rat whole brain was biochemically most affected, being reduced to 12 per cent of the control level, while dopamine was reduced to 51 per cent and 5-HT to 54 per cent, respectively.

The disappearance of fluorescent fibres after Oxypertin treatment does not occur with equal ease in all brain areas. In the rat hypothalamus a considerable number of nerve fibres with weak or medium fluorescence were still demonstrated after a relatively large dosage of Oxypertin (Fuxe *et al.* 1967).

MATERIAL AND METHODS

Fifty-seven adult, male rats of about 300 g weight were used in this part of the study. Bilateral cervical sympathectomy was performed on ten rats. They were sacrificed 5 days after operation.

Twelve rats received reserpine (Serpasil^{R}, Ciba AG) 3 mg/kg i.p. on four consecutive days before killing. Twelve further rats received Oxypertin (Winthrop, London) 25 mg/kg, 50 mg/kg and 75 mg/kg i.p. Oxypertin was dissolved in lactic acid diluted with distilled water and injected immediately after preparing the solution. The rats were killed 3 hours after the injection. Sympathectomy controls received no treatment. Drug controls were injected with physiological saline i.p.

For fluorescence microscopy, the rats were decapitated under Nembutal anaesthesia. About 2 mm thick hypothalamic slices including the SON areas were frozen, immediately after removal, in isopentane and liquid nitrogen. The tissue pieces were freeze-dried for seven days *in vacuo* at —35°C, according to the method described by Eränkö (1961, 1967). The frozen-dried tissue blocks were treated for one hour at 80°C with formaldehyde gas prepared from paraformaldehyde equilibrated with 60 % humidity; infiltrated with paraffin *in vacuo* and cut at 5 μ. Sections were mounted in xylene, examined, and the fluorescence was photographed with a Wild fluorescence microscope equipped with a high-pressure mercury lamp HBO-200 (Osram). The light was filtered through a Schott K2 heat-absorbing filter and Schott BG12 filter. The stop filters in the tube were Leitz Euphos and Schott OG1.

The sections were examined by light microscope after toluidine blue staining to make sure of the proper area.

For elecetron microscopy, the rats were perfusion-fixed in Nembutal anaesthesia with 2.5 % glutaraldehyde in 0.1 M phosphate buffer, pH 6.9. The 15 minutes' perfusion fixation was further prolonged with 3 hours' immersion fixation in the same fixative and two hours' in OsO_4 (Rhodin 1954). Dehydration, embedding and trimming of the blocks were performed as described in Chapter I. The thin sections were poststained with uranyl acetate and lead citrate. The sections were examined and photographed with Philips EM 200 at 80 kV.

The number of large granular vesicles in the control and experimental specimens was counted in 50 synaptic terminals using photographs with the same magnification.

RESULTS

After sympathectomy ptosis and miosis of the eyes were always observed.

After four daily reserpine injections the rats were heavily sedated, as they were shortly after injection with 50 mg/kg and 70 mg/kg of Oxypertin. The sedative effect of Oxypertin was noticed as early as 5—10 minutes after the injection.

FLUORESCENCE MICROSCOPY

The fluorescence microscopic observations in rats after sympathectomy, reserpine and Oxypertin treatments are reported in Table I.

TABLE I. *Fluorescence intensity in the nerve terminals of SON*

	Nerve cells	Nerve fibres
Control specimens	—	+++
After sympathectomy	—	+++
After reserpine	—	—
After Oxypertin	—	+

— = no fluorescence
\+ = weak fluorescence
++ = medium fluorescence
+++ = strong fluorescence

ELECTRON MICROSCOPIC OBSERVATIONS

Neurosecretory cells

No changes were noticed in the neurosecretory cells of the sympathectomized rats when compared to the controls.

After reserpine treatment, the number of small inclusion bodies decreased in both the light and the dark neurosecretory cells. This decrease is illustrated in Figs 20 and 21. Empty vesicles of the same size as the small inclusion bodies



Fig. 20
A light neurosecretory cell of the SON after Serpasil (3 mg/kg four times) treatment.
Note almost complete loss of small inclusion bodies (neurosecretory granules). The amount of large inclusion bodies and ribosomes corresponds to that seen in Fig. 21. Glutaraldehyde perfused and immersion fixed, refixed in OsO_4. Poststained with uranyl acetate and lead citrate. × 10 200

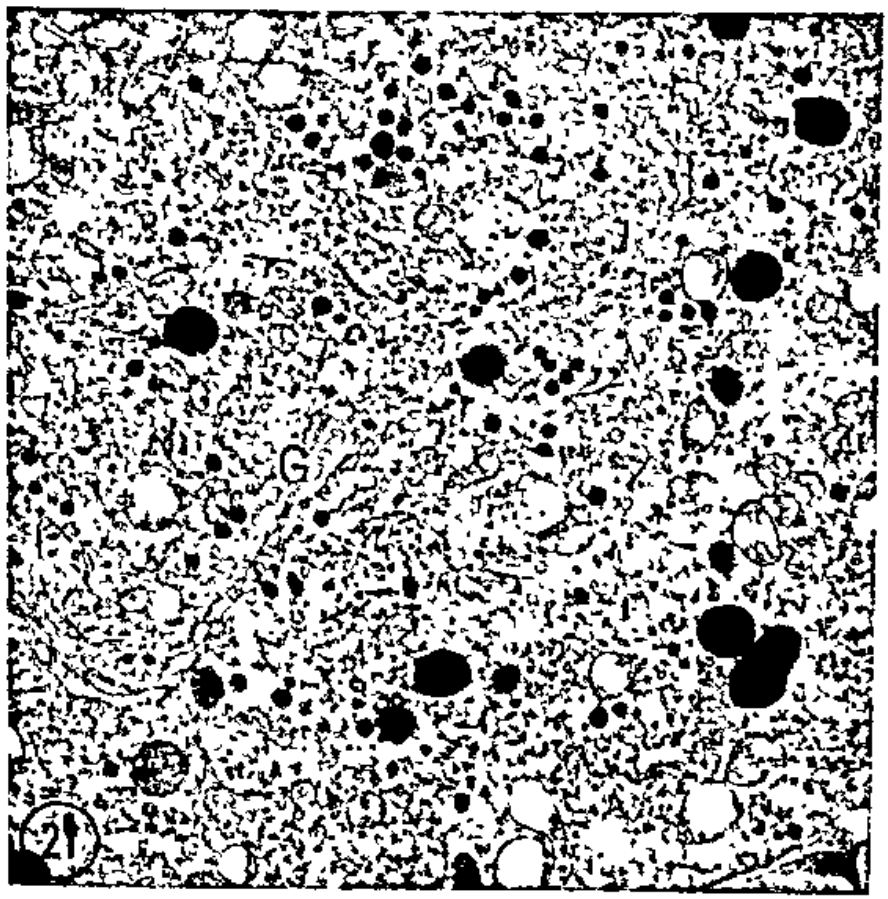


Fig. 21
Electron microscopic view of a light neurosecretory cell of control rat SON.
Note the amount of small inclusion bodies when compared to Fig. 20 and 23. Specimen processed as in Fig. 20. × 10 200

were found near the flattened Golgi cisternae, as seen in Fig. 22. The number of ribosomes and large inclusion bodies did not show essential changes (Figs. 20 and 21).

All the Oxypertin doses employed had the same effect on the neurosecretory cells as reserpine, the main effect being a decrease in the number of small inclusion bodies (*cf.* Figs. 23 and 21).

Synapses

With combined glutaraldehyde and OsO_4 fixation, only the large type of granular vesicles was demonstrable in axon terminals, among the numerous small agranular vesicles. Neither sympathectomy nor reserpine or Oxypertin treatments had any effect on the number of these large granular vesicles in the synaptic endings and axons. The size of the granular vesicles was also approximately equal to that in the control animals, and the same applies to the electron density of the central core. The localization of the large granular vesicles in the granular vesicle population in presynaptic endings was found to correspond to that of the controls. Axo-somatic, axo-dendritic and axo-axonal synapses after the experimental procedures are seen in Figs. 24, 25 and 26. The appearance of large granular vesicles can be compared to the control specimen in Fig. 27.

DISCUSSION

The histochemical fluorescence method developed and introduced by Eränkö (1951c, 1955, 1967), Falck *et al.* (1962), Corrodi and Hillarp (1963) and Corrodi and Jonsson (1967) has proved very sensitive and specific for monoamines in nervous tissue. With this valid method it was possible to demonstrate the fine, varicose fluorescent nerve fibre net round the non-fluorescent nerve cells of the rat SON. On the basis of several histochemical and pharmacological studies it has been firmly established that these greenish fluorescent fibres are evidently noradrenaline-containing nerve terminals (Carlsson *et al.* 1962, Fuxe 1965).

Carlsson *et al.* (1962) suggested that the superior cervical ganglia were not the source of monoamine terminals to the rat SON. The present writer's observations agreed with his. There was no detectable change in the fluorescence intensity of the fibres between the operated and control animals. At ultrastructural level no changes were seen in the morphology of the neurosecretory cells or in the synaptic endings. The pons and medulla oblongata possibly contain the neuronal cell bodies of the nerve terminals found in the rat SON (Fuxe 1968). This assumption is based on the stereotactic studies carried out by Andén *et al.* (1966 a, 1966b).

The fluorescent fibres of the rat SON are known to be very sensitive to reserpine when observed by fluorescent microscopy. After a single dose of 0.5 mg/kg i.p. reserpine no fluorescence could be detected in this area (Carlsson *et al.* 1962). Thus, it was not surprising that fluorescence was completely abolished by the four repeated doses 3 mg/kg i.p. of reserpine here employed.

At ultrastructural level no change was noticed in the amount or density of the large granular vesicles in the three types of synapses, respectively. This observation is in agreement with the reports of Fuxe *et al.* (1965, 1966) and Bloom and Agha-

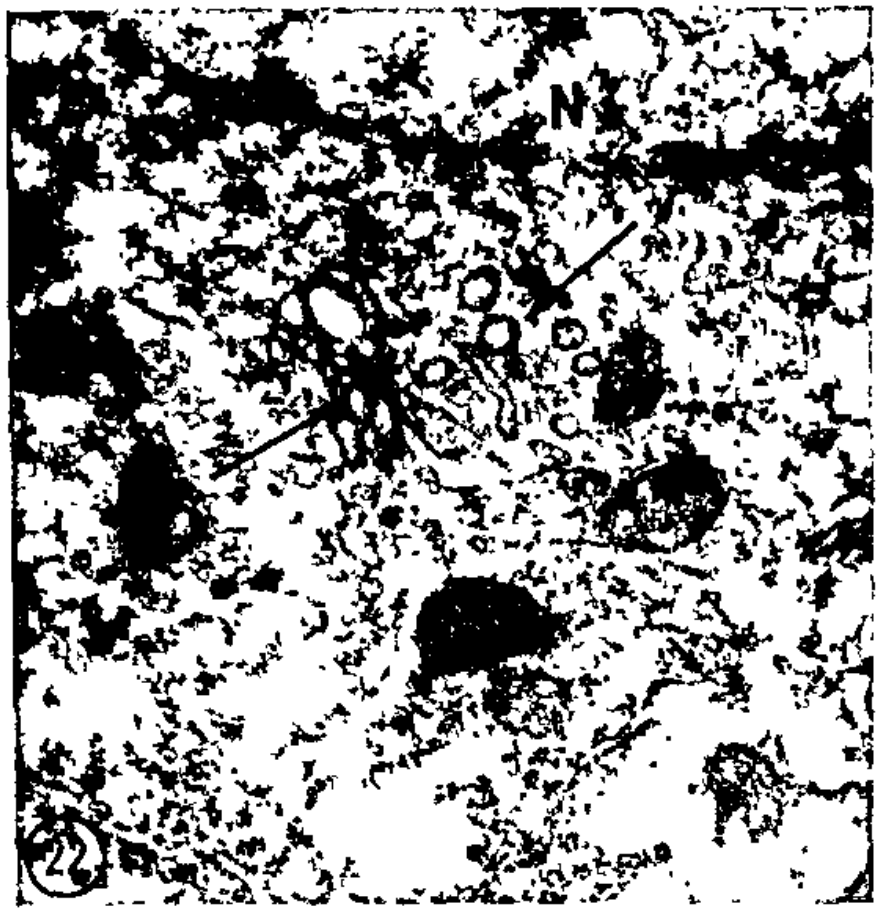


Fig. 22
A light neurosecretory cell of the SON after Serpasil (3 mg/kg four times) treatment.
Empty vesicles of 700 Å size, indicated by arrows, are seen near the Golgi apparatus. Specimen processed as in Fig. 20.
× 39 000



Fig. 23
A light neurosecretory cell of the SON after Oxypertin (70 mg/kg) treatment.
Several Golgi complexes are seen, in their vicinity only few small inclusion bodies are observed. Specimen processed as in Fig. 20.
× 10 200



Fig. 24 General view of the neuropile and a neurosecretory cell after bilateral cervical sympathectomy. *Three types of synapses are seen. In all these synapses there are* large granular vesicles. One arrow points to an axo-somatic synapsis containing several large granular vesicles. Two arrows point to an axo-dendritic synapsis, a small arrow to an axo-axonal synapsis containg one large granular vesicle. Specimen processed as in Fig. 20. × 17 000



Fig. 25 Axo-dendritic synapses after Serpasil (3mg/kg four times) treatments. Among agranular vesicles there are large granular vesicles of the same size and shade as in Fig 27. Proced res as in Fig. 20. × 25 500

Fig. 26 Synaptic terminals after Oxypertin (70 mg/kg) treatment. Several large granular vesicles are seen in two axon terminals in the middle and at the left. The largest terminal at the right contains only agranular vesicles. Procedures as in Fig. 20. × 25 500

janian (1967, 1968). According to them reserpine had no notable effect on the large granular vesicles in the rat hypothalamic nerve terminals.

It is a matter of dispute whether the large granular vesicles in brain tissue are catecholamine storage granules at all. However, several studies favour the view that in hypothalamic areas they form at least a part of the total monoamine reservoir. The number of large granular vesicles corresponds well with the biochemically determined concentration of noradrenaline (Ishii *et al.* 1965, De Robertis *et al.* 1965, De Robertis 1966). Aghajanian and Bloom (1966) found that, on autoradiography, tritiated H-noradrenaline accumulates on those hypothalamic nerve endings which contain large granular vesicles. Pharmacological studies with reserpine have also shown changes in amount and density of the large granular vesicles in rabbit (Shimizu and Ishii 1964) and in rat (Hökfelt 1968). As fixative Hökfelt used potassium permanganate by which he was able to demonstrate both small and large granular vesicles. Reserpine abolished both these types of vesicles in rat hypothalamus.

In the light of the recently accumulated extensive literature (cited by Hökfelt 1968) it seems evident that many factors are involved in the depletion of large granular vesicles. Reserpine sensitivity differs in the various animal species, in various brain areas, and is dependent on the doses employed. In addition, the controversial results are due to the different fixatives used. The mechanisms by which the fixatives react with the central cores in the large granular vesicles are of importance. Glutaraldehyde is known to react with proteins, while, on the other hand, potassium permanganate dissolves them (Hake 1965). In the present study, only glutaraldehyde-OsO_4 combination was used as fixative. With this fixation the central cores of the large granular vesicles were unchanged after a relatively large reserpine dose which histochemically abolished the fluorescence in the nerve fibres.

The small inclusion bodies were markedly reduced in the neurosecretory cells after reserpine and Oxypertin treatments. The noradrenaline containing nerve terminals are assumed to be inhibitory in the rat SON (Fuxe and Hökfelt 1967). Stimulation of antidiuretic hormone secretion after reserpine treatment was reported by Gaunt *et al.* (1955) and Gabe *et al.* (1961). The disappearance of the small inclusion bodies from the neurosecretory cells seems appropriate, because monoamine inhibition is blocked by reserpine and Oxypertin.

After Oxypertin treatments with doses 25 mg/kg, 50 mg/kg and 75 mg/kg i.p. there remained a faint but distinct fluorescent fibre net around the SON nerve cells. The same observations were reported by Fuxe *et al.* (1967) for rat hypothalamic noradrenaline containing fluorescent fibres.

The electron microscopic findings concerning the amount and density of the large granular vesicles in synaptic endings were similar to those after reserpinization. There was no difference in the effect of various doses of Oxypertin. It was believed that Oxypertin depleted selectively the different monoamines in brain tissue (Matsuoka *et al.* 1965, Bak and Hassler 1968). These authors reported a decrease in the number of large granular vesicles in hypothalamic areas, while no effect was noticed for instance in substantia nigra and nucleus caudatus, which have a high dopamine content (Bak 1967). Further it was suggested that the lower doses up to 35 mg/kg i.p. of Oxypertin depleted only noradrenaline, whereas higher doses had an equal effect on dopamine and serotonin (Makin

1968). Oxypertin acts *in vivo* as an uptake-storage mechanism blocker of the granular pool, and its effect is mainly the same as that of reserpine. In the present study the observations after Oxypertin treatment were independent of the doses employed.

It seems that in the rat SON the large granular vesicles in the synaptic terminals and axons at ultrastructural level are resistant to the depletory substances, reserpine and Oxypertin.



Fig. 27 A control section of synaptic terminals.
The arrows point to two axon terminals containing several large granular vesicles.
Procedures as in Fig. 20. × 25 500

III. CHOLINESTERASE ACTIVITY IN THE SUPRA-OPTIC NUCLEUS OF NORMAL AND DEHYDRATED RATS

REVIEW OF THE LITERATURE

In 1939 and 1947 Pickford suggested that cholinergic synapses might be involved in the release of the neurohypophyseal hormones in the pituitary of the dog. This was later confirmed by Duke *et al.* (1950), Abrahams and Pickford (1954, 1956).

LIGHT MICROSCOPIC OBSERVATIONS

Several light microscopic studies have been published on the histochemical demonstration of ChEs in the neurosecretory systems of various animal species under normal conditions. The first report was published by Koelle (1954); he found a moderate ChE activity in the rat SON. The enzyme activity was confined to the neurons and fibres.

Abrahams *et al.* (1957) mapped the cholinergic areas of the dog hypothalamus. Three nuclei of the anterior hypothalamus were found to contain AChE: the SON, the paraventricular and the suprachiasmatic nuclei. In the SON, AChE was located in the perikarya of the neurons and in the very proximal parts of the axonal and dendritic fibres. Ns. ChE appeared to be localized as dense rings between the neurosecretory cells.

Uemura (1965) studied systematically the distribution of these enzymes in several animal species: frog, newt, turtle, bird and rat. He found a relatively strong enzyme activity in rat neurosecretory cells using acetylthiocholine as substrate and no activity at all with butyrylthiocholine. Ns. ChE activity was demonstrated in the capillaries of the neurosecretory areas.

There have been several reports of ChE in the neurosecretory nuclei of other animals: in the ox, sheep, pig, rabbit, fowl and pigeon (Arvy 1962) and in the sparrow (Kobayashi and Farner 1964). A moderate ChE activity was reported in the monkey SON by Iijima *et al.* (1967).

In 1957, Pepler and Pearse reported their observations that after 14 days' dehydration the neurosecretory cells in the SON of rats showed a decrease in the intracellular non-specific esterase activity. At the same time a rise in AChE activity in the cells was demonstrated. Ns. ChE and possible changes in its activity were not reported in this study.

ELECTRON MICROSCOPIC OBSERVATIONS

The present author (Rechardt 1967) published a short report of electron microscopic demonstration of ChE in the rat SON. Up to the present time there have been no extensive studies concerning ChE localizations at ultrastructural level in either normal or experimental conditions.

MATERIAL AND METHODS

Fifty-five adult male rats of about 300 g weight were used; 12 rats were kept without water for 7 days. The rest served as controls.

The rats were perfused under ether anaesthesia *via* the abdominal aorta. The fixation fluid consisted of 3.5 % formaldehyde in 0.3 M sucrose, buffered with 0.075 M phosphate to pH 7.2. The brain was dissected out after 15 minutes' perfusion and the hypothalamic area was immersed in the same fixative overnight. The tissue piece was then washed during two hours in several changes of ice-cold 0.075 M phosphate buffered 0.3 M sucrose pH 7.2. For light microscopy, 10 μ thick sections were cut with a freezing microtome. For electron microscopy, both SONs were dissected under a stereo microscope and cut with a razor blade in thin slices. Two methods for light microscopy were employed: the Koelle method (1951) as modified by Gomori (1952), and the copper-ferrocyanide method described by Karnovsky and Roots (1964). For electron microscopy, only the latter method was employed.

In the Koelle-Gomori method the incubation was carried out at 37°C. Preincubation without substrate but with various inhibitors lasted 20 minutes and incubation with inhibitors and substrates 4 hours. The pH was adjusted to 6.0.

In the Karnovsky-Roots method for electron microscopy the incubation was carried out at 0°C for 20 minutes and with substrates for 60 minutes. No sucrose was added to the incubation medium. The tissue slices were all handled free-floating.

The substrates used were acetylthiocholine iodide and butyrylthiocholine iodide (Fluka AG., Buchs). The inhibitors were 10^{-5} M eserine, 10^{-4} M, 10^{-5} M and 10^{-6} M iso-OMPA (tetra-isopropylpyrophosphoramide) (L. Light & Co. Ltd., Colnbrook, England) and 10^{-5} M 284C51 [1.5-bis(4-allyldimethylammoniumphenyl) pentan-3-one dibromide] (Wellcome Research Laboratories, England).

Some incubations were carried out without substrate in otherwise complete incubation medium.

For electron microscopy, the tissue pieces were washed after incubation in four changes of ice-cold 0.3 M sucrose and refixed for one hour in 1 % OsO_4 (Rhodin 1954). Dehydration was carried out in graded ethanol series and the pieces were embedded in Epon. Thick (1 μ) sections were stained with toluidine blue and examined under a light microscope to ascertain the proper SON area, which was further trimmed. Silver to grey sections were cut at the depth of 5—10 μ from the surface of the tissue piece. Sections were poststained with lead citrate for 5 minutes (Reynolds 1963). The specimens were viewed and photographed with Siemens Elmiskop I or Philips EM 200 electron microscopes.

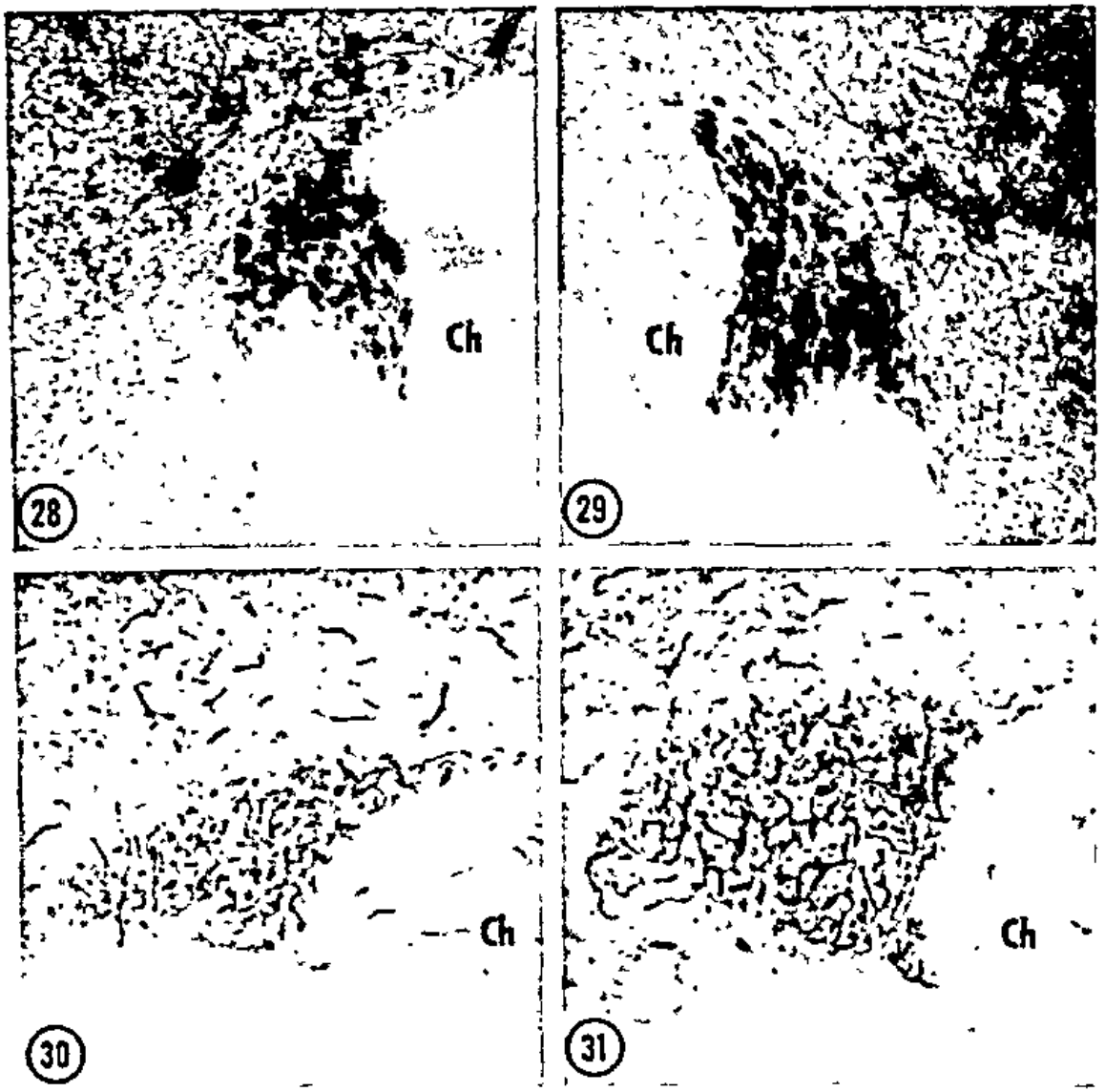


Fig. 28 A light microscopic view of AChE activity in control rat SON. A moderate activity is found in the neurosecretory cells. Some variations in the activity are noticed among the cells. The neuropile and capillaries appear negative.
Note the strongly positive neurons with related fibres in the accessory SON above. Formaldehyde perfused specimen incubated for 3 hours at +37°C in Koelle-Gomori medium acetylthiocholine as substrate and with iso-OMPA 10^{-4} M. Ch (chiasma opticorum) × 65

Fig. 29 A light microscopic view of AChE activity in rat SON after 7 days' dehydration. The reaction product is of equal amount and distribution as in the control specimens. *Cf.* Figs. 28 and 29.
Specimen processed identically to that in Fig. 28. Ch (chiasma opticorum) × 65

Fig. 30 A light microscopic view of ns. ChE activity in control rat SON. The capillary walls show a moderate activity. The reaction product in the capillary walls is of the same amount as in the surrounding hypothalamic capillaries. The specimen was handled as in Fig. 28, but butyrylthiocholine was used as substrate and 284C51 10^{-4} M as inhibitor. Ch (chiasma opticorum) × 65

Fig. 31 A light microscopic view of ns. ChE activity in rat SON after 7 days' dehydration.
The capillary walls show more reaction product than in the control specimens. *Cf.* Fig. 30. There is a distinct difference in activity between the capillaries of the SON and those of the surrounding hypothalamic areas, which show weaker activity.
Specimen processed as in Fig. 30. Ch (chiasma opticorum) × 65

RESULTS

A. AChE ACTIVITY

LIGHT MICROSCOPIC OBSERVATIONS

The reaction product was seen in the perikarya of nerve cells of the SON. The neuropile appeared negative, as did the capillaries, when iso-OMPA in 10^{-5} M concentration was used as inhibitor. When iso-OMPA 10^{-6} M was used, a weak capillary activity was noticed.

The AChE activity in the main SON was moderate but it was much stronger in the accessory SON neurons after one hour incubation in Karnovsky-Roots medium, or 4 hours' incubation in Koelle-Gomori medium (Fig. 28).

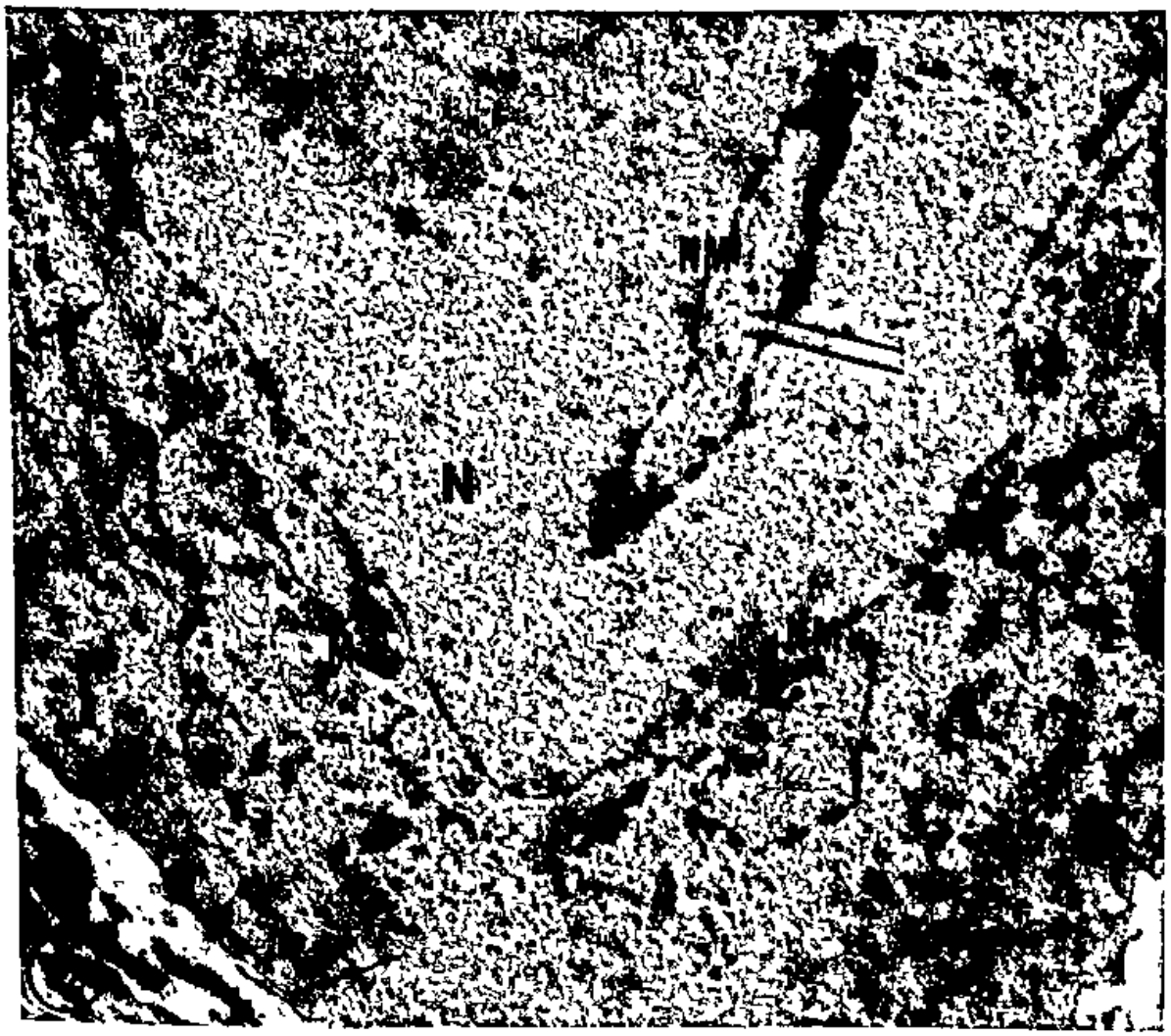


Fig. 32 AChE activity in the neurosecretory cell of control rat SON. The reaction product is localized in the rough surfaced endoplasmic reticulum (ER) and in the nuclear membrane (NM). A typical invagination of the nuclear membrane (NM) with reaction product is observed (double arrow). The Golgi apparatus, indicated by arrows, is negative.
Formaldehyde perfusion fixation, acetylthiocholine as substrate, iso-OMPA 10^{-5} M as inhibitor. Refixed in OsO_4 and poststained with lead citrate. × 11 300

After 7 days' dehydration the neurosecretory cells appeared larger than in the control sections. The reaction product was localized in the cytoplasm of the nerve cells without any other demonstrable localizations. The intensity of enzymatic activity was equal to that found in the control specimens (*cf.* Figs. 29 and 28).

ELECTRON MICROSCOPIC OBSERVATIONS

Considerable variation was observed in the activity of individual neurosecretory cells. The reaction product occurred in the nuclear membrane as a discontinuous line and in the rough surfaced endoplasmic reticulum cisternae at the periphery

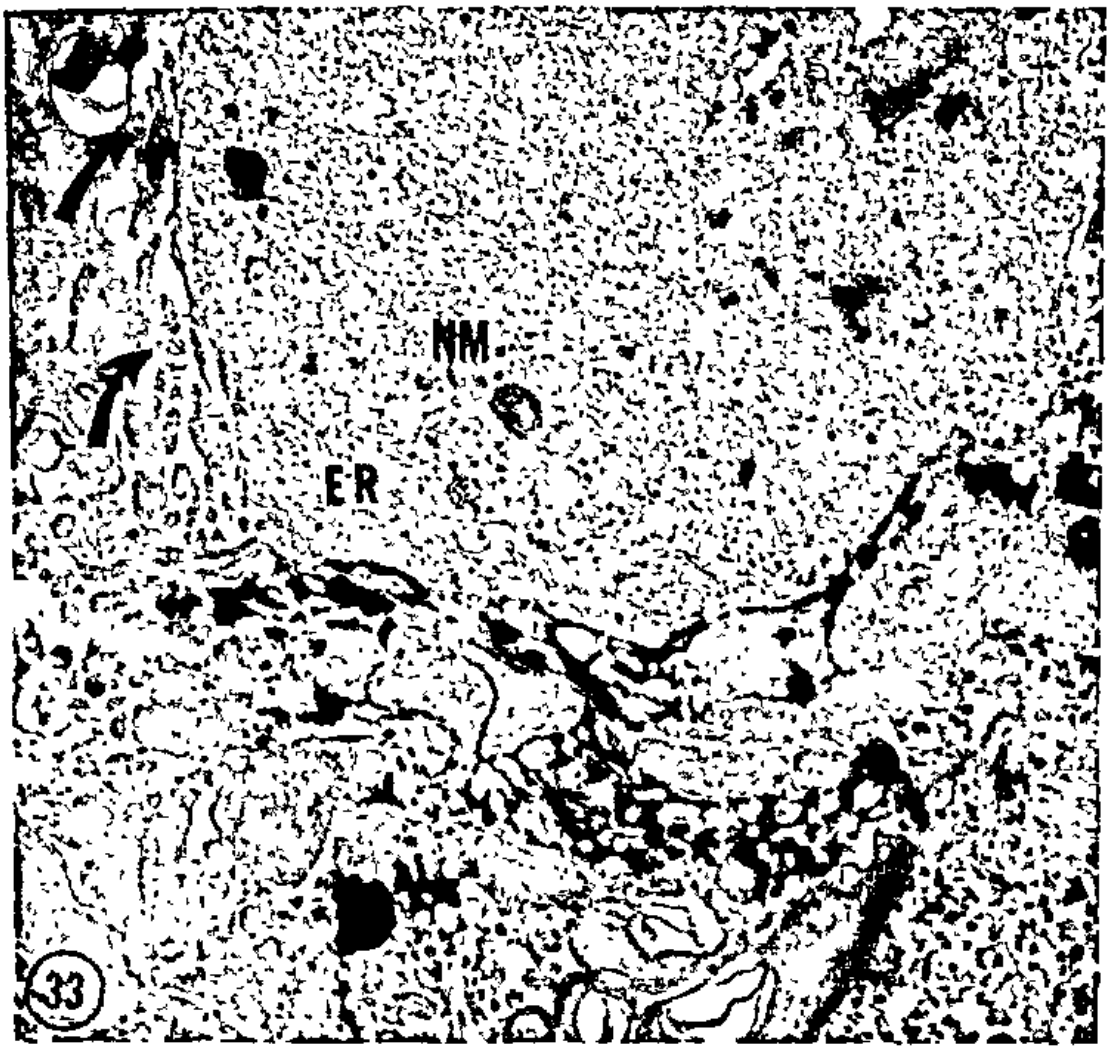


Fig. 33 AChE activity in the neuropile which adjoins an almost negative neurosecretory cell of the SON. The nerve fibres are surrounded by reaction product, which also occurs between them and the cell membrane. No reaction product is found in the cell membrane when adjoining a negative neuropile indicated by arrows. Specimen processed as in Fig. 32. · 11 300

of the cells (Fig. 32). The nucleus, the Golgi apparatus, the mitochondria and the small and large inclusion bodies were always non-reactive. The reaction product sometimes had affinity to the nucleolus. Among the reactive cells, like those seen in Fig. 32, there were always a few almost non-reactive cells (Fig. 33). Round these non-reactive cells, nerve fibres encircled by dense reaction product could be detected.

When the specific inhibitor of ns. ChE, iso-OMPA, was employed in concentrations 10^{-5} M and 10^{-6} M, a weak but distinct activity was seen in the capillary walls, the reaction product being randomly scattered in the cytoplasm of the endothelial cells. When the iso-OMPA concentration was raised to 10^{-4} M, no reaction product was found in capillaries, while the other sites were still reacting.

Glial cells remained non-reactive even when using only 10^{-6} M iso-OMPA.

Eserine essentially abolished the reaction product, so that only a few randomly scattered crystals were found. These had no special affinity to any structures

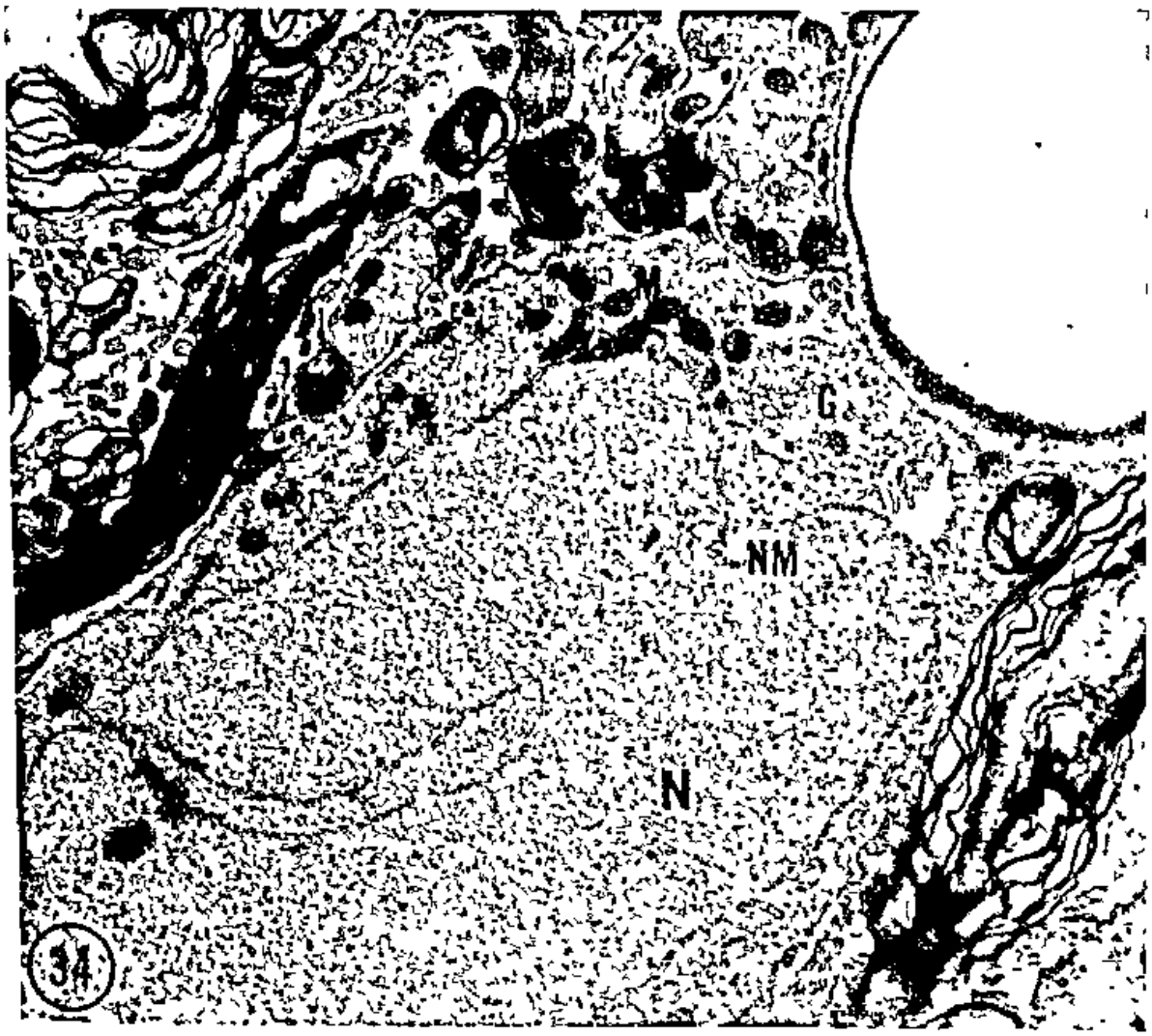


Fig. 34 An almost negative neurosecretory cell of the SON with eserine 10^{-5} M in the incubation medium. Some reaction product crystals are seen without special affinity for any structures.
Procedures as in Fig. 32, eserine 10^{-5} M was used instead of iso-OMPA. × 11300

(Fig. 34). When acetylthiocholine iodide was omitted from the incubation medium, no reaction product was obtained (Fig. 35).

No change was noticed in the distribution and amount of AChE activity even after 7 days' dehydration.

B. Ns.ChE ACTIVITY

LIGHT MICROSCOPIC OBSERVATIONS

The numerous capillaries of the SON exhibited moderate reaction (Fig. 30). The other structures appeared non-reactive.

After 7 days' dehydration, the capillaries appeared widened and their network denser than in the control sections. More reaction product was demonstrated in the walls of these enlarged capillaries (Fig. 31).



Fig. 35 A control section of the SON incubated without substrate. No reaction product is visible. Procedures as in Fig. 32, except that acetylthiocholine was omitted. × 11 300

ELECTRON MICROSCOPIC OBSERVATIONS

The reaction product was localized in the endothelial cells, especially in its nuclear membrane. The basal lamina showed no reaction (Fig. 36). The glial cells showed a weak activity in the cytoplasm and the nuclear membrane (Fig. 38). The neurosecretory cells and the nerve fibres between them were completely non-reactive.

The capillary walls showed an increased amount of reaction product after 7 days' dehydration when butyrylthiocholine was used as substrate and 284C51 10^{-5} M as inhibitor (Fig. 37).

DISCUSSION

TECHNIQUES

Perfusion fixation and the use of unfrozen sections proved essential for a satisfactory ultrastructural preservation. The moderate ChE activity of the rat SON cells favoured the choice of formaldehyde fixation instead of glutaraldehyde, which was tried in preliminary studies and found inhibitory. In this respect the present results agree with those of Brzin *et al.* (1966) and Anderson (1967).

Methodological problems with the Karnovsky-Roots technique have been discussed in detail (Eränkö *et al.* 1967). Especially in the brain, poor penetration of the reagents was hard to overcome. When unfrozen sections were used, an osmotic shock was necessary to demonstrate intracellular enzyme activity. This was also emphasized by Bell (1966) and Grant *et al.* (1967) at light microscopic level. The omission of sucrose from the incubation medium proved favourable in this respect.

A. AChE ACTIVITY

In light microscopic specimens AChE activity was detected only in the neuronal cell bodies when iso-OMPA in 10^{-6} M, 10^{-5} M or 10^{-4} M concentrations were used. This localization is in agreement with the findings of Pepler and Pearse (1957) and Uemura (1965) in the rat SON. Using his own technique, Koelle described AChE activity also in the adjacent nerve fibres, axons and dendrites of the neurosecretory cells in the rat (Koelle 1954) and in the dog (Abrahams *et al.* 1957). Some activity has been reported in the neuropile of monkey SON (Iijima *et al.* 1967).

After 7 days' water deprivation, no demonstrable increase was observed in AChE activity in the neuronal cell bodies, such as was reported by Pepler and Pearse (1957) and Pearse (1958). In their experiments, however, the rats were kept on a salt diet for 14 days, which may partly explain the different results.

The cytoplasmic localization of the reaction end product, copper-ferro cyanide, corresponds well to the findings at fine structural level reported by several authors. A reaction in the nuclear envelope and in the cisternae of the rough-surfaced endoplasmic reticulum has been noted previously in the following sites: Koelle and Foroglou-Kerameos (1965) found such a reaction in the sympathetic ganglion cells of the rat (gold-thiolacetic acid method); Brzin *et al.* (1966) in the sym-





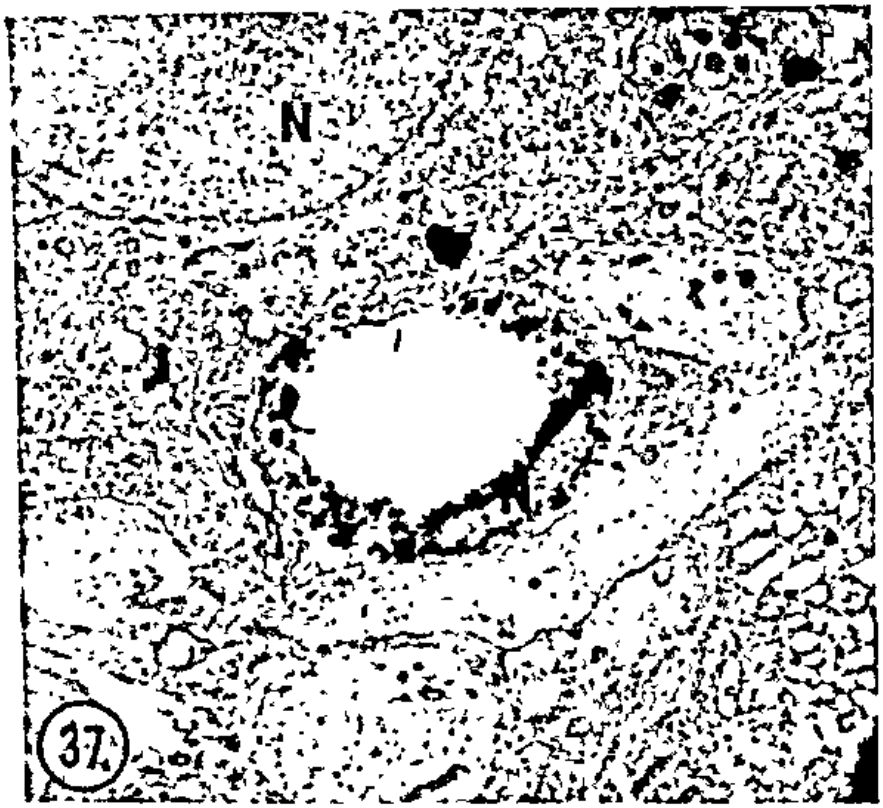


Fig. 37 Ns.ChE activity in the capillary wall of rat SON after 7 days' dehydration. A stronger activity is found, especially in the swollen cytoplasm of the endothelial cell, than in the control specimen, *cf* Fig. 36.
The neurosecretory cell with its nucleus (N) in the left corner above are negative. Procedures as in Fig. 36 × 6 600

pathetic ganglion cells of the frog (modified Koelle method); Lewis and Shute (1966) in ventral horn cells from the cervical cord of the rat (their own method); Shute and Lewis (1966) in the hippocampus cells of the rat; Bloom and Barrnett (1967) in the sympathetic ganglion cells of the rat (Lewis and Shute method); Erānkō *et al.* (1967) in the ventral horn cells of the rat (Karnovsky-Roots method), and Palkama (1967) in the adrenomedullary cells of the rat (modified Levis and Shute method). End product crystals were found with higher magnifications in the nuclear envelope between the two membranes and in the endoplasmic cisternae as also reported by Lewis and Shute with their thiocholine modification, which yields end product of very fine crystal size. In contrast to the findings of Kása and Csernovszky (1967), no activity related to ribosomes was ever obtained in the present work.

The present electron microscopic observation of AChE-positive fibres round the nerve cells of the SON suggests that at least some neurosecretory cells are innervated by cholinergic nerve fibres. The origin of these fibres is unknown, and stereotactic studies would be needed for further elucidation. Fibres coming to or running through the SON originate from several different brain areas: neocortical fibres mainly from the amygdaleum, and indirect fibres from fronto-cortical areas, fibres from the ventral thalamic nucleus and possibly from the retina. In addition, all the hypothalamic nuclei are closely connected to each other by fibres (see *e.g.* Krieg 1953 and Diepen 1962a).

AChE has been found in at least three of the sites where the fibres presumably originate. The paraventricular and suprachiasmatic nuclei in the hypothalamus and nucleus amygdaleum contain AChE-positive neurons (Koelle 1954, Abrahams *et al.* 1957 and Ishii 1957). The terminal stria, in which part of the cortical fibres run, contains also ChE-positive fibres (Shute and Lewis 1963).

Koelle and Geesey (1961) demonstrated a faint but distinct AChE activity throughout the neurohypophyseal tract and neurohypophysis in the cat. On the other hand, Abrahams *et al.* (1957) did not detect any activity in the dog's neurohypophyseal tract. Koelle and Geesey (1961) suggested that acetylcholine, in addition to neurosecretory products, was liberated in the nerve terminals of the neurohypophysis. The rate of hormone release was, according to him, under cholinergic control. Gerschenfeld *et al.* (1960) also proposed that the small agranular vesicles, which were assumed to contain acetylcholine and were found in the nerve terminals of the neurohypophysis, might be involved in the liberation of neurohypophyseal hormones.

The presence of cholinergic mechanisms is further supported by biochemical observations demonstrating acetylcholine, as well as AChE and choline acetyl-

Fig. 36 Ns.ChE activity in the capillary wall of control rat SON. The reaction product occurs randomly in the endothelial cell, and as a continuous line in the nuclear membrane (NM).
Note the markedly better preservation of the structures when butyrylthiocholine was used as a substrate instead of acetylthiocholine. 284C51 was used as inhibitor, other procedures as in Fig. 32. × 8 600

Fig. 38 Ns.ChE activity in the glial cell (oligodendrocyte) of the control rat SON. The activity is weak in the cytoplasm of this oligodendrocyte but more prominent in the nuclear membrane (NM). Procedures as in Fig. 36. × 9 600

transferase activities in the neurohypophysis (Lederis and Livingston 1966, Livingston 1966, LaBella and Shin 1968).

Because the SON neurons are only moderately positive, AChE might be needed for purposes other than neuronal transmission. The enzyme could be involved in the metabolic procedures of these highly active cells (Eränkö 1968, Welsch and Pearse 1969).

B. Ns. ChE ACTIVITY

It has long been known that blood vessels and glial cells in central nervous tissue contain ns. ChE (see Koelle 1954, 1955). When AChE inhibitor 284C51 was used in a concentration of 10^{-5} M, a distinct capillary activity, due to ns.ChE, was detected. After 7 days' dehydration the ns. ChE activity in capillaries was highly increased — a new observation of considerable interest.

The scattered reaction product crystals in the endothelial cells were unrelated to any special cytoplasmic organelle. They are probably localized in the intra-endothelial pinocytotic vesicles, as was also suggested by Joó and Csillik (1966).

The ns. ChE activity has been found in the blood-brain barrier type capillaries (Koelle 1954, Gerebtzoff 1959, Joó and Csillik 1966). It has been supposed to be related to their barrier function. The mechanism is, however, not understood. Eserine and acetylcholine have been reported to increase the permeability of the blood-brain barrier (Greig and Holland 1949). On the other hand, it has been suggested that ns. ChE in brain tissue inhibits the entrance of the excess acetylcholine, not hydrolysed by AChE, into the cerebrospinal fluid space (Abrahams *et al.* 1957).

The increase of ns. ChE activity after dehydration, as observed in the present study, would fit well into both of these concepts. It would be needed to stabilize the permeability after dehydration stress and to hydrolyse the increased amounts of acetylcholine in the SON after dehydration.

IV. ACID PHOSPHATASE ACTIVITY IN THE SUPRAOPTIC NUCLEUS OF NORMAL AND DEHYRATED RATS

REVIEW OF THE LITERATURE

LIGHT MICROSCOPIC OBSERVATIONS

The first enzyme ever demonstrated histochemically in the SON was acid phosphatase (AcPh) (Eränkö 1951a). The neurosecretory cells of the rat SON showed a very strong activity. Since then, several observations on AcPh in the rat SON have been reported (Eichner 1958, Kivalo *et al.* 1958, Rinne and Kivalo 1958, Samorajski and Fitz 1960). Strong activity was also found in the SON of other species: in cow embryo (Talanti *et al.* 1958), in ox, pig, sheep, rabbit, pigeon and domestic fowl (Arvy 1962), in guinea pig (Felgenhauer 1963) and in monkey (Friede and Knoller 1965, Iijima *et al.* 1967).

Only few reports deal with AcPh in the rat SON after dehydration. Rinne and Kivalo (1958) and Pilgrim (1967) found an increased activity after dehydration stress. Biochemical quantitations of hypothalamic AcPh activity have, however, been made by Kobayashi *et al.* (1962) and Ferreyra Moyano (1967) in dehydrated rats. Kobayashi *et al.* detected no change in hypothalamic AcPh activity but a significant increase of AcPh in the neurohypophysis, wheras Ferreyra Moyano (1967) reported a marked increase in the AcPh activity in the hypothalamic neurosecretery areas after 7 days' dehydration.

ELECTRON MICROSCOPIC OBSERVATIONS

Osinchak (1964a) found AcPh in the large inclusion bodies of 0.4—1.0 μ size and in some Golgi cisternae of the rat SON. Since AcPh has been widely used as a marker for lysosomes, he related these active bodies to the lysosomes of other cells.

No previous reports on electron microscopic findings concerning the AcPh activity in the SON of dehydrated rats have been published so far.

MATERIAL AND METHODS

Thirty-two male rats, about 300 g in weight, were perfused under ether anaesthesia as described in the first part of the present paper. 3.5 % formaldehyde in 0.1 M cacodylate buffer, pH 7.2, was used as fixative. Ten adult male

rats weighing about 300 g were kept without water for seven days and perfused with the controls.

After perfusion, the hypothalamic slices containing each SON were immersed for 4 hours at 4°C in the perfusion fluid. The slices were then washed overnight in Holt's (1958) gum-sucrose buffered with 0.1 M cacodylate buffer to pH 7.2. After freezing the tissues quickly in Holt's gum-sucrose, frozen sections 10 μ and 80 μ thick were cut with a freezing microtome. The sections were incubated free.

Two modifications of Gomori's (1941b) original AcPh lead method were applied, those of Eränkö (1951a) and of Miller (1962), this latter described by Ericsson and Trump (1965). The substrates, α- and β-glycerophosphates, were of Grade I purity (Sigma Chemical Company, St.Louis, Missouri). The pH of the incubation media was 5.0—5.1 in both modifications. The incubations were carried out at 0°C temperature for 30 minutes. In some incubations 0.3 M sucrose was included. In control studies, the substrates were omitted or 0.01 M sodium fluoride was added to the incubation medium.

The sections were rinsed for 15 minutes in three changes of 0.05 M acetate buffer to which 0.3 M sucrose had been added. For light microscopy the sections were treated with dilute ammonium sulphide for a few seconds. SON areas were dissected under a stereo microscope and refixation in 1 % OsO_4 (Rhodin 1954), embedding, dehydration and sectioning were performed as described in the previous chapter. No poststaining was used. The sections were examined and photographed with Philips EM 200.

RESULTS

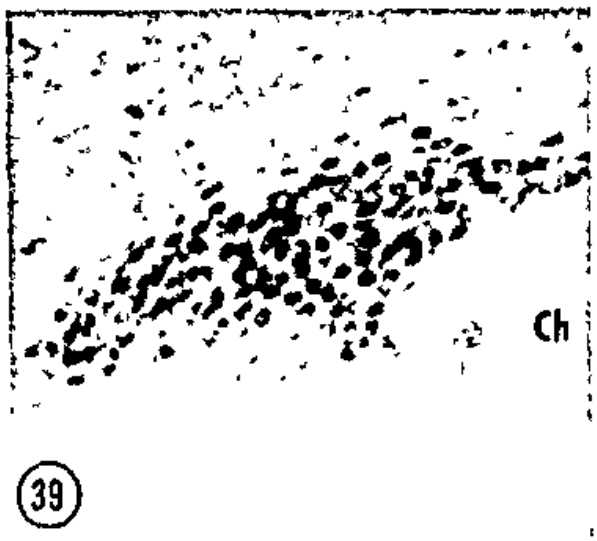
TECHNIQUES

After preliminary trials, 3.5 % formaldehyde proved to be the best fixative. It gave the most reproducible results. When glutaraldehyde was used alone or combined with formaldehyde the incubation time increased and the ultrastructure suffered. Eränkö's (1951a) modification was chosen, and it was also employed in electron microscopy. Miller's (1962) modification proved to be very sensitive to overincubation in a tissue with an intense enzyme activity, such as the SON, often yielding nuclear staining.

LIGHT MICROSCOPIC OBSERVATIONS

In the light microscopic specimens a strong granular reaction product filled the cytoplasm. The activity varied from cell to cell to some extent. No capillary activity was noticed. Using 30 minutes' incubation at 0°C, non-specific nuclear staining was totally avoided.

After 7 days' dehydration, the enzyme activity increased in all nerve cells and the granular reaction product was observed not only in the cytoplasm of the neurosecretory cells but also in the nuclei. The intervening neuropile and the capillaries were negative. The difference in overall AcPh reactivity in the SON after dehydration is demonstrated in Figs. 39 and 40.



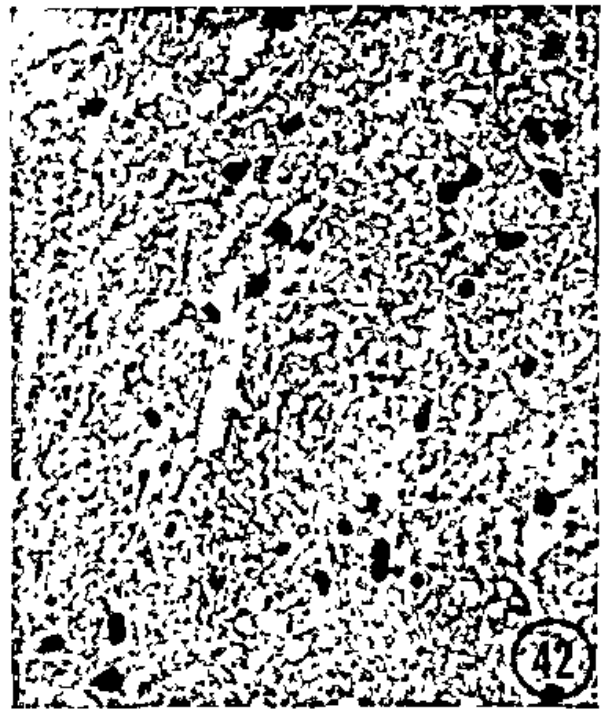
39



40



41



42

Fig. 39 A light microscopic view of AcPh activity in control rat SON. The reaction product is confined to the neurosecretory cells. It appears in the cytoplasm of the cells, which vary in enzyme activity. The specimen was formaldehyde perfused, incubated for 30 min. at 0°C in Gomori's medium modified by Erankö. Ch (chiasma opticorum) × 60

Fig. 40 A light microscopic view of AcPh activity in rat SON after 7 days' dehydration. A very strong AcPh reaction product covers the neurosecretory cells. Note the increase in the size of cells and the whole nucleus. Specimen processed as shown in Fig. 39. Ch (chiasma opticorum) × 60

Fig. 41 AcPh activity in the light neurosecretory cell of control rat SON. Large inclusion bodies show reaction product, which is also found in some Golgi lamellae, indicated by arrows. A higher magnification of the reactive Golgi lamellae is seen in the inset picture. A negative capillary wall is seen in upper left corner. Formaldehyde perfusion fixation, 30 min. incubation. Refixation in OsO_4 and no poststaining. × 5 600
× 17 000

Fig. 42 AcPh activity in the light neurosecretory cell of rat SON after 7 days' dehydration. Note increased number of large inclusion bodies with more abundant reaction product than seen in Fig. 41. Specimen processed identically to that shown in Fig. 41. × 5 600

ELECTRON MICROSCOPIC OBSERVATIONS

In electron microscopic specimens, which were always examined unstained, the large inclusion bodies, 0.5—1 μ in size, contained a very fine granular precipitate. There were variations among these bodies in amount of reaction product, as seen in Fig. 43. Some activity was noticed in the lamellar Golgi cisternae (Fig. 41). Neurosecretory elementary granules were negative, as were the granular vesicles in the nerve endings and the capillary walls (Fig. 41).

When the substrates were omitted or 0.01 M sodium fluoride was included in the otherwise complete incubation medium, no reaction product was visible.

After 7 days' dehydration there was an increase in the number of large inclusion bodies filled with very dense reaction product in all neurosecretory cells. The increase was even more marked in the small dark cells, where only a few non-reactive, large inclusion bodies were seen, as shown in Fig. 42. Reaction product was also demonstrated in the lamellar Golgi cisternae, especially in the dark cells (Fig. 44). The Golgi lamellae showed more abundant reaction product in the neurosecretory cells of dehydrated rats than of the controls. Compare Figs. 44 and 41.

DISCUSSION

After preliminary experiments with glutaraldehyde-containing fixatives, known to give excellent preservation of fine structural details, 3.5 % buffered formaldehyde was chosen as fixative.Glutaraldehyde proved to be so inhibitory for enzyme activity that incubation times had to be doubled after fixation with it. So the benefit of better structure preservation was lost because of longer incubations in the acid milieu. It is known that some part of the brain AcPh is especially sensitive to this dialdehyde (Torack 1965). Glutaraldehyde from Taab Laboratories is routinely used in this laboratory without previous distillations or other purification procedures. This stock solution may therefore contain inhibitory impurities. Inhibitory effects of commercial glutaraldehyde solutions on AcPh activities have been reported in other tissues by Smith and Farquhar (1966) and Fahimi and Drochmans (1968).

Washing and freezing in Holt's gum-sucrose proved favourable for both enzymatic activity and ultrastructural preservation.

The SON area showed strikingly intense AcPh activity compared with other brain regions, as has been unanimously stated by all previous authors (Eränkö 1951a, Leduc and Wislocki 1952, Scharrer and Scharrer 1954, Cohn and Richter 1956, Kivalo *et al.* 1958, Pilgrim 1967).

In the present work, a heavy brownish black final reaction product was observed in all cell bodies of the neurosecretory cells and the differences between activities of individual cells had disappeared after 7 days' dehydration. All the cells showed more intense AcPh activity than the most active cells in the control sections. Pilgrim (1967) also demonstrated increased activity after 5—6 days' water withdrawal. However, he differentiated two types of neurosecretory cells

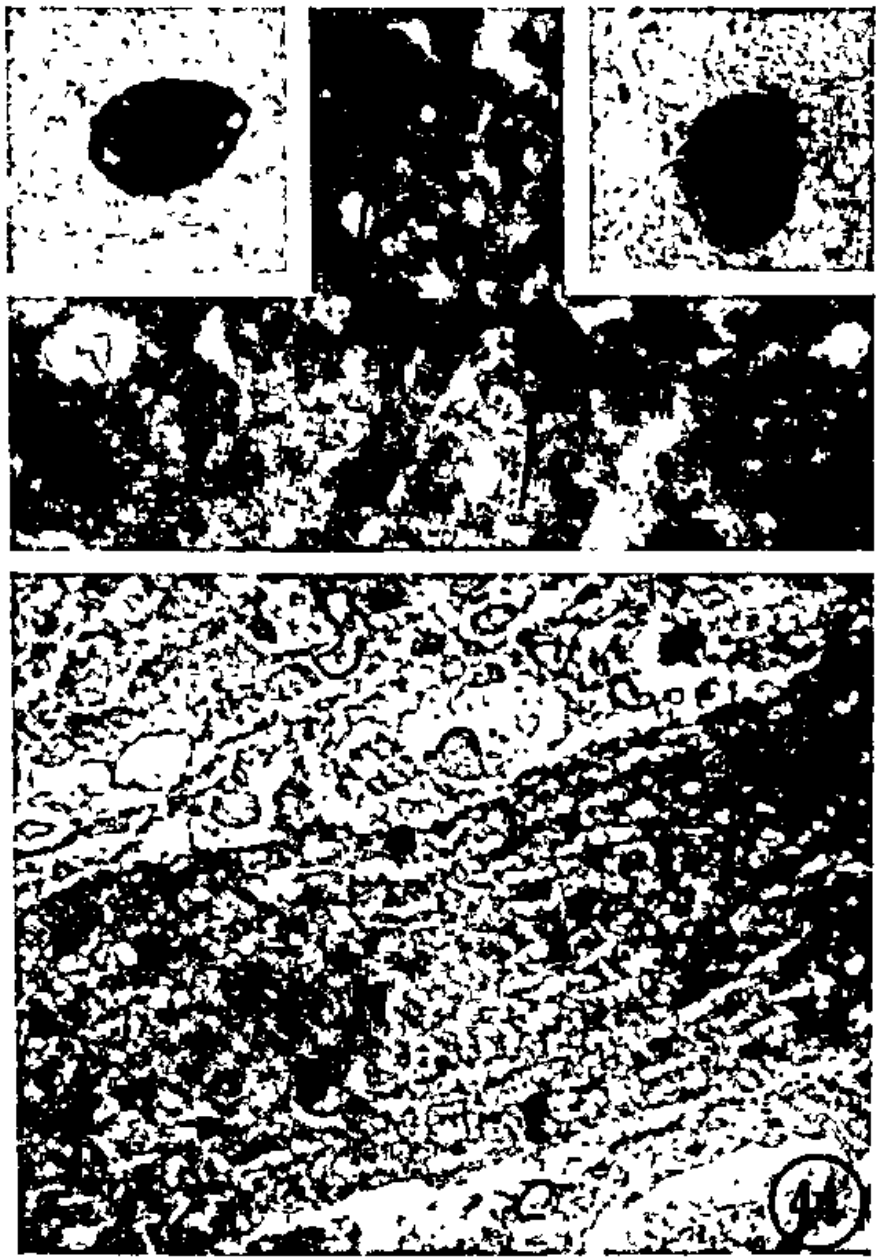


Fig. 43 AcPh activity in the large inclusion bodies of a light neurosecretory cell in control rat SON. Two large inclusion bodies (arrows), which are seen vary in the amount of reaction product. The lead salt is very finely granular as seen in the inset pictures with higher magnification. Procedures as in Fig. 41. × 25 500
× 39 000

Fig. 44 AcPh activity in the dark neurosecretory cell of rat SON after 7 days' dehydration. The large inclusion bodies are heavily covered with lead precepitate. The Golgi lamellae (G) are also reactive. Specimen processed as in Fig. 41. × 6 600

according to their AcPh reactivity. The small, dense-looking cells were more active than the larger, more vacuolated cells.

On the basis of electron microscopy, the reaction product was found in the large inclusion bodies of both light and dark neurosecretory cells. There were always non-reactive large inclusion bodies among the reactive ones, both in the light and the dark cells. In the control animals, some flattened Golgi cisternae showed reaction product, but the small inclusion bodies were as a rule negative. These observations are in agreement with those of Osinchak (1964a). He was the first to propose that the large inclusion bodies were identical with the lysosomes of other nerve cells and different from the small inclusion bodies. According to Novikoff (1961), the bodies limited by single membranes and showing AcPh activity fulfill the criteria of lysosomes.

After 7 days' dehydration, the neurosecretory cells showed an increased number of reactive lysosomes and more reaction product in the flattened Golgi cisternae than in the control animals. Individual lysosomes also had more reactive material inside the matrix.

High AcPh activity has often been related to lytic events and degenerative changes of the cell (Novikoff 1961, Bitensky 1963, Söderholm 1965). Such an explanation does not seem likely in the neurosecretory cells under dehydration, in which increased protein synthesis can be expected.

Indeed, high AcPh activity outside the hypothalamic neurosecretory cells has been related to increased protein synthesis for increased metabolic demands (see *e.g.* Novikoff 1963 and Kokko 1965). The appearance of AcPh in non-lytic sites like the Golgi cisternae was reported by Smith and Farquhar (1966) in the hormone-producing cells of the rat adenohypophysis and by Holtzman and Dominitz (1968) in rat adrenal medullary cells. Smith and Farquhar (1966) suggested that lysosomes are needed to abolish the overproduction of the secretory products serving as a protein-degrading apparatus. Increased activity of AcPh was also reported by Szabó *et al.* (1967) in the lysosomes of stimulated adrenal cortex cells. Recently, Gordon *et al.* (1968) demonstrated synaptic lysosomes with both histochemical and biochemical AcPh activity in the cortical terminals of the rat brain. They suggested that *via* proteolysis these presynaptic lysosomes produced amino acids for fresh protein synthesis.

In the light of the present observations it is conceivable that increased AcPh activity in the neurosecretory cells under dehydration stress may well reflect an activated polypeptide hormone synthesis. The increased activity of Golgi apparatus cisternae of the SON, first observed in the present study, may be related to increased lysosomal activity or is directly involved in the packing and releasing mechanism of the neurosecretory granules.

V. NUCLEOSIDE PHOSPHATASE ACTIVITY IN THE SUPRAOPTIC NUCLEUS OF NORMAL AND DEHYDRATED RATS

REVIEW OF THE LITERATURE

On the basis of biochemical assays the existence of at least two different ATPases in nerve tissue is fairly well established: (1) the Na^+ -K^+-dependent Mg^{++} activated ATPase, which is related to the membrane structures and inhibited by g-strophantin (ouabain) (Skou 1957, 1960, 1965, Bonting *et al.* 1961, Järnefelt 1961, Lewin and Hess 1964, Fried 1965, Samaha 1967, Fahn and Côté 1968), and (2) the Mg^{++} -activated and Na^+ -K^+ -independent ATPase, which is related to mitochondria and blood vessels and is not inhibited by g-strophantin (see *e.g.* Lehninger 1964, Parsons 1965).

LIGHT MICROSCOPIC OBSERVATIONS

Light microscopic observations of ATPase activity in nervous tissue were made by McClurkin 1964, Fried 1965, Söderholm 1965, Iijima *et al.* 1967, and Sabatini *et al.* 1968. Of these authors, Iijima *et al.* (1967) studied the magnocellular nuclei of hypothalamus and reported ATPase activity in almost all of its structures: in nuclei, nucleoli, capillaries, neuropile and glial cells.

ELECTRON MICROSCOPIC OBSERVATIONS

Only a few electron microscopic studies have been published on ATPase activity in nervous tissue (Torack and Barrnett 1963, Torack 1965, Rechardt and Kokko 1967, Sabatini *et al.* 1968). Mitochondrial ATPase activity has been demonstrated in the mitochondria of many other tissues (Lazarus and Barden 1962, Ashworth *et al.* 1963, Otero-Vilardebó *et al.* 1963, Essner *et al.* 1965, Moses *et al.* 1966, Wood 1967).

Of the histochemical methods for demonstration of ATPase the lead method introduced by Wachstein and Meisel (1957) is suitable for electron microscopic purposes. Recently, however, severe criticism has been directed against the lead methods for demonstration of ATPase (Moses *et al.* 1966, Moses and Rosenthal 1968, Rosenthal *et al.* 1966, Palkama and Uusitalo, 1968).

MATERIAL AND METHODS

Forty adult male control rats about 300 g in weight, and 10 rats of equal weight kept 7 days without water, were perfused under ether anaesthesia *via* the left cardiac ventricle with 0.075 M cacodylate-buffered 3.5 % formaldehyde in 0.3 M sucrose, pH 7.2. Perfusion time was 10 minutes. The hypothalamic area was excised and the tissue piece washed overnight in cacodylate-buffered gum-sucrose, pH 7.2 (Holt 1958). Frozen sections, cut with the freezing microtome at 10 μ for light microscopy and at 80 μ for electron microscopy, were incubated free-floating for 30 minutes at 0°C. The standard incubation medium consisted of ATP *disodium salt (C. F. Boehringer & Soehne, Mannheim)* or *dibarium salt* (Sigma Chemical Company, St.Louis, Missouri) 8.3×10^{-5} M, Tris-maleate buffer pH 7.2 0.08 M, $MgCl_2$ 0.05 M, $Pb(NO_3)_2$ 2.5×10^{-3} M, sucrose 0.3 M was added and the pH was adjusted to 7.2 (Otero-Vilardebó *et al.* 1963). After incubation the sections were rinsed in 4 changes of ice-cold 0.3 M sucrose for 20 minutes. Light microscopic specimens were treated with diluted ammonium sulphide.

Controls were made by incubating the sections either without the activator and substrate, or by substituting β-glycerophosphate, ADP, IDP, ITP, TPP or UDP for ATP, respectively. The effects of 0.01 M sodium fluoride and of ouabain 3×10^{-3} M (Fluka AG,Buchs SG) were studied, as well as that of 0.1 M Na^+ and 0.02 M K^+.

For electron microscopy, the SON areas of incubated sections were dissected under a stereo-microscope and the pieces were fixed in 1 % OsO_4 for one hour. Dehydration, embedding and sectioning were performed as described in Chapter III. The thin sectios were examined unstained and photographed with Siemens Elmiskop I and Philips EM 200 operated at 80 kV.

RESULTS

LIGHT MICROSCOPIC OBSERVATIONS

Compared with the other hypothalamic areas, a strong nucleoside phosphatase activity was demonstrated in the SON area with disodium ATP as substrate. The negative neurosecretory cells were surrounded by a strongly positive network consisting of capillaries and a scanty neuropile between the nerve cells (Fig. 45). Pia arachnoidea, which usually was attached to the lower surface of the nucleus, showed a very strong activity. Reaction product was also detected in a few blood vessels in the optic chiasma.

After 7 days' dehydration the localization of reaction product was unchanged but the overall activity was weaker in all sites (Fig. 46).

ELECTRON MICROSCOPIC OBSERVATIONS

Neurosecretory cells

In the neurosecretory cells, finely granular reaction product was localized between the endoplasmic cisternae both with sodium- and barium-ATP salts and ITP as substrates. The localization was the same as that of free ribosomes (Fig. 47).

In some cells, the large inclusion bodies showed a certain affinity to the reaction product, but the Golgi apparatus was negative in all cells. The mitochondria were also negative. Neither did the nuclear and plasma membranes show any reaction product, not even when K^+ and Na^+ ions were added to the incubation medium.

When ADP, IDP or UDP was used as substrate, the reaction product was localized in the Golgi apparatus, mainly in the flattened cisternae and also in the large inclusion bodies. This kind of localization was more prominent in the dark cells (Fig. 48). Using TPP as substrate, the only localization was the Golgi apparatus.

After 7 days' dehydration, ATPase and ITPase activity was noticed in the flattened Golgi cisternae in the form of dense granules. Some large inclusion bodies also showed reaction product, as seen in Fig. 49.

The nerve fibres

Both myelinated and unmyelinated nerve fibres contained mitochondria which showed nucleoside triphosphatase activity in the matrix between the cristae

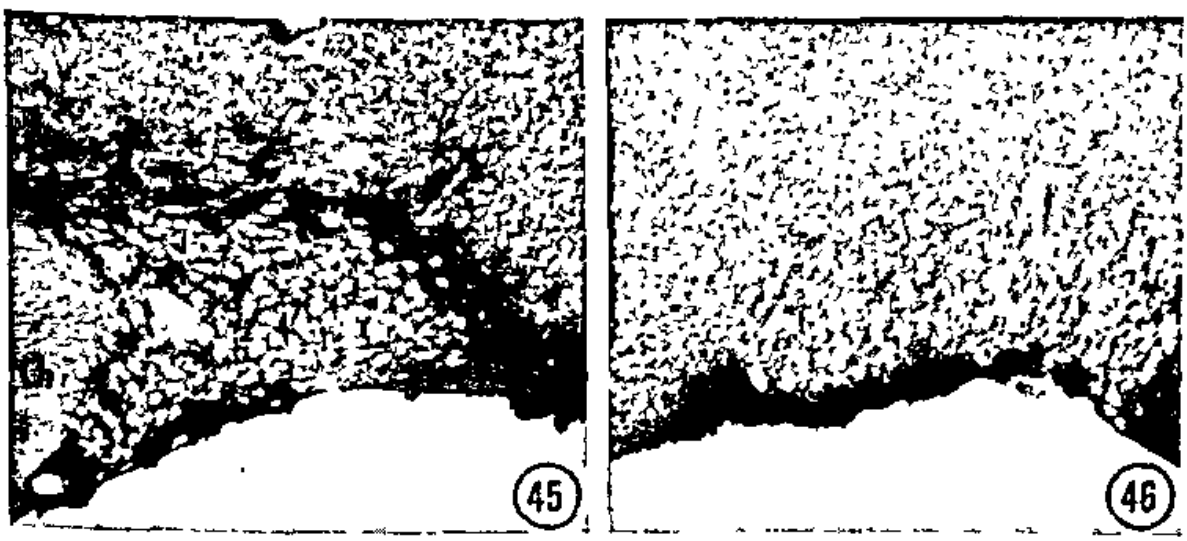


Fig. 45 A light microscopic view of nucleoside phosphatase activity in control rat SON. Enzyme activity is noticed round the neurosecretory cells, which appear negative. 10 minutes' formaldehyde perfusion fixation, incubated 30 min. with ATP disodium salt as substrate. Ch (chiasma opticorum) × 65

Fig. 46 A light microscopic view of the nucleoside phosphatase activity in rat SON after 7 days' dehydration. The enzymatic activity is weaker than in the control specimen. *Cf.* Fig. 45. Procedures as in Fig. 45. Ch (chiasma opticorum) × 65

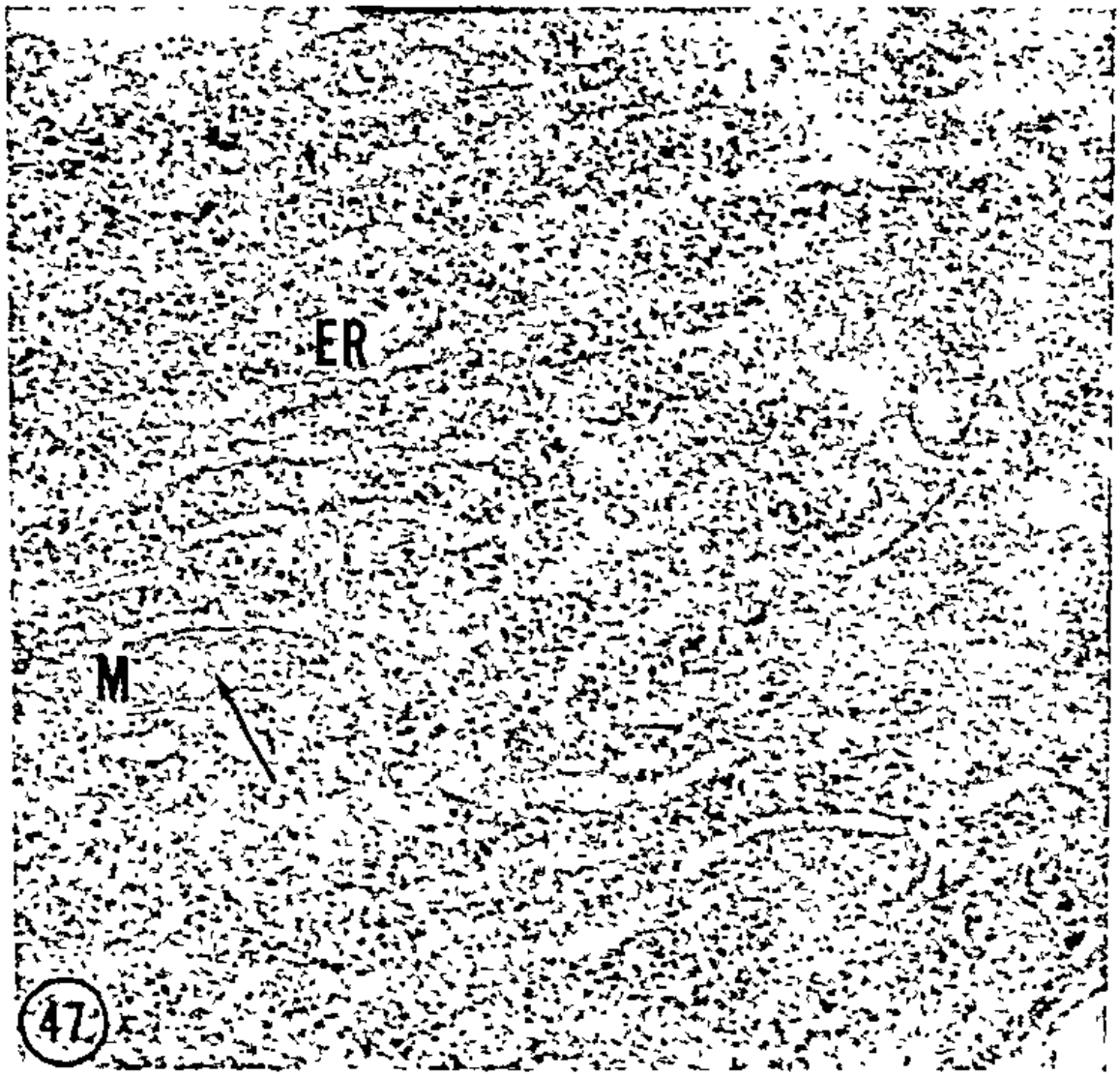
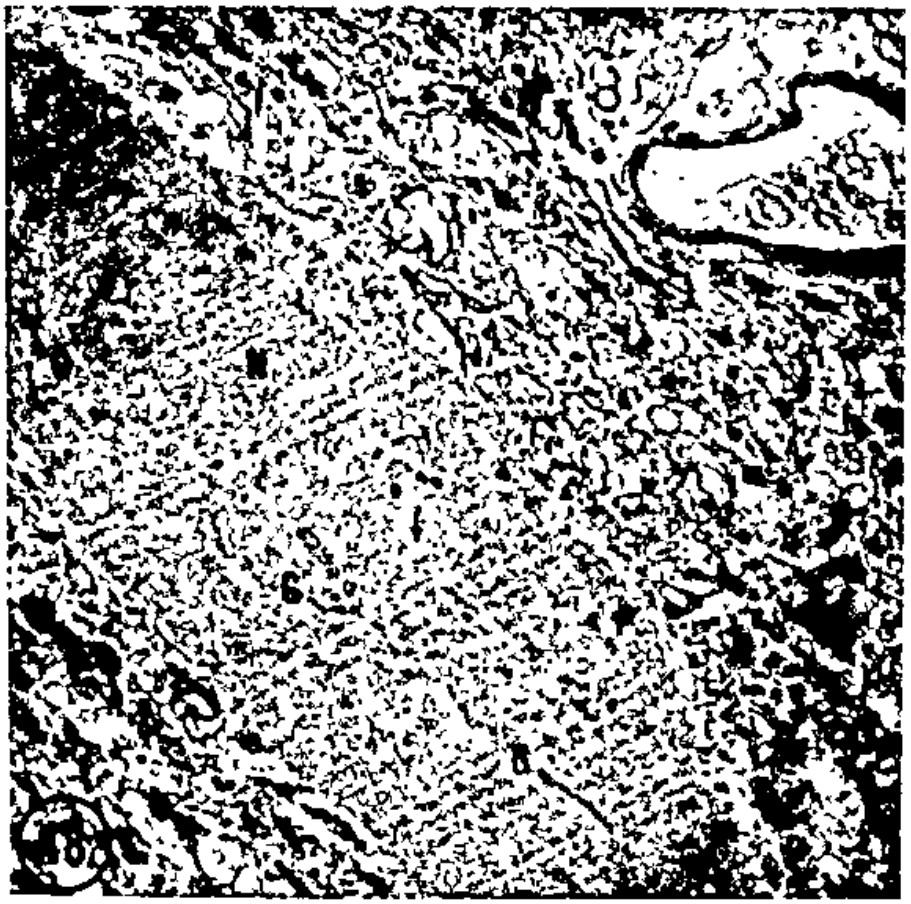


Fig. 47 Nucleoside triphosphatase activity in the light neurosecretory cell of control rat SON.
Finely granular precipitate is found between the endoplasmic cisternae inside the cell. An arrow points to a negative mitochondrion (M).
10 minutes' formaldehyde perfusion, incubated with ATP barium salt as substrate. OsO_4 refixation and no poststaining. × 43 200

Fig. 48 Nucleoside diphosphatase activity in a dark neurosecretory cell of control rat SON. The reaction product is found in the flattened cisternae of the Golgi apparatus (G). A weak activity is noticed in the capillary wall.
Procedures as in Fig. 47, except for IDP being used as substrate. × 5 100

Fig. 49 Nucleoside triphosphatase activity in a light neurosecretory cell of rat SON after 7 days' dehydration. Reaction product is demonstrated in the flattened Golgi cisternae (G) and in some large inclusion bodies (arrows).
Procedures as in Fig. 47, except for ATP di-sodium salt being employed. × 7 900





49

(Fig. 50). Non-reactive mitochondria were always found among reactive ones. The axon terminals in synapses did not contain more reactive mitochondria than the other fibres. No reaction product was demonstrated in the neuroglial processes around the capillaries.

The same kind of mitochondrial activity was obtained with ITP as substrate. When ADP, IDP or UDP were employed no mitochondrial activity could be detected.

There was no change after 7 days' dehydration in the amount and distribution of the reaction product in the mitochondria. The mitochondria in the neurosecretory cells remained negative as in the control specimens.

Capillaries

Both finely and coarsely granular reaction was found in all capillaries of the SON. The strongest activity was localized in the basal lamina as a continuous or interrupted line (Fig. 51). The cytoplasm of the endothelial cell showed only a few randomly scattered granules. The mitochondria of the endothelial cells were negative.

The glial elements round the capillaries were negative.

When ITP was substituted for ATP, the amount and localization of the reaction product were unchanged. With *ADP*, *IDP* or *UDP* as substrate the enzyme reaction was distinctly weaker in the capillaries and the reaction product was of greater amount in the endothelial cytoplasm than in the basal lamina.

After 7 days' dehydration, a decrease was noticed in nucleoside phosphatase activity. Electron microscopy showed this decrease to be due to a loss of reaction product from the capillary walls.

The basal laminae of the capillaries of the dehydrated rats did not show as much precipitate as in the control capillaries (Figs. 51 and 52).

CONTROL STUDIES

When the substrates were omitted from the incubation medium, no reaction product was visible in any structure, except for some finely granular precipitate occasionally seen in the nuclei of the endothelial cells. No reaction product was demonstrated in the cytoplasm of the cells, neuropile or capillaries when β-glycerophosphate was used as substrate. Sodium fluoride had a slight inhibitory effect especially on mitochondrial activity but capillary activity was equally strong as without any inhibitors. Ouabain had no inhibitory effect in any localizations. The addition of Na^+ - and K^+ -ions to the incubation medium did not affect the distribution of the end product. Without Mg^{++} -ions no activity could be demonstrated.



Fig. 50 General view of the nucleoside triphosphatase activity in the mitochondria (M_1) of the nerve fibres. Both myelinated and unmyelinated nerve fibres, axons (Ax) and dendrites (D) contain mitochondria with reaction product. One mitochondrion (M_2) without any reaction product is noted in the vicinity of an active mitochondrion. The reaction product is localized between the cristae in the matrix part. Specimen processed as in Fig. 47. × 28 200

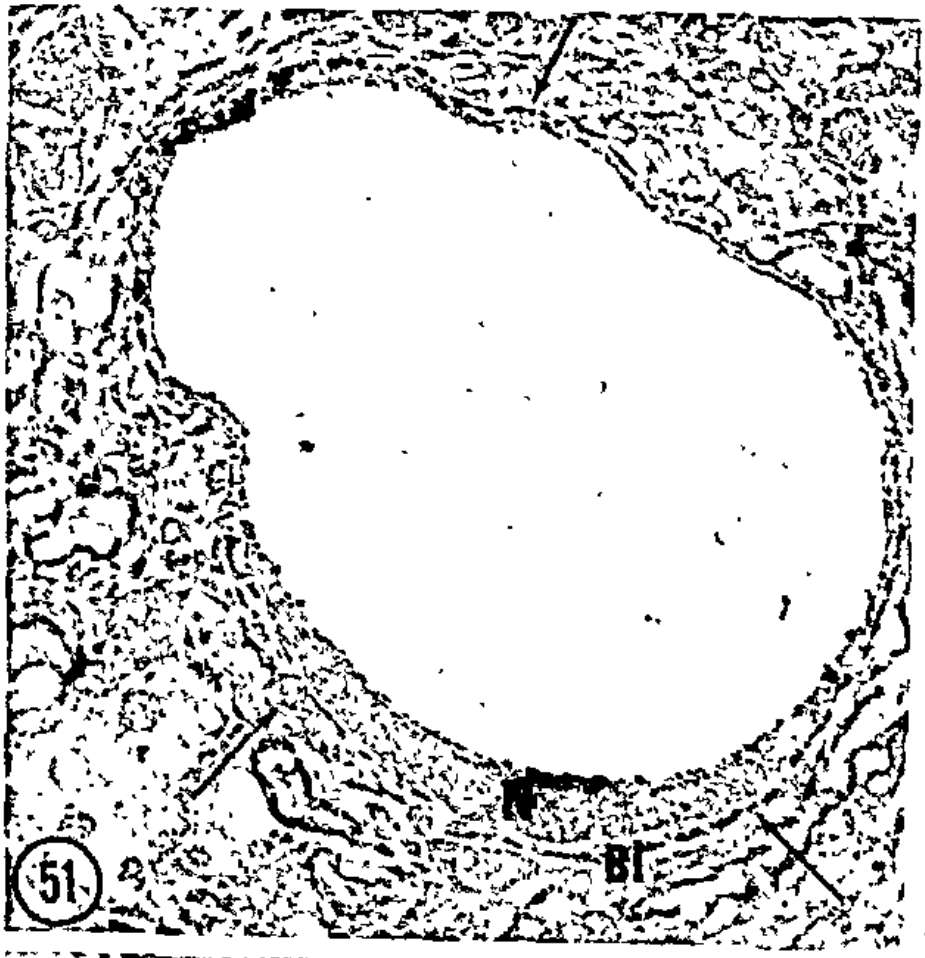


Fig. 51 Nucleoside triphosphatase activity in the capillary wall of control rat SON. The reaction product (arrows) is localized mainly to the basal lamina (Bl). Some reaction product is also demonstrated in the endothelial cell. Very fine nonspecific precipitate covers the endothelial cell nucleus. Procedures as in Fig. 47. × 11 800

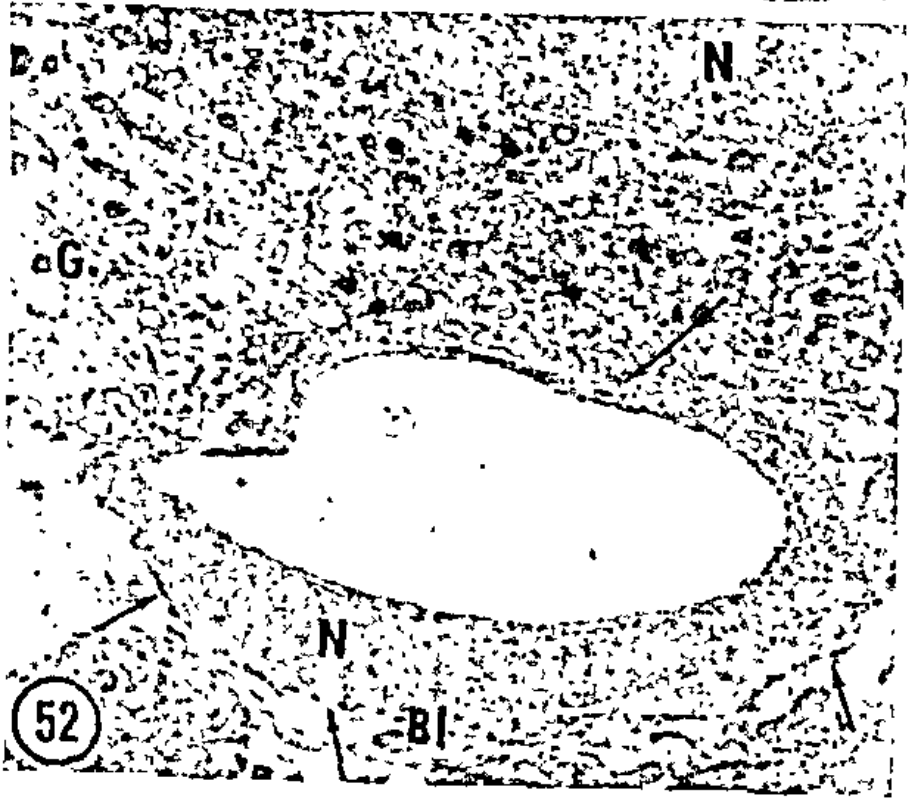


Fig. 52 Nucleoside triphosphatase activity in the capillary wall of rat SON after 7 days' dehydration. The reaction product now occurs as a weaker and discontinuous line (arrows) in the basal lamina (Bl) when compared to Fig. 51. Procedures as in Fig. 47. × 11 900

DISCUSSION

Of the methods available for demonstration of nucleoside phosphatases, the lead method introduced by Wachstein and Meisel (1957) is most widely used. Its advantage for electron microscopic purposes is that the end product, lead phosphate, is highly electron-dense. The method has, however, many pitfalls which restrict its validity (*e.g.* Moses and Rosenthal 1968). These authors have biochemically shown that the lead concentrations used in the Wachstein-Meisel method inhibit nucleoside phosphatase activity and, moreover, favour non-enzymatic hydrolysis of the substrates (Moses *et al.* 1966, Moses and Rosenthal 1967). On the other hand, Novikoff (1967) has presented observations indicating non-enzymatic hydrolysis of ATP after long incubations. However, he also showed that such hydrolysis can be avoided by using appropriate fixation and incubation conditions. Modifications, mainly by changing reagent concentrations have been developed for histochemical demonstration of nucleoside phosphatases (Otero-Vilardebó *et al.* 1963, McClurkin 1964).

Recently Palkama and Uusitalo (1968, 1969), using a technique further modified from that of Wachstein and Meisel (1957) have bio- and histochemically demonstrated ouabain-sensitive Mg^{++} —activated and Na^{+} -K^{+} -dependent adenosine triphosphatase in the rabbit ciliary processes, although this nucleoside phosphatase has been reported to be especially sensitive to lead ions and fixatives (Gordon and Torack 1967, Moses and Rosenthal 1968).

In the modification of the Wachstein-Meisel method used in the present study, the concentration of lead was reduced from the original, and Mg^{++} concentration was increased (Otero-Vilardebó *et al.* 1963). The mitochondrial nucleoside phosphatase activity is known to be especially sensitive to fixatives (Essner *et al.* 1965). With very brief perfusion fixation it was possible, however, to demonstrate the mitochondrial activity in nervous tissue. The methodological problems have been discussed in detail in a previous paper (Rechardt and Kokko 1967).

Both myelinated and unmyelinated nerve fibres showed mitochondria in which the granular end product was localized in the matrix between the cristae. Similar intercristal localization was reported by Lazarus and Barden (1962) in rabbit pancreas, by Ashworth *et al.* (1963) in rat intestine, kidney and liver, by Otero-Vilardebó *et al.* (1963) in rat colonic epithelial cells, by Essner *et al.* (1965) in cultured human amnion cells and by Grossman and Heitkamp (1968) in rabbit's skeletal muscle mitochondria.

The nerve fibre mitochondria showed heterogeneity in reactivity. There were always negative mitochondria in the immediate vicinity of positive ones.

It is interesting that the mitochondria in the nerve cell bodies were negative. No activity could be detected with any of the substrates employed. A similar observation was made by Rechardt and Kokko (1967) in the mitochondria of the motor nerve cells of rat's spinal cord. The possibility that the mitochondria inside the neurons contain the enzyme although its activity is not demonstrable with the present method cannot be excluded. These mitochondria may be more resistant to freezing and thawing, which is necessary for the penetration of the reagents and thus for nucleoside phosphatase demonstration. It is also possible that their metabolism is different or that formaldehyde inhibits their activity.

In the present study, di- and triphosphatase activity was observed between the endoplasmic cisternae of the neurosecretory cells. This localization was reported in the secretory cells of rat and rabbit pancreas with various triphosphates as substrates (Wachstein and Fernandez 1964).

After 7 days' dehydration, reaction product was found in the flattened Golgi cisternae when ATP or ITP was used as substrate. During dehydration the Golgi apparatus of the neurosecretory cell is possibly the most active cell organelle, packing and liberating the neurosecretory granules. However, no activity was observed in the neurosecretory granules of these neurosecretory cells, although adenosine triphosphatase activity has been biochemically determined in the neurosecretory granules from the bovine neurohypophysis (Poisner and Douglas 1968). Nucleoside phosphatases may be involved in the release of the neurosecretory product, as has been suggested in the case of the chromaffin granules in the adrenal gland (Oka *et al.* 1965, Poisner and Trifaró 1968).

The reaction in the capillary wall was similar with all the substrates except β-glycerophosphate, with which no activity was observed in the present study. Similar observations were reported by Torack and Barrnett (1964). It seems that the capillary wall activity is not due to alkaline phosphatase activity, but to an enzyme or enzymes splitting several di- and triphosphates.

With the aid of electron microscopy reaction product was found mainly in the basal lamina though some activity was detected in the endothelial cells, presumably in pinocytotic vesicles. The sparse, fine granules in the endothelial cell nuclei were considered non-specific. Nucleoside phosphatase activity in the basal lamina has been related to the blood-brain barrier function of the blood vessels (Becker *et al.* 1960, Torack and Barrnett 1964, Torack 1965). In brain areas in which there is no blood-brain barrier the basal lamina is non-reactive and the endothelial cells show nucleoside phosphatase activity in their pinocytotic vesicles. Torack and Barrnett (1964) described a weak activity also in the plasma membranes of the glial end feet and oligodendrocytes, which, however, was not confirmed in the present study.

After 7 days' dehydration, activity of the basal lamina was reduced, while more activity was noticed in the endothelial cells. If nucleoside phosphatase activity is related to the barrier function, the decreased activity of the basal lamina is suggestive of a change in permeability after dehydration stress. There might be a loss in the barrier function. However, a change in nucleoside phosphatase activity could be explained also on the basis of the theory that nucleoside phosphatases are involved in the synthesis and degradation of mucopolysaccharides in the basal lamina, which as such is an extracellular structure (Campbell 1968). Mucopolysaccharide synthesis possibly undergoes changes during dehydration.

SUMMARY AND CONCLUSIONS

The present investigation was carried out to study the SON of the rat with fluorescent and electron microscopic methods, especially to examine hitherto controversial views and to use new techniques not yet applied to this nucleus. In Chapter I the effect of dehydration on the fine structure of the SON was studied. The behaviour of adrenergic nerve fibres after experimental conditions was studied in Chapter II. Some hydrolytic enzymes demonstrable electron microscopically were studied in Chapters III—V in the normal SON, and after increased hormone production due to water deprivation.

I. Two distinct types of neurosecretory cells, dark and light, were observed without doubt by both light and electron microscopy in the SON using material fixed by perfusion with formaldehyde and glutaraldehyde. The difference between the dark and light cells depended on the great difference in the number of free ribosomes, as was seen by electron microscopy. Dehydration caused a further distinct increase in the number of ribosomes in the dark cells, while in the light cells such a change was less evident. While the presence of dark and light nerve cells in the SON has often been considered artifactual due to poor preservation and handling of the tissue, it can be concluded from the present study that two different types of neurosecretory cells really exist.

Small inclusion bodies, also called neurosecretory granules, were observed in both types of cells. Dehydration caused a loss of these bodies, and the loss was almost complete after 7 days in both types of cells. In such cells, empty vesicles of the same size were found near the Golgi apparatus. No changes due to dehydration were noticed in the amount or appearance of mitochondria or lysosomes.

Three kinds of synaptic vesicles were observed in the axo-somatic, axo-dendritic and axo-axonal synapses: small agranular, small granular and large granular vesicles. The small granular vesicles were observed only in material fixed by perfusion with potassium permanganate. Such vesicles, related to adrenergic neuronal transmission, were in the present study demonstrated for the first time in the axo-somatic and axo-dendritic synapses of rat's SON area. Dehydration had no effect on the appearance or number of the vesicles.

The capillaries were of the blood-brain barrier type with few pinocytotic vesicles in the endothelial cells. In normal rats, intimate contacts between the nerve cells and the capillaries were found. After dehydration, the glial processes were hyper-

trophic and swollen, and the distance between the neurosecretory cells and capillaries was increased. These new observations suggest participation of the glial tissue in the osmoregulatory function of the SON.

II. The effect of sympathectomy and of administration of reserpine or Oxypertin, not earlier examined in the rat SON, was studied by fluorescence and electron microscopy. Bilateral cervical sympathectomy had no effect on the fluorescent nerve net round the non-fluorescent nerve cells, whereas reserpine treatment totally abolished the fluorescence and Oxypertin treatment diminished it.

Bilateral cervical sympathectomy affected neither the neurosecretory cells nor the large granular synaptic vesicles. Reserpine and Oxypertin treatments caused a loss of neurosecretory granules from the cells but had no noticeable effect on the large granular vesicles in any of the three types of synapses.

III. Cholinesterases were demonstrated using the acetylthiocholine method of Koelle and Gomori and that of Karnovsky and Roots, together with the specific inhibitors iso-OMPA and 284 C51. Moderate acetylcholinesterase activity was observed light and electron microscopically in the neurosecretory cells. The relatively weak enzyme reaction in the neurosecretory cells suggests that this enzyme is needed for other purposes than neuronal transmission, possibly for metabolic demands. Only few unmyelinated nerve fibres and some synaptic endings on the neurosecretory cells showed a reaction, located in the cell membrane. After 7 days' dehydration, no change was noticed in the acetylcholinesterase activity of any tissue.

The non-specific cholinesterase activity was localized light microscopically in the capillary walls only. Electron microscopically, the reaction was found both in the endothelial cells and in the glial cells. After dehydration, the intensity of the reaction increased in the endothelial cells, suggesting a change in their metabolism.

IV. Acid phosphatase activity was demontsrated with glycerophosphate as a substrate using Gomori's lead method, as modified by Eränkö. Strong acid phosphatase activity was detected in the neurosecretory cells and the intensity of this activity increased after dehydration. The reaction product was localized electron microscopically in the large inclusion bodies of both types, *i.e.* the lysosomes and the multivesicular bodies, as well as in some Golgi lamellae. After 7 days' dehydration acid phosphatase activity increased markedly, and in electron microscopic specimens the number of reactive lysosomes was found to be increased suggesting increased cellular turnover rate. The intensity of the reaction also increased in individual lysosomes and in the Golgi apparatus.

Pronounced acid and nucleoside (see V below) phosphatase activity in the Golgi apparatus and intense acid phosphatase activity in the lysosomes, as well as abundant ribosomal content and intracisternal material suggest that contrary to earlier assumptions, the dark neurosecretory cells are not resting cells but metabolically active cells, which react during dehydration.

V. Nucleoside phosphatase activity not previously examined in the SON, was demonstrated with the lead method introduced by Wachstein and Meisel using

ADP, IDP, UDP, ATP, ITP, TPP and β-glycerophosphate as substrates. Light microscopically, the reaction with di- and triphosphates was observed round the nerve cells, which were themselves non-reactive, in the capillaries and in the neuropile.

Electron microscopically, triphosphatase activity was found in the mitochondria of the nerve fibres and in the basal laminae of the capillaries. In the neurosecretory cells, triphosphatase activity was localized between the endoplasmic cisternae.

After 7 days' dehydration mitochondrial triphosphatase activity was unchanged, but the basal laminae showed reduced activity in all capillaries. The change in the triphosphatase activity in the capillary wall, *i.e.* basal lamina, after dehydration is suggestive of altered capillary function comparable to that indicated by increase in the non-specific cholinesterase activity, both enzymes being apparently related to the blood-brain barrier function.

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SUBJECT INDEX

The figures refer to the abstract numbers

SYMPOSIA

Physiological adjustments and limiting factors at maximal work	1–10
Secretion	11–17
Uptake, storage and release of biogenic amines	18–34
Excitation-contraction coupling	35–39
Motor cortex-afferents and efferents	40–45

INVITED LECTURES

Transfer of signals from muscle stretch receptors	46
Neural control mechanisms determining pace and depth of respiratory movements	47
Etiological and curative aspects on obesity	48
Photochemical aspects of the receptor process in vision	49
The molecular basis for the active transport of Na^+ and K^+	50
The role of gastrointestinal wall in drug metabolism	51
The dynamics of aqueous humor	52
On the role of central monoamine neurons in neuroendocrine mechanisms	53
Plasma kinins in physiology and pathophysiology	54
Reception of sound and vibrations by the fish ear	55
Functional organization of the monkey visual cortex	56
The blood-brain barrier	57

FREE COMMUNICATIONS

Amine storing cells	58–63
Nerve degeneration, supersensitivity	64–68
Release and metabolism of amines	69–79
Behaviour	80–88
Pharmacokinetics	89–95
Gastric secretion	96–97

Endocrinology	98–102
Metabolism: general aspects	103–109
Muscle metabolism	110–114
Respiration	115–119
Exercise physiology	120–132
Central haemodynamics; circulatory control	133–146
Regional circulation	147–156
Capillary exchange	157–162
Membranes and active transport	163–170
Muscle physiology	171–179
Central nervous system	180–188
Receptors and sensory transmission	189–196

Hohwü-Christensen,E.(Department of Physiology, Gymnastik-och idrottshögskolan, Stockholm, Sweden): INTRODUCTION. 1

How can the working organism solve the problems of energy delivery when a motoreffect of 3 to 5 HP are needed for some 10 sec. maximal effort during a 100 m run, or when a total of 1500 kcal per hour are consumed during 6 hours (Vasaloppet)? When are the energy stores in the working muscles adequate and sufficient, and when will a certain supply from other stores have to be transported with the blood to the muscles? How many kcal are available for muscular work as ATP and CrP, and how fast can these energy quantities be released and replaced? Is the limitation for speed during a 100 m run due to lack of acceptable, fast energy sources, due to an accumulation of waste products or are there mechanical limitations? At which level of metabolic turn over rate have calories from fat to be replaced entirely by those from carbohydrate to secure sufficient fast energy delivery for the resynthesis of ATP? Which role plays the local oxygen tension in this "regulation" of muscle metabolism? How do we solve the varying local oxygen supply? Which are the lowest acceptable oxygen tensions in the blood capillaries in the muscles if anaerobic metabolism shall be omitted? How many ml of oxygen per g and min can be delivered to the working muscles, and where are the bottle necks in the oxygen transport system from the air via the lungs and blood to the mitochondria? Is the oxygen flow limited by the bellow function of the pulmonary system, the pulmonary capillary membranes, or is it limited by the pumping capacity of the heart? What are the pro and contra as to respiratory work as a limiting factor when pulmonary ventilation surpasses 150 to 200 litres per min? How far and how can the blood flow-oxygen flow - be directed towards the working muscles and away from less "important" tissues? What extra load is involved in the elimination of waste products, including heat from the working muscles? How many litres of the cardiac output must the organism spend for heat transport to the skin, and under which conditions may this extra load on the blood circulation limit work performance?

Those are some of the questions which I do hope will get an answer in this symposium. What we want to be informed about is: What do we actually know today and where are the main lacks in our knowledge? Where are the front lines of research in this rather complex field of physiology?

2 Asmussen, E. (Laboratory for the theory of gymnastics A, University of Copenhagen, Denmark): RESPIRATION.

During exercise the pulmonary ventilation ($\dot{V}E$) increases in proportion to the increase in aerobic metabolism ($\dot{V}O_2$) up to a certain value, corresponding to about 60% of $\dot{V}O_2$ max, after which $\dot{V}E$ increases at a relatively higher rate. This is effected by an increase in depth and in respiratory frequency, but neither of these reach the highest possible values in exercise. Exercise results in an increase in the overall $\dot{V}A/Q$-ratio, and the distribution of $\dot{V}A$ and $\dot{Q}$ in the different parts of the lungs becomes less uneven (West & Dollery, J.Appl.Physiol. 1960. 15. 405). This would tend to diminish the $P(A-a)O_2$-difference. The increased gradient found at maximal exercise (fig.1) may, therefore, be due to the shortened time for reaching diffusion equilibrium in the alveoli. The increased $P(A-a)O_2$-difference is mainly due to an increased PAO_2 at maximal work, the PaO_2 remaining nearly constant. $\dot{V}E$ is hardly the limiting factor in hard exercise at normoxia: Each liter of inspired air will deliver 30 to 40 ml O_2 to the blood in hard exercise, and the extra cost of breathing in the interval 120 to 150 l/min is only about 10 ml O_2 per extra liter ventilation (Bartlett, Brubach & Specht, J.Appl.Physiol. 1958. 12. 413). Local fatigue due to anaerobiosis in the respiratory muscles may, however, play a role: Eldridge (J.Appl.Physiol. 1966. 21. 853) has shown that increased ventilation against an added inspiratory resistance may cause increases in blood lactate and pyruvate. By substituting $He-O_2$ mixtures for air in the inspired air, Murphy, Clark, Bucking & Young, (J.Appl.Physiol. 1969. 26. 303) found in hard exercise that $\dot{V}E$ remained the same at the same external work loads in spite of a drop in $\dot{V}O_2$, caused by the diminished air density. The different steps in the O_2-transport from atmosphere to tissues at increasing $\dot{V}E$ are illustrated by the composite diagram in fig.1. The data are from young average students. A $\dot{V}O_2$ of about 3.5 l/min must be considered maximal in their case.

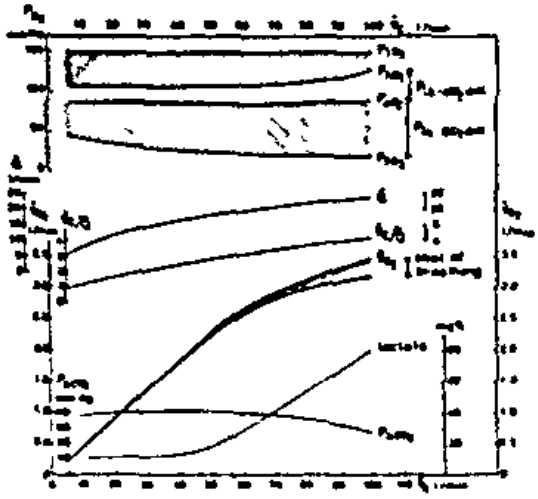


fig.1.

Saltin, B. (Department of Physiology, GIH, Stockholm, Sweden): 3

CENTRAL CIRCULATION AND BLOOD FLOW DISTRIBUTION.

Methods: A comparison of the direct Fick and the dye-dilution technique for determination of the cardiac output in the range from 4 to 32 l/min demonstrated no systematic difference.

Maximal exercise (3-10 min): When the dye-dilution technique was applied in three subjects, who on separate days exercised at a maximal (6-10 min) and a super maximal (3-5 min) work load on a bicycle ergometer, 1.5-4 l/min higher cardiac outputs were observed in the longest run. Maximal oxygen uptakes were the same in both runs. Hence, arterio-venous oxygen difference was less in the long run. The higher oxygen tension in the brachial vein, found in the longest maximal run may indicate a larger skin blood flow, which in this situation may be necessary to avoid overheating. An additional explanation for the observed difference in cardiac output may be that in a super maximal effort the pressure within the muscle may inhibit a maximal muscle blood flow to occur. Perfusion pressure is, however, in the super maximal effort higher, as maximal arterial pressure (mean) is related to the work load. The finding that maximal oxygen uptake can be achieved with different "maximal" cardiac outputs may indicate that the central circulation is not a limiting factor in this type of exercise. On the other hand the increase in maximal oxygen uptakes caused by physical training in young men is almost directly related to the increase in maximal cardiac output (stroke volume).

Maximal values for	Two months training		Several years training	Athletes
	Before	After		
Oxygen uptake l/min	2.52	3.41	4.93	5.57
Arterio-venous oxygen diff. vol%	14.6	17.0	17.1	15.6
Cardiac output l/min	17.2	20.0	28.9	36.0
Heart rate beats/min	192	191	186	190
Stroke volume ml	90	105	155	189
Heart volume ml	740	830	1070	1140

Maximal exercise (30-180 min): During prolonged heavy exercise cardiac output is rather constant after 10-15 minutes of exercise. Heart rate, however, increaseseven after this time with a corresponding reduction in stroke volume. Increased venous pooling of blood and reduced ventricular filling has been suggested, but as exercise in supine position does not change the heart rate/stroke volume relationship, this possibility seems unlikely. A changed balance in the autonomic nervous system with a reduced sympathetic activity (mean arterial blood pressure goes down) on the heart may be excluded as this ought to have influenced also the heart rate. The vagus on the other hand, has mainly a chronotropic effect on the heart and the increase in heart rate may be due to a gradual reduction in vagus activity. When atropin is given before a prolonged exercise period a constant heart rate has been observed between 8 and 60 minutes of exercise. It can be concluded, however, that an impaired central circulation is not a primary factor limiting prolonged heavy exercise.

4 Bevegård, S. (Laboratory of Clinical Physiology, Södersjukhuset, Stockholm, Sweden): CENTRAL CIRCULATION AND BLOOD FLOW DISTRIBUTION.

The capacity of the cardiovascular system to transport oxygen, measured as maximal aerobic capacity or as submaximal exercise tolerance at a given heart rate is closely correlated to static dimensions of the various links of the oxygen transport chain such as total amount of hemoglobin, blood volume and heart volume. These correlations are helpful in defining the borderlines between normal and abnormal and for prediction of functional capacity from static dimensions.

Up to maximal levels of work, heart rate and cardiac output increase as approximately linear functions of oxygen uptake. In order to effect an increase in oxygen transport of 1 liter/min. the cardiac output increases on average by 6 liters/min. During supine leg exercise the stroke volume may increase by 10-20% of the resting value. This is true for healthy males and females, athletes and nonathletes, regardless of the type of supine exercise carried out and the number of muscles involved. Heart volume remains practically unchanged or decreases slightly during supine leg exercise.

Since cardiac output and heart rate during work increase as linear functions of oxygen uptake the arterio-venous oxygen difference is a rectangular hyperbolic function of both oxygen uptake and heart rate.

With transition from supine to upright position at rest about 20% of the blood volume in the heart and lungs is shifted to the lower part of the body. This hydrostatic shift of blood results in decreased filling of the heart and stroke volume decreases. On transition from rest to exercise in the erect position the mechanical effect of the leg muscles redistributes blood centrally and heart volume and stroke volume increase according to the Frank-Starling mechanism. For the same amount of submaximal work performed in the upright compared with the supine position the cardiac output is less because of a smaller stroke volume. From the viewpoint of oxygen transport the difference in stroke volume between the two body positions is compensated for by a higher arterio-venous oxygen difference in the upright position.

With increasing work systemic arterial and pulmonary arterial mean pressures increase approximately linearly in relation to the increase in cardiac output. There is no significant change in pulmonary arterial wedge pressure with submaximal exercise in ordinary subjects. In athletes higher pulmonary arterial wedge pressures have been measured during heavy exercise. Right ventricular end-diastolic pressure remains unchanged or decreases slightly. The increase in blood pressure and heart rate during exercise is tempered by the arterial baroreceptors. They continue to oppose the increase but cannot prevent it.

Efficient distribution of left ventricular output according to metabolic needs and temperature regulation is provided through reflex constriction of resistance vessels. The effects of the increased noradrenergic activity in the exercising muscles are overcome by the local vasodilator mechanism.

The circulatory adaptation to exercise varies according to the muscles involved and whether the work is carried out rhythmically or by isometric contractions. Arm work results in higher heart rate and blood pressure than leg work at a given oxygen uptake.

The increase in cardiac output with exercise is linked to the vasodilation in active muscles and hence to metabolism. Superimposed on this basic relationship is a reflex activation of the sympathetic nervous system to the heart and systemic resistance and capacitance vessels.

Mellander, S. (Department of Physiology, University of Lund, Sweden): CIRCULATION IN SKELETAL MUSCLES.

The increased metabolic demand of skeletal muscle during exercise is met by adjustments of tone in resistance vessels determining blood flow and capillary filtration pressure, in precapillary sphincters determining size of functional capillary surface, and in capacitance vessels influencing venous return. Such vascular adjustments can be evaluated quantitatively and simultaneously by recording blood flow, arterial and venous pressures, capillary filtration or diffusion capacities, tissue volume, and regional blood volume. The local chemical factors (e.g. K^+, hypoxia, and hyperosmolality) can relax precapillary vessels completely at maximal work. In "white" skeletal muscle, this provides for an increase of flow from a resting value of < 50 to > 500 ml/kg tissue x min, and a threefold increase of functional capillary surface, or from about 2 to 7 m^2/kg tissue. The corresponding values are about three times larger in "red" muscle, both at rest and maximal dilatation. Local chemicals cause no active dilatation of capacitance vessels, but decrease the pre-/postcapillary resistance ratio. This leads to net transcapillary fluid loss, in initial stages of exercise at a rate of 5 to 10 ml/kg x min, part of which is regained via the lymph vessels. Other control systems, and mechanical factors, can modify this metabolic pattern of response. Cholinergic dilator fibres, if engaged in exercise, and release of adrenaline (β-adrenergic effect), would contribute to the precapillary vessel dilatation. Increased constrictor fibre discharge, and noradrenaline release, during exercise lead mainly to constriction of capacitance vessels. Rhythmic tetanic muscle contractions can impede muscle flow significantly due to mechanical occlusion of vessels, but also promote venous return by compression of veins. In dependent limbs this latter effect can cause marked phasic reduction of venous pressure, and hence increase the pressure gradient to such an extent that average flow approaches that in an unobstructed, maximally dilated muscle vascular bed. The high transmural pressure in dependent exercising limbs leads to myogenic constriction of precapillary sphincters which is an important reaction to prevent gross oedema formation. It thus appears that during rhythmic exercise, dependent muscle regions are characterized by high blood flows and restricted capillary flow distribution, whereas regions at, or above, heart level receive smaller flows which are distributed over the entire capillary network. This might help to explain the greater solute extractions noted in the latter regions. With regard to transport, limitation in strenuous exercise may be related not only to inadequate supply of O_2 and substrates via the blood stream, but also to exchange problems across the barriers in the tissue, for instance in the interstitial compartment. There is evidence to indicate that the net transcapillary fluid movement occurring in muscle during exercise can help to improve solute transfer through this space.

6 Pernow, B. (Department of Clinical Physiology, Serafimerlasarettet, Karolinska Institute, Stockholm, Sweden): CIRCULATION IN SKELETAL MUSCLES.

The blood flow through resting skeletal muscle amounts to about 3 - 5 ml blood/100 g tissue · min. During maximal muscular exercise the corresponding value is 50 - 85 ml/100 g · min. During both rhythmic isometric and isotonic exercise at submaximal levels the blood flow and oxygen uptake rise in linear proportion to the work intensity, the arteriovenous oxygen difference remaining unchanged. During exercise above 80 % of the maximal work load no further increase in blood flow seems to occur. The size of the blood flow during exercise is dependent on the position of the exercising muscles in relation to the heart level. Studies on forearm blood flow have thus shown lower blood flow values during exercise with the arm vertically elevated than with the arm horizontal. This difference in flow was partly compensated for by a larger arteriovenous oxygen difference resulting in an almost identical oxygen uptake. Production of lactate was, however, significantly greater at work performed with the elevated arm, indicating a greater anaerobic component of the total muscular metabolism in this position.

Muscular exercise elicits an increase in sympathetic adrenergic vasomotor tone, resulting in a constriction of the resistance vessels in skeletal muscle, skin and in the splanchnic region, the increase in vasomotor tone being related to the relative intensity of the work performed. In the exercising muscles this increase in resistance is, however, overcome by a local metabolic vasodilating mechanism, which results in a distribution of blood flow to the exercising muscles well adapted to meet the metabolic demand. Thus, during leg exercise blood flow in the forearm is markedly reduced. The oxygen saturation of femoral venous blood of the exercising leg is decreased in proportion to the work intensity down to values of less than 10 % at maximal levels, indicating a distribution of blood from non-muscular tissues to the exercising muscles. Studies on exercise with arms or legs have shown that an identical external work performed with small muscle groups is accompanied by higher heart rate, arterial blood pressure and pulmonary ventilation, indicating a higher sympathetic tone.

Nielsen, Bodil (Laboratory for the Theory of Gymnastics, University of Copenhagen, Denmark): HEAT REGULATION. 7

In homeothermic organisms the deep body temperature is maintained at a constant level securing a constant high rate for the chemical and physical processes in the body. The temperature distribution in the body is the outcome of a balance between the local heat productions and losses. Due to the forceful stirring of the body core by the circulation the local variations within the core are, however, small. During muscular exercise the temperature of the whole body core is maintained at a higher level than during rest, independent of wide variations in ambient temperature and heat production. The plateau levels of core temperature seem to be a function of the oxygen uptake, and further to be correlated with the individuals maximum oxygen intake capacity.

The regulated core temperature is the result of the action of the temperature centres which regulate the rate of sweat secretion so that the total heat loss by convection, radiation plus by evaporation of sweat equals the heat production.

The possible mechanisms for the temperature setting and for the sweat regulation will be subjects for the discussion.

Nielsen, B.: Thermoregulation in rest and exercise. Acta physiol. scand. 1969. Suppl. no. 323.

10 Karlsson, J., S. Rosell and B. Saltin. (Dept. of Physiology, Gymnastik- & Idrottshögskolan, Stockholm and Dept. of Pharmacology, Karolinska Institutet, Stockholm, Sweden): BLOOD-BORNE SUBSTRATES.

Glucose and free fatty acids (FFA) can be mobilized within minutes from the storage sites in the liver and adipose tissue. It has also been proposed that muscular activity promotes the uptake of blood glucose into skeletal muscle. Therefore, the substrates required for muscular activity may be supplied from the blood rather than from storage sites within the muscle.

In order to clarify the quantitative aspects of the uptake of blood-borne substrates during muscular activity, experiments were performed on canine gracilis muscle. After a resting period of 20-30 min the mixed nerve to the muscle was stimulated electrically for 60 min with 5 imp x sec^{-1}, duration 0.1 msec. This resulted in a 30-fold increase in O_2-uptake, indicating heavy muscular contractions.

During rest the blood flow averaged 7 ml (all values refer to 100g x min^{-1}), oxygen uptake 7 µmoles and uptake of glucose 0.8 µmoles. FFA showed no significant uptake or release. Lactate production averaged 1.1 µmoles.

During muscular contractions there was a pronounced increase in O_2-uptake and lactate production. A small but significant rise in uptake of glucose occurred.

The results indicate that blood-borne FFA was not a major source for increased substrate consumption. The total consumption of lipids could not be calculated since the changes in the lipids stored in the muscle tissue was not determined.

The RQ values indicated a predominating carbohydrate metabolism during muscular contractions. However, the uptake of blood glucose could only account for approximately 20-30 per cent of the total calculated carbohydrate metabolism. The major part of the carbohydrates thus seemed to be derived from glycogen stored in the muscle. This suggestion was supported by measured decrease in the glycogen depots. Thus, blood-borne substrates seemed to be of minor importance during heavy muscular contractions.

Thorn, N.A. (Institute of Medical Physiology C, University of Copenhagen, Denmark): CURRENT PROBLEMS CONCERNING THE MECHANISM OF SECRETION OF WATER AND ELECTROLYTES BY EXOCRINE GLANDS. 11

Studies in recent years have demonstrated the importance of the duct system in the formation of glandular secretions. On the basis of indirect studies Thaysen, Thorn and Schwartz (Amer.J.Physiol. 1954.178.155) formed the hypothesis that parotid saliva is formed as a precurser fluid isotonic with plasma and that sodium is reabsorbed actively in the duct system. This reabsorption seemed to be influenced by aldosterone (Thorn, Schwartz and Thaysen, J.Appl.Physiol. 1956.9.477.) The hypothesis has been confirmed by more direct studies, comprising duct destruction and micropunctures (see Young & Schögel, Pflügers Arch. 1966.291.85.) Such studies have also revealed passive exchange over the duct epithelium (reequilibration processes) at low flow rates. Evidence is now accumulating that the duct system of the pancreas also participates in the formation of the final secretory product (Case, Harper & Scratcherd, J.Physiol. 1969.2o1.335).

As regards the formation of the primary saliva there is still much ignorance concerning the time relations of the different partial processes as well as their causal interrelations. Most evidence seems to be in agreement with the hypothesis of a non-electrogenic pump transporting Na and Cl across the acinar part. It seems that the secretory potential over the basal membrane is more likely due to a transport of cations from cell to blood than to transport of anions in the opposite direction. The secretory potential demonstrated by Lundberg (Physiol.Rev. 1958.38.21) may be a step in the activation of the secretion, but is probably not an expression of an ion transport quantitatively significant in the secretion proper (Petersen & Poulsen, Experientia 1968.24.919). Recent years have brought other interesting information concerning the activation of the secretory process. It has been demonstrated that calcium lack inhibits the secretion (Douglas & Poisner, J.Physiol.(Lond.) 1963.165.528). The fact that the secretory potential was nearly unchanged as was the K-loss to the perfusion fluid in glands perfused with a Ca-free-fluid (Petersen, Poulsen & Thorn, Acta Physiol.Scand. 1967.71.2o3, Proc.Int.Union Physiol.Sci. 1968, vol. VII.345), would seem to be in accordance with the hypothesis that the activation of the basal cell membrane is normal when Ca is lacking, and that Ca is essential for the link between activation and secretion. This is analagous to conditions in certain neurosecretory systems (Berlind & Cooke, Gen.Comp.Endocrinol. 1968.11. 458). In the initial state of secretion seemingly correlated transient changes in conc. of K^+ and Na^+ in saliva occur (Petersen & Poulsen, Acta Physiol.Scand. 1968.73.93) which seem to reflect special conditions before a steady state is reached.

12 Emmelin,N. (Institute of Physiology, University of Lund, Sweden): CURRENT PROBLEMS CONCERNING INNERVATION OF SALIVARY GLANDS.

Morphological observations on salivary glands show that the various effectors, secretory, vascular and myoepithelial, receive nerves (Garrett,J.R., J.R.microsc.Soc. 1966.85.149); this is supported by physiological experiments. The secretory cells are mainly activated by parasympathetic impulses at a rate normally seldom exceeding 8/sec. Some glands are activated by sympathetic impulses also, acting on α-receptors (cat submaxillary), β-receptors (dog submaxillary) or both (rat submaxillary). From the nerves transmitter leaks, normally causing no secretion but lowering the sensitivity of the cells; after nerve section a transiently increased leak causes "degeneration secretion". Some glands secrete spontaneously but most of them only when acted on by the transmitters or related drugs. Certain polypeptides (physalaemin, substance P, sialotonin) may also evoke secretion but it is unknown whether polypeptides physiologically activate the gland cells. Moderate doses of kallidin or bradykinin cause no salivation. Sympathetic nerves constrict the blood vessels and recent experiments suggest that the vasodilatation during parasympathetic stimulation is in fact due both to vasodilator nerves and a kinin mechanism (Gautvik,K., personal communication). The myoepithelial cells receive both sympathetic and parasympathetic motor nerves (see Emmelin,N., Garrett,J.R. and Ohlin,P., J.Physiol. 1969.200.539). Stimulation of such nerves causes a pressure rise in the salivary duct even without secretion, e.g. when single shocks are applied or secretion is abolished by drugs. Thus in the dog submaxillary gland sympathetic stimulation raises the pressure even after propranolol, but not after an α-blocker also. Kallidin and bradykinin contract the myoepithelial cells but it is unknown if this has any physiological significance.

Stimulation experiments thus suggest that the various effectors are innervated, often both by sympathetic and parasympathetic nerves; but this does not necessarily imply that specific secretory, vasomotor and motor nerves exist. The electron microscopical studies show that the salivary nerves enter into contact with the effectors by en-passant contacts. A single axon might thus affect several secretory cells, perhaps by a more or less close contact, but might possibly also affect different types of effectors. The existence of separate sympathetic glandular vasoconstrictors is suggested by the fact that bleeding causing a marked vasoconstriction in the cat submaxillary gland is not accompanied by salivary secretion which occurs when similar vasoconstriction is produced by electrical stimulation of the sympathetic (Emmelin,N. and Engström,J., J.Physiol. 1960.153.1); this specificity is reasonable in view of the general functions of the vasoconstrictors. But secretion and myoepithelial contraction may be caused by the same sympathetic axon. Similarly, impulses in one parasympathetic axon might start secretion, open up blood vessels to increase the blood supply and contract myoepithelial cells to promote salivary flow. At the same time excitation of the secretory cell starts the kinin mechanism, causing vasodilatation and perhaps myoepithelial contraction.

Flemström, G. (Institute of Physiology and Medical Biophysics, University of Uppsala, Sweden): **13**

IN VITRO STUDIES ON GASTRIC MUCOSA.

Sodium and hydrogen ion transport and some electrical properties of frog gastric mucosa (Rana temporaria) in vitro were investigated with different oxygen tensions in the bathing solutions. Experiments were performed under voltage clamp conditions. ^{22}Na and ^{24}Na were used as tracers.

Active transport of sodium ions from the mucosal to the serosal side was 0.12 uEqv/hr,cm^2 with a pO_2 of 700 mm Hg, 0.41 uEqv/hr,cm^2 at 300 mm Hg and 0.05 uEqv/hr,cm^2 at 50 mm Hg. This transport contributed to 5%, 20% and 4% respectively of the total shortcircuit current.

Reduction of pO_2 to 300 mm Hg or less gave rise to an decrease of the hydrogen ion transport not completely reversible. The ratio between active transport of hydrogen ions and chloride ions remained the same at oxygen tensions between 700 and 150 mm Hg (chloride transport calculated as short-circuit current + hydrogen transport - sodium transport).

It is concluded that a degree of hypoxia which reduces hydrogen ion transport also stimulates sodium ion transport in the frog gastric mucosa. Recently sodium transport was demonstrated in the mammalian gastric mucosa in vitro. As it is difficult to obtain satisfactory oxygenation in isolated mammalian tissues this transport may be at least in part due to hypoxia in the mucosa.

The hydrogen and chloride ion transport mechanisms showed the same sensitivity to hypoxia which supports the suggestion (Forte, J.G., Am. J. Physiol. 1969.216.167) that they are mutually coupled biochemically or electrically.

14 Helander, H.F. (Department of Anatomy, University of Umeå, Sweden): *MORPHOLOGICAL ASPECTS OF GASTRIC SECRETION*

Five different types of secretory cells are intermingled in the fundus glands of the mammalian gastric mucosa. The zymogen cells produce pepsinogen and are characterized by their basophilia and their secretory (zymogen)granules. Correspondingly, in the electron microscope these cells exhibit a large granular endoplasmic reticulum, mainly concentrated to the basal part of the cytoplasm, and numerous secretory granules in the apical part. The Golgi apparatus is also fairly large. During secretion there is an increase in the RNA content of these cells, and the cisternae of the endoplasmic reticulum become dilated, probably as a result of an accumulation of secretory material. The Golgi apparatus expands, but the mean size of the zymogen granules is reduced. Emptying of zymogen granules into the gland lumen is accomplished by a granulocrine type of secretion. Inhibition of the gastric secretion by atropine, results in a piling up of zymogen granules, and a concomitant increase in the peptic activity of gastric mucosa homogenates.

Although it has not definitely been proved, it is generally accepted that the parietal cells secrete HCl. These cells are acidophilic and contain very little RNA. Their granular endoplasmic reticulum is very sparse, and the Golgi apparatus discrete. Mature parietal cells contain no secretory granules. A very large number of mitochondria indicates a high respiratory capacity . Another striking feature is the presence of cytoplasmic vacuoles, mainly accumulated in the apical half of the cytoplasm. During ontogeny these vacuoles appear at about the same time as the production of HCl starts. The most unique characteristics of the parietal cells are the intracellular canaliculi which start deep in the cytoplasm and open onto the apical cell surface. These canaliculi with their numerous microvilli greatly increase the "secretory surface" of the parietal cells. During secretion, the cytoplasmic vacuoles become less numerous and the microvilli of the secretory surface increase greatly in number, so that the canaliculi become almost obliterated.

The mucoid cells of the fundus glands, i.e. the surface epithelial cells and the mucous neck cells generally exhibit little endoplasmic reticulum, a moderately sized Golgi apparatus, and a varying number of secretory granules. The surface epithelial cells are tall columnar cells, whereas the mucous neck cells often are pear-shaped. The larger number of secretory granules in the surface epithelial cells may also serve to distinguish these two cell types from each other. There are no greater changes in the morphology of the mucoid cells after stimulation by feeding.

Argyrophile cells occur in small numbers, mainly concentrated to the basal half of the gastric mucosa. Since they hardly ever reach the glandular lumen, they have been ascribed an endocrine function. They are rather similar to other cells of the enterochromaffine system, and may be the source of gastrin.

Poulsen, J.H. (Institute of Medical Physiology C, University of Copenhagen, Denmark): THE NATURE OF THE RESTING MEMBRANE POTENTIAL IN THE ACINAR CELLS OF THE CAT SUBMANDIBULAR GLAND. 15

While the nature of the secretory transmembrane potential in salivary glands has been the subject of a number of investigations, only little attention has been paid to the corresponding resting potential. As the size of the resting membrane potential of the acinar cells in the cat submandibular gland is comparatively small (about -2o mV) it seems likely that the mechanism underlying it, differs from the one of the much more thoroughly investigated nerve- and muscle cells.

The submandibular gland of the cat was artificially perfused by Locke's solutions of different ion composition. Transmembrane potentials were recorded by use of ordinary glass microelectrodes filled by either 3M MCl or 3M NH_4NO_3. The tip potentials were always less negative than -5 mV.

The resting membrane potential was found to vary inversely approximately with the logarithm of the extracellular potassium concentration, but the slope was very small (about 6 mV per lo-fold increase in potassium concentration). Substituting lithium for sodium did not change the resting potential. However, substituting tetraethylammonia for sodium increased the potential. A similar effect was obtained by substituting sulphate for chloride, whereas substituting nitrate for chloride did not change the membrane potential significantly.

It is concluded that both potassium, sodium, and possibly chloride contribute to the maintenance of the resting membrane potential. Similar results and conclusions were reached by Douglas, W.W., T. Kanno, and S.R. Sampson, J.Physiol. 1967 191.1o7. in studies on the adrenal chromaffin cells.

16 Petersen, O.H. (Institute of Medical Physiology C, University of Copenhagen, Denmark): THE MECHANISM OF THE LOSS OF POTASSIUM FROM SALIVARY GLANDS AFTER STIMULATION WITH ACETYLCHOLINE.

When salivary glands are stimulated to secrete, their cells lose potassium (K^+) to both the saliva and the blood. This loss is followed by a reuptake of K^+ from the blood to the gland cells (Burgen, A.S.V., J.Physiol., 1956. 132. 2o). Little is known about the mechanisms underlying these transports.

In the present study the K^+ transport between perfused cat submandibular glands and the perfusion fluid was investigated. The flow of perfusion fluid from a cannula in the gland vein was recorded and the concentration of K^+ in the perfusion fluid coming from the gland vein cannula was measured by flame photometry. Injections of lo µg acetylcholine (ACh) were given intraarterially and the relationship between the secretion (number of drops of saliva coming from a cannula inserted into the submandibular duct) and the loss of potassium to the perfusion fluid was studied.

During perfusion with sodium-free (Na^+-free) lithium (Li^+) Locke's solution the secretion was much more reduced than was the loss of K^+, while the gland was unable to take up K^+ from the perfusion fluid. After having returned to Na^+ Locke's solution the secretory ability was only partially restored while the loss of K^+ on stimulation was of the same size as before the perfusion with Li^+ Locke's solution. The ability to take up K^+ from the perfusion fluid was immediately restored by returning from Li^+ to Na^+ Locke's solution.

lo min after start of perfusion with Na^+ Locke's solution containing dinitrophenol (DNP) (10^{-4}M) the loss of K^+ on stimulation was uninhibited while the secretion was reduced. To secure that this apparently lacking effect on the K^+ loss was not due to simultaneous effects on K^+ loss and uptake, obscuring an effect on the K^+ loss, the effect of perfusion with DNP for 15 min was investigated after 2 min of perfusion with K^+-free Locke's solution. Also in this case DNP had a much smaller effect on the K^+ loss after stimulation than on the secretion.

Generally the effect of ACh on cell membranes is to increase the permeability to ions such as K^+. As K^+ is not in thermodynamic equilibrium across the acinar cell membranes of the salivary glands, but has a marked tendency to escape from the cells, this effect of ACh could explain the K^+ loss from salivary glands after stimulation with ACh.

Frederiksen,O. & P.P.Leyssac. (Inst.f. exper.Medicine, University of Copenhagen. Denmark). ISOSMOTIC TRANSPORT ACROSS THE GALL BLADDER EPITHELIUM. 17

It is well established that the in vitro gall bladder preparation from the lumen absorbs NaCl and water in isosmotic proportions between identical bathing solutions in the absence of impermeant solutes. Net transport of Na and Cl are accomplished by an active electrically neutral transfer process.

Taking advantage of the high rates of solute-and water transfer and energy consumption characteristic for the gall bladder epithelium some general assumptions regarding the mechanism of solute transfer could be investigated. Net fluid absorption was measured by weighing the bladder; net Na-transport was calculated from the water transport and the Na-concentration of the bathing media, and oxygen consumption was measured with an oxygen electrode.

The acute effects resulting from abrupt changes in NaCl concentration of the bathing media indicated that fluid volume transfer remained unaffected while Na-transport changed abruptly in direct proportion to the NaCl concentration. Transport-linked oxygen consumption was linearly related to net fluid volume transfer and oxygen uptake per eqv. Na transported varied in inverse proportion to the NaCl concentration of the media.

It is concluded that the range of absorption rates of isosmotic fluid represents a range of energy-requiring capacities for transcellular transfer of fluid volume units.

18 Uvnäs, B. (Department of Pharmacology, Karolinska Institutet, Stockholm, Sweden): INTRODUCTION.

Storage in subcellular elements is a common way by which living organisms harbour biologically-active substances in an inactive form. From such intracellular depots the substances can be rapidly released in an active form. Several biogenic amines are localized to granules or vesicles in adrenal medullary cells, in mast cells, in thrombocytes, in enterochromaffin cells, in peripheral and central neurones and nerve terminals etc. From their stores the biogenic amines can be released by chemical and physical stimuli under physiological and pathological conditions, as well as by various drugs. It was thought that a comparative analysis of the morphological and chemical features of the subcellular depots, the mechanisms of uptake, storage and release of the active substances from these depots into the extracellular milieux should contribute to a wider understanding of these release processes. Today's symposium is designed to give various specialists in the Scandinavian countries the possibility of presenting and collating their knowledge and experience within this field which is of the utmost importance to both physiologists and pharmacologists. I hope their contributions will stimulate a lively and constructive discussion.

19 Hökfelt, T. (Department of Histology, Karolinska Institutet, Stockholm, Sweden): ULTRASTRUCTURAL STUDIES ON MONOAMINE STORAGE VESICLES.

Using various types of fixatives (e.g. potassium permanganate) the ultrastructural localization of certain monoamines in peripheral and central neurons has been studied. Evidence was obtained that two types of storage vesicles exist in peripheral noradrenaline as well as in central dopamine, noradrenaline and 5-hydroxytryptamine neurons. The first type, the small granular vesicles, have a diameter of about 500 Å and the second type, the large granular vesicles, have a diameter of about 1000 Å. Both types are limited by a triple-layered membrane and contain an electron dense core or granule, which probably represents the reaction product between the amine and the fixative. The small and large granular vesicles are found in all parts of the neurons, although the highest numbers occur in the terminal parts of the axons, mainly localized to axonal enlargements often called varicosities. The small granular vesicles are mostly more numerous than the large ones. Thus, in certain peripheral adrenergic neurons about 98 per cent of all vesicles are of the small type. Results obtained after electric stimulation and the intraneuronal localization of the vesicles may suggest that the small granular vesicles are of immediate importance for the release of monoamines at the synaptic cleft.

Bloom, G.D. and Ö. Hägermark (Department of Histology, University of Umeå, Umeå and Department of Dermatology, Karolinska Institutet, Stockholm, Sweden): ON THE ULTRASTRUCTURE OF MAST CELL GRANULES. **20**

The ultrastructure of mast cells is well preserved after fixation in glutaraldehyde and osmium tetroxide. In electron micrographs of thin sections stained with uranyl acetate and lead citrate mast cells are filled with electron dense granules, about 0.5 μ in diameter. The fine structure of the granules varies in different animal species. The granules are surrounded by a closely adhering unit membrane; sometimes an electron lucid space separates the granular matrix from the surrounding membrane.

Histamine release and the concomitant morphological changes were studied in rat mast cells under various conditions. Some liberators, e.g. compound 48/80, seem to trigger an active cell process with extrusion of mast granules as a final result. After treatment with such liberators the number of altered, less dense granules increases in the cells. The granular extrusion seems to occur by exocytosis, whereby the perigranular membrane is left behind in the cell. Thus the released granules are devoid of a membrane. Surface active agents and hyposmosis bring about histamine release by affecting the integrity of mast cell structure resulting in a breakdown of plasma membrane and cellular compartments.

Helle, Karen B. (Institute of Physiology, University of Bergen, Norway): THE CHEMICAL COMPOSITION OF THE CHROMAFFIN GRANULE. **21**

Chromaffin granules of bovine adrenal medulla have been shown to contain 35% protein, 22% lipid, 20.5% catecholamines and 15% ATP (Hillarp, N.-Å., Acta physiol. scand. 1959.47.271). The lipid fraction consists mainly of phospholipids and cholesterol (Winkler, H. et al. Arch. exp. Path. Pharmak. 1967.256.407). Chromogranin, the major protein of the chromaffin granule, has been shown to be present in the membrane as well as in the lysate fraction of the granules (Helle, K.B. and Serck-Hanssen, G., Pharmacol. Res. Comm. 1969. 1). Chromogranin has been isolated as a lipoprotein of very high density, containing 0.25μmoles lipid phosphate/mg protein (Helle, K.B., Biochem. J., 1968.109.43P), particularly rich in glutamyl and prolyl residues but low in cysteine and cystine (Helle, K.B., Mol. Pharmacol. 2. 298. 1966). Associated with the granule membrane are the enzymes dopamine-β-hydroxylase (Kirshner, N., Pharmacol. Rev. 11.350. 1959) and ATP-ase, plus a cytochrome of the b-type (Banks, P., Biochem.J. 1965.95.490.

Chromaffin granules of splenic nerves have been shown to contain in addition to noradrenaline and ATP (Euler, U.S.v. and Hillarp, N.-Å., Nature (Lond.) 1956.177.44) also the specific granule protein, chromogranin (Banks, P. et al., Mol. Pharmacol. 1969.5.210) as well as the enzyme dopamine-β-hydroxylase (Livett, G.B. et al., Biochem. Pharm. 1969. 18.923).

22 Bergqvist, Ulla (Department of Pharmacology, Karolinska Institutet, Stockholm, Sweden): *CHEMISTRY OF MAST CELL GRANULES.*

In rat peritoneal mast cells histamine is stored in intracellular granules consisting mainly of a protein-heparin complex. Since it has been proposed (Åborg, C.-H., J. Novotný and B. Uvnäs, Acta physiol. scand. 1967, 69, 276-283) that the amine is bound electrostatically to carboxyl groups in the protein part of the complex we have attempted to isolate and identify this protein.

Most of the protein-heparin linkage appears be ionic and mast cell granules may be dissolved in M NaCl. The major part of the protein can then be separated from heparin by running such a solution through a Dowex 1-X2 column. Subsequent elution with 2 M NaCl yields a fraction containing heparin and a small amount of protein which is probably covalently bound to it.

The free protein can be separated into water-soluble and water-insoluble fractions. Both are homogenous on disc electrophoresis. Their amino acid compositions are similar, each containing 16 different amino acids. The proteins are both basic with an IP on Cellogel strips of 10.5-11.0. The percentages of the dicarboxylic acids Glu. and Asp. are high enough to account for the amine binding capacity of the granules if it is assumed that the amines are bound to free carboxyl groups in the protein.

The molecular weight of the soluble protein, determined by equilibrium centrifugation was 5,600. The two proteins have high chymase activities, which is rather surprising considering their low molecular weights.

23 Björklund, A. (Institute of Anatomy and Histology, University of Lund, Lund, Sweden) HISTOCHEMICAL DEMONSTRATION OF TRYPTAMINE.

A sensitive fluorescence method has been devised for the demonstration of tryptamine on the cellular level by formaldehyde condensation. This gives a 13-18 fold increase in the fluorescence yield of tryptamine compared with the conventional Falck-Hillarp method. The conditions of the conventional procedure were modified in one of two ways: (1) A gaseous oxidant (ozone) was introduced into the reaction vessel. Up to a certain concentration of ozone, the yield of the formaldehyde-induced tryptamine fluorescence was markedly increased, probably because of an increased oxidative dehydrogenation of the initial condensation product. In the combined formaldehyde-ozon procedure, the fluorescence yields of catecholamines and 5-HT were reduced to about 20% compared with the yields in the conventional formaldehyde treatment. (2) Acidification after conventional formaldehyde treatment produced a similar high fluorescence yield.

The histochemical and microspectrofluorimetric characteristics of the fluorophores formed from tryptamine under these conditions differ clearly from those of the fluorophores formed from catecholamines, 5-HT, and other indole compounds tested. These procedures, which were worked out on histochemical models, have successfully been applied also on tissues incubated with tryptamine. Thus, the findings seem to offer the possibility of demonstrating tissue tryptamine with high specificity and sensitivity. A substance behaving like tryptamine, according to the above-mentioned criteria, has been found in cells of the pars intermedia and pars distalis of the pituitary gland.

Owman, Ch. and Håkanson, R. (Departments of Histology and Pharmacology, University of Lund, Sweden): FLUORESCENCE MICROSCOPY OF NON-MAST CELL HISTAMINE IN MURINE GASTRIC MUCOSA. 24

Histamine in freeze-dried cryostat sections (Håkanson and Owman, Life Sci, 1967, 6, 759; Thunberg, Exp. Cell Res. 1967, 47, 108) or paraffin sections (Ehinger, Håkanson, Owman and Sporrong, Biochem. Pharmacol. 1968, 17, 1997) forms fluorophores with o-phthaldialdehyde vapours. Microspectrographic analysis of the fluorophores from authentic histamine on thin-layer silica gel chromatoplates (Håkanson, Juhlin, Owman and Sporrong, to be published) shows activation peaks at 365 mμ and 405 mμ. The emisson maximum changes with the concentration of histamine, from yellow (about 550 mμ) at high concentrations towards a blue fluorescence at low concentrations.

The bulk of gastric histamine in mouse and rat is stored in a large system of blue-fluorescent, enterochromaffin-like cells in the mucosa of the oxyntic gland area; the cells are argyrophilic but non-argentaffin (Håkanson and Owman, Experientia, in press). Histamine also occurs in the mast cells, which are the only storage site for gastric histamine in all other mammalian species analyzed. Gastric histamine is resistant towards 48/80 (Aures, Håkanson, Owman and Sporrong, Life Sci, 1968, 17, 1147).

The epithelial histamine cells in rat gastric mucosa are probably endocrine. Their distribution agrees with that of vitamin B_{12}-binding protein; they may thus be the storage site of the antipernicious principle (Håkanson, Lindstrand, Nordgren and Owman, to be published).

von Euler, U.S. (Department of Physiology, Karolinska Institutet, Stockholm, Sweden): FACTORS INFLUENCING UPTAKE AND RELEASE OF TRANSMITTER IN ADRENERGIC NERVE GRANULES. 25

Isolated adrenergic nerve granules suspended in phosphate buffer or sucrose give off noradrenaline (NA) at a rate which is dependent on pH, temperature and the NA concentration in the medium. Concomitantly with the release, reuptake occurs, beginning at about 10^{-7} M NA. At 10^{-4} M NA reuptake balances release and no net change of NA occurs. In the presence of ATP in the medium, release is balanced by uptake even at 10^{-6} M NA.

Characteristic changes in the release rate and uptake power are observed with various groups of drugs. Indirectly acting amines (tyramine, phenethylamine, amphetamine) enhance the release and inhibit the uptake of NA. Only part of this effect is due to exchange.

Adrenergic blocking agents, particularly of the α-type, inhibit both release and uptake of NA. The possible analogy of the uptake processes in granules and in effector cell receptors has been suggested (Euler and Lishajko 1967).

Various metabolic inhibitors influence the release and uptake of NA in granules. Oligomycin inhibits both processes, while antimycin A in 10^{-5} M blocks uptake almost completely. Uncouplers of various kinds (DNP, CCP, pentachlorphenol and desaspidin) all enhance the release. It is suggested that uptake and binding require the presence of ATP, and that release ensues in the absence of ATP or during increased ATP-ase action.

26 Lishajko, F. (Department of Physiology, Karolinska Institutet, Stockholm, Sweden): RELEASE AND REUPTAKE OF CATECHOLAMINES IN ISOLATED ADRENAL MEDULLARY GRANULES.

Isolated granules from sheep and bovine adrenal medullary cells release dopamine (DA), noradrenaline (NA) and adrenaline (A) at characteristically different rates. Thus DA and A follow an exponential course, while NA is initially more rapidly released and thereafter exponentially at a slower rate in 0.13 M K-phosphate pH 7.5 at 37°C. The rapidly released NA was localized in the low speed sediment, while in microsome fractions NA and A are released exponentially. Addition of DA to the incubation fluid decreases the net loss of DA in the sediment after incubation, owing to reuptake.

Addition of ATP-Mg^{2+} inhibits the release of DA as well as that of A and NA, presumably owing to inhibition of release and increased reuptake. However, in the presence of chloride ions above 40 mM in the incubation media, ATP-Mg^{2+} accelerates the release of NA and A. The influence of Cl^- ions on release of catecholamines will be discussed. No net uptake of NA and A was observed in partially depleted granules in the presence of ATP-Mg^{2+}, while the granules take up DA up to the original amount. Addition of ATP-Mg^{2+} also inhibits the release of soluble proteins in parallel with the catecholamines. The increased release of catecholamines caused by prenylamine is also accompanied by a release of soluble protein.

27 Uvnäs, B. and Åborg, C.-H. (Department of Pharmacology, Karolinska Institutet, Stockholm, Sweden): STORAGE AND RELEASE OF HISTAMINE IN ISOLATED MAST CELL GRANULES.

Granules isolated from water-lysed mast cells retain their histamine when suspended in distilled water. The histamine is released on suspension of the granules in sodium chloride solutions, the release occurring as an equivalent ionic exchange between Na^+ and Hi^+. The influence of pH on the histamine storage indicates an ionic linkage to COO^- groups. The uptake capacity is around 1000 mμeq/mg granules (dry weight). Titrations of the granules between pHs 3 and 7 indicate the presence of weakly acid (COO^-) groups capable of binding around 1000 mμeq. of cations per mg. in the protein part of the granule heparin protein complex.

The cation binding properties of the granules are similar to those of a weak cation exchange resin. On exposure to cations in concentrations normally present in intra- and extra-cellular milieux they will release all their histamine.

Hamberger, B. and G. Jonsson (Department of Histology, Karolinska Institutet, Stockholm, Sweden). UPTAKE OF NORADRENALINE IN ADRENERGIC NERVES. **28**
Adrenergic nerve terminals have a great ability to take up and accumulate catecholamines. It is now generally accepted from histochemical and biochemical experiments that at least two mechanisms exist for this uptake and storage in the isolated rat iris. The membrane pump seems to be the most important mechanism for the initial uptake while the granular uptake mechanism is important for the storage and retention intraneuronally. Reserpine pretreatment does not affect the initial uptake rate and noradrenaline can accumulate intraneuronally outside the usual storage sites provided that the action of monoamine oxidase has eliminated. After pretreatment with the catecholamine synthesis inhibitor H 44/68, the initial uptake is not influenced and the total storage capacity is only increased 10-20% compared to the untreated and the intraneuronal localization seems to be the same. The results indicate that the depleted noradrenaline stores cannot be completely refilled with exogenous noradrenaline after depletion with a synthesis inhibitor. Uptake of noradrenaline to any significant degree outside the adrenergic nerves only occurs with high concentrations (10^{-3}M or more) of the amine in the medium and can in these cases be located both diffusely and to special cells.

Stjärne, L. (Department of Physiology, Karolinska Institutet, Stockholm, Sweden): RELEASE OF CATECHOLAMINES FROM ADRENAL MEDULLA AS COMPARED TO THAT FROM SYMPATHETIC NERVES. **29**

Hormone secretion from the adrenal medulla seems to occur by exocytosis. Secretion is initiated by acetylcholine (Ach) released from the secretory nerve terminals, which exhibit both temporal and spatial summation. Ach causes partial local depolarization of the adrenal medullary cells accompanied, but not necessarily causally related to, increased Ca^{++} influx, which in its turn mediates secretion of all the soluble components of the amine storage vesicles directly into the extracellular space. There is good experimental support that this sequence of events can take place. However, final proof that this is the physiological mechanism for catecholamine (CA) secretion from the medulla is still lacking.

Concerning neurotransmitter release from sympathetic nerves theoretical considerations as well as available experimental data rather contradict the probability for exocytosis. Thus CA turnover in the nerves is much more rapid than in adrenal medulla. It seems unlikely that protein turnover could be equally fast, as required by the exocytosis hypothesis. Moreover, the half life of nerve granules is considerably longer than that of the granular amines. Experimentally, there is so far no indication that the noradrenaline (NA) released by nerve impulses is accompanied by other soluble constituents of the nerve granules. Results recently obtained at this laboratory give no indication that adenine nucleotide material is released concomitantly with NA from the sympathetic nerves of the cat spleen. The evidence thus points towards basic differences in mechanisms of hormone secretion and neurotransmitter release.

30 Paasonen, M. K. and Solatunturi, E. (Department of Pharmacology, University of Helsinki, Finland): RELEASE OF 5-HYDROXYTRYPTAMINE (5HT) FROM BLOOD PLATELETS.

5HT is stored in mammalian blood platelets in highly osmiophilic organelles, which also contain ATP and, in the platelets of rabbit, histamine. The uptake of 5HT by platelets against a concentration gradient can be inhibited at the platelet membrane by, e.g., imipramine, and at the intracellular stores by reserpine-like drugs.

The release of 5HT from platelets is induced by reserpine and, at high concentrations, by many drugs, which, at lower concentrations inhibit the uptake of 5HT. Chlorpromazine (CPZ) and other related phenothiazine derivatives cause a depletion of 5HT, histamine, potassium and ATP from rabbit platelets in vitro. When platelets were incubated with $3x10^{-4}M$ or higher concentration of CPZ in plasma containing ^{3}H-methoxyinulin the amount of inulin in the subsequently separated platelet pellet was about doubled when compared with inulin in the control pellet. This indicates that CPZ causes membrane damage allowing inulin to penetrate into platelets. Desmonomethyl--CPZ, a more potent 5HT releaser than CPZ, was more potent in this experiment, too, but reserpine had no effect. The membrane damage seems not to be necessary for the 5HT release induced by CPZ-sulphoxide. This phenothiazine has only weak central action, and, unlike CPZ and other related drugs, it neither inhibits glucose oxidation nor decreases ATP content in platelets.

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31 Slorach, S.A. (Department of Pharmacology, Karolinska Institutet, Stockholm, Sweden): RELEASE OF HISTAMINE FROM MAST CELLS INDUCED BY DEGRANULATING AGENTS.

Histamine in mast cells is stored in discrete intracellular granules, consisting of a protein-heparin complex, and may be released by suspending the granules in cation-containing media. It is probable that histamine release from such cells involves a cation exchange, either extra- or intra-cellular, at some stage.

A study of the amounts of histamine, heparin and granule protein released on exposure of the cells to the degranulating agent compound 48/80 showed that there was a good correlation between the three release curves. From these results and further studies on the uptake of sodium by compound 48/80-treated cells it is concluded that the principal mechanism of histamine release induced by this degranulating agent involves an initial extrusion of histamine-containing granules. This is followed by an exchange of histamine in the extruded granules for cations in the extracellular medium.

Diamant, B.(Dept. of Pharmacology, Karolinska Institutet, Stockholm, Sweden): HISTAMINE RELEASE FROM MAST CELLS INDUCED BY ATP. 32

ATP induces histamine release from mast cells by a non-degranulating mechanism. A 1:1 molar relationship has been established between histamine release and hydrolysis of extracellular ATP by an ATP-hydrolysing enzyme in the mast cell membrane. The difference in morphology of mast cells after local application of ATP and compound 48/80 to the cell membrane will be demonstrated. The two mechanisms also differ in regard to time course and effects of divalent cations. Common features of the two mechanisms are their dependence on pH and temperature as well as on endogenous ATP. With antimycin and oligomycin (10^{-7}M) histamine release is inhibited concomitantly with a decrease of intracellular ATP; in the presence of glucose the decrease of endogenous ATP is counteracted and at the same time the effect of antimycin on histamine release is reversed in the case of 48/80 but not in the case of ATP. This indicates that 48/80-induced histamine release can utilize endogenous ATP derived from glycolysis whereas ATP-induced histamine release depends on oxidative phosphorylation. Due to differences in the time course of the two release mechanisms, mast cells need preincubation with antimycin for inhibition of 48/80-induced histamine release whereas this is not necessary for release induced by ATP.

The results indicate the existence of two different mechanisms by which histamine can be released from mast cells.

Häggendal, J.(Dept. of Pharmacology, University of Göteborg, Sweden): ON RELEASE OF TRANSMITTER FROM ADRENERGIC NERVE TERMINALS AT NERVE ACTIVITY. 33

Transmitters are generally assumed to be released in the form of "quantal packets" (cf. Eccles, J.C., The physiol. of synapses. Springer-Verlag. 1964). The transmitter content in one vesicle or granule is suggested to correspond to one quantal packet which can produce a subthreshold miniature potential. - Since the amine storage granules have a life-span of several weeks (Dahlström, A. and Häggendal, J., Acta physiol. scand. 1966. 67. 278), whereas the nerve terminal turnover of noradrenaline (NA) appears to be at most a few days, it seems improbable that whole granules would be released. - Considering the inactivating mechanisms at the nerve terminal-effector cell level of adrenergic vasoconstrictor nerves, the amount of NA, released to the blood stream, was found rather low per nerve impulse as compared to the tissue NA content (Folkow, B., Häggendal, J. and Lisander, B., Acta physiol. scand. 1967. Suppl. 307). Assuming that one nerve impulse may release NA from almost every varicosity along the nerve terminals, the figure for NA release was calculated to about 400 molecules/varicosity/stimulus, corresponding to only a few per cent of the estimated NA content in one granule. Obviously this appears to contradict the view that one transmitter quantum corresponds to the NA content in one granule. - The concept of "quantal release" appears, however, to be compatible with the release of a small NA pool of the granule content, or release from a hypothetical NA pool attached to sites on the nerve terminal membrane. - On the other hand recent results together with B. Folkow may suggest that the amount of released NA to some extent also depends on the reactive state of the effector cell. Therefore, a hypothetical feed back mechanism, operating via the synaptic gap may be considered.

34 Andén, N.-E. (Department of Pharmacology, University of Göteborg, Sweden): MONOAMINE RELEASE FROM NERVE TERMINALS.

Noradrenaline (NA) is released from the postganglionic sympathetic nerve terminals by nerve impulses (electrical stimulation, ganglionic stimulating drugs) or by indirectly acting sympathomimetics (tyramine and amphetamine).

After reserpine treatment the granules are gradually depleted of NA. The functional effect of nerve stimulation is not impaired until the NA is lowered by approximately 90 per cent. The responses to nerve stimulation recover when NA levels still are very low. The uptake of NA in the granules is, however, partially restored at the time of functional recovery indicating that the release by the nerve impulses is dependent on the ability of the granules to incorporate NA in a small fraction immediately important for the function: This fraction may consist of granules synthetized in the cell body after the reserpine treatment and transported to the nerve terminals.

Reserpine treatment also abolishes the functional effects of indirectly acting sympathomimetics. The responses to tyramine and amphetamine are easily restored by administration of NA. These drugs probably act by release of NA from extragranular stores.

The NA in the granules can be replaced by analogs like α-methyl NA and metaraminol which also can be released by nerve impulses ("false transmitters"). Though these amines have a somewhat weaker action on the receptors, they do not produce a pronounced impairment of the adrenergic transmission. The NA may be released normally due to weak inhibition of the granular uptake mechanism.

35 Cedergren, E. and Thornell, L.E. (Department of Anatomy, University of Umeå, Umeå, Sweden): ULTRASTRUCTURE OF MUSCLE COMPONENTS INVOLVED IN EXCITATION-CONTRACTION COUPLING.

It will be necessary to limit the subject considerably as almost any structure in the muscle cells can be said to be more or less involved in the excitation-contraction coupling process. The plasmamembranes and intracellular membranes belonging to the sarcotubular system - sarcoplasmic reticulum - or analogous structures are the main components which will be concerned about. The organization and ultrastructure of the sarcotubules and the triad and diad configurations appearing in different kinds of striated muscle, heart muscle and smooth muscle will be described. Experiments concerning the influence of different ionic and enzymatic conditions on the membranous structures will be related. Special attention will be given to the T-tubule and the relation of the plasmamembranes to the intracellular membranes.

The cellular membrane at contacts in ordinary and specialized heart muscle cells will be described and recent observations on the complexity of these structures will be reported as well as changes at the cell attachments induced by different enzymes.

Frankenhaeuser, B. (Nobel Institute for Neurophysiology, Karolinska Institutet, S-104 01 Stockholm 60, Sweden): **36** THE EFFECT OF CALCIUM AND OF LOCAL CURRENTS ON TWITCH MUSCLE FIBRES.

It is commonly held that calcium, released inside the fibre, during membrane excitation, causes the fibre to contract (e.g. Ebashi, S. & Endo, M., Progr. Biophys. Mol. Biol. 1968. 18). Calcium, however, also acts on the muscle fibre membrane. It shifts the curve relating contraction and relaxation to membrane potential along the potential axis. These effects have striking similarities with the effects of calcium on the specific sodium permeability mechanism in nerve. A consequence of the membrane effects is that a muscle fibre in Ca-free solution does not contract. Magnesium mimics the actions of calcium on the muscle membrane.

The whole cross-section of a fibre is activated in a shorter time than that required by an activating substance to move by free diffusion from the surface to the centre of the fibre. Experimental findings indicate that the contraction activating substance is released from the sarcoplasmic reticulum and that it is released during activity from a store firmly bound in the reticulum since: (a) a muscle contracts for a considerable time in a Ca-free medium; (b) caffeine causes a contraction even when it is applied to a muscle held so long a time in Ca-free medium that it does not respond mechanically to polarization.

Release of an activator for the mechanical system is associated with positive membrane polarizations. The contracture during lasting polarization is transient but during caffeine application and during calcium injection it is lasting. Therefore it is evident that the release of activator during polarization is transient. The polarization of the membrane (including the transverse tubular system) may either control the calcium permeability of the membrane, or the polarization may activate a transport system in which the transport itself is independent of potential as driving force. In the former case the transport is by free diffusion in the electric field through the membrane, and the net transport changes direction from inward to outward at the equilibrium potential for calcium.

Experiments were made with the aim to decide whether or not Ca^{++} moves passively through the polarized membrane during excitation. The sarcomeres were measured cinephotographically during local polarization of the fibre by current through an external electrode (5-20 μm diameter) applied close to the fibre. Large positive step polarizations were associated with contractions also very close to the electrode. Since these potential steps were large ($>$ 200 mV) they exclude a passive inward movement of free calcium through the membrane in the vicinity of the electrode. A similar polarizing current efficiently blocked calcium entry through a ruptured membrane and the fibre remained in a relaxed state. It is consequently concluded that the idea is excluded that Ca^{++} moves passively through a membrane in which the calcium permeability is controlled by the membrane potential.

37 Lännergren, J. (Nobel Institute for Neurophysiology, Karolinska Institutet, S-104 01 Stockholm 60, Sweden): MECHANICAL PROPERTIES OF ISOLATED AMPHIBIAN SLOW MUSCLE FIBRES.

On account of differences both in morphology, innervation pattern, membrane response and contractile properties one type of skeletal muscle fibre can be discerned - the slow muscle fibre (s.m.f.) which differs from the more familiar twitch fibre in several respects: small diameter, scanty and irregularly arranged sarcoplasmic reticulum, small capacity for energy production, multiple innervation distributed along the length of fibre, lack of regenerative membrane activity. The mechanical response is characterized by slow development of tension on stimulation and ability to maintain a high level of tension for long periods of time (Kuffler, S. W. & Vaughan Williams, E. M., J. Physiol. (Lond.) 1953. 121. 318).

Even in Amphibia, where s.m.f. are relatively numerous, they are by far outnumbered by twitch fibres which makes results obtained on whole muscles difficult to interpret. This difficulty is circumvented by the use of single fibres. Single s.m.f.:s from the iliofibularis muscle of the clawed toad, Xenopus laevis, have been shown to give long-lasting contractures in response to KCl, ACh and choline. The relation between membrane potential and tension production is very steep (Lännergren, J. Acta physiol. scand., 1967. 69. 362). That the 'slowness' of the s.m.f. is a property of the contractile system itself has been demonstrated by Costatin et al. (J. Physiol. (Lond.) 1967. 188. 261) who showed that local Ca application to skinned twitch and slow m.f. resulted in a much slower sarcomere shortening in the latter.

Previous experiments on Xenopus s.m.f.:s showed that the tension response to local electrical stimulation was much larger at a just subthreshold [K] than in Ringer solution. This indicated that the stiffness of the fibre increased as a result of depolarization even if there was no active tension. Results from experiments on twitch fibres have also been claimed to show that stiffness may vary independently of active tension (Frank, G. B. Acta physiol. scand. 1965. 65. 1). The experiments to be presented were undertaken to test this assumption.

Small ($< 1\%$ of L_o), constant-speed extensions of isolated Xenopus s.m.f.:s in Ringer solution gave a small tension response consisting of a steep initial phase (up to 50 Å extension/sarcomere = elastic limit) and a second phase of much lower slope, just as for a whole sartorius muscle (Hill, D. K., J. Physiol. (Lond.) 1969. 199. 637) or a single tw. m.f. The values for the elastic modulus (E) and the elastic limit were about the same for the two types ($E \simeq 0.7$ kg/cm^2; el. limit $\simeq 2$ g/cm^2). This response was little changed at subthreshold [K].

Long extensions (up to $L = 140\%$ L_o) at subthreshold [K] gave rise to large tension responses. A tension level, close to that which the fibre would reach when fully activated, could be attained. This is not a simple 'passive' tension, however, but more likely the combined result of a moderate stretch activation and the great change of slope of the force-velocity curve when forced lengthening is compared to active shortening (Katz, B., J. Physiol. (Lond.) 1939. 96. 45).

Edman, K.A.P.(Department of Pharmacology, University of Lund, Lund, Sweden): 38
ASPECTS UPON THE EXCITATION-CONTRACTION COUPLING IN CARDIAC MUSCLE.

A great number of observations support the view that the contractile process in cardiac muscle, similar to the situation in skeletal muscle, is governed by means of an activator agent, probably calcium, which is released into the myofibrillar space in consequence of excitation of the plasma membrane (for references, e.g. Edman, K.A.P., Ann. Rev. Pharmacol. 1965. 5. 99-118 and Langer, G.A., Physiol. Rev. 1968. 48. 708-757). Only recently, however, has it become possible, by analysis of the active state of the myocardium, to evaluate the kinetics of the activation of cardiac muscle, and hence the kinetics of the hypothetical activator in the myofibrillar space(Edman, K.A.P., D.W. Grieve and E.Nilsson, Pflügers Arch. 1966. 290. 320-334; Brady, A.J., J. Physiol. 1966. 184. 560-580; Sonnenblick, E.H., J.gen. Physiol. 1967. 50. 661) As demonstrated in these studies the capacity of the heart muscle to produce tension and the capacity to produce motion undergo parallel changes during the course of contraction. These findings are fully consistent with the view that the two parameters of myocardial activity, force and shortening velocity, are governed by a common control function in the excitation-contraction coupling (Edman, K.A.P. and E. Nilsson, Acta physiol. scand. 1968. 72. 205-219).

The time course of the active state in myocardium is markedly different from that in skeletal muscle. Thus whilst the rising phase of the active state in skeletal muscle is a matter of a few milliseconds, there is a slow development of the activity in cardiac muscle, 1/3-1/2 of the total active state time being required for attainment of maximum. Furthermore, in contrast to the situation in skeletal muscle, the maximal intensity of the myocardial active state is a variable. These differences would seem to imply that the rate of release (or inflow) and the rate of elimination of the activator agent are different in the two types of muscle. The data from skeletal muscle suggest that there is a very rapid increase of the activator concentration and that, furthermore, the amount of activator calcium released is greater than needed for complete activation of the contractile system. In the myocardial fibre, on the other hand, there is probably a relatively slow inflow of activator, and the concentration of activator does not, under ordinary conditions, reach the level for mechanical saturation of the contractile element. The implication of these facts is that every change in concentration of the activator around the myofibrils (above a certain threshold concentration) will manifest itself as a mechanical response in the myocardium. In accordance, the analysis of the active state provides strong support for the idea that the inotropic changes produced by altered stimulation frequency, digitalis, adrenaline and calcium are due to altered kinetics of the activator mechanism in the excitation-contraction coupling (Edman, K.A.P. and E. Nilsson, Acta physiol. scand. 1969, in press). The origin of the calcium which activates the contractile system is not yet defined. An inward calcium current across the plasma membrane appears to take place during excitation of the myocardial fibre. Most of the evidence would seem to indicate, however, that the calcium immediately involved in the excitation-contraction coupling is released from an intracellular site, most likely the sarcoplasmic reticulum. The same structures are also, conceivably, responsible for the sequestration of calcium from the myofibrillar space (for references, see Langer, G.A., Physiol. Rev. 1968. 48. 708-757).

39 Axelsson, J. (Dept. of Physiology, University of Iceland): POTENTIAL CHANGE AND CONTRACTION.

Investigations of the activation of the contractile mechanism in polarized muscles have shown that there is, contrary to what was commonly believed, no certain correlation between transmembrane potential changes and mechanical activity. This has recently become obvious in several types of smooth muscle. Thus simultaneous records of electrical and mechanical activity in the portal vein have been digitized and the transfer function between them studied with the result that great dissociation between electrical and mechanical activity can now be clearly demonstrated. For example, the increase in tension in response to noradrenaline is not accompanied by a corresponding increase in measurable electrical activity.

In the portal vein as elsewhere, calcium appears to be an indispensable link in the chains of events leading from the stimulation of the muscle to eventual contraction. This suggests the hypothesis with regard to activation of the contractile mechanism by means of noradrenaline without corresponding changes in transmembrane potential that the action of this drug is mediated by an increased influx of calcium ions and/or liberation of inactive or bound calcium in intracellular or membrane comportments.

In some types of smooth muscle activation of the contractile mechanism not correlated to transmembrane potential changes may play a physiological role.

Landgren, S. (Department of Physiology, University of Umeå, Sweden): PROJECTION OF GROUP I MUSCLE AFFERENTS TO THE CEREBRAL CORTEX. 40

A study of afferent and efferent connections has been one of the approaches to an understanding of the function of the cerebral cortex. The classical outlining of the somatosensory projection areas (SI and SII) and the cortical motor areas (M_1 and M_2) is an example of a functional framework resulting from this approach. These homologues are, however, too simple to accommodate the present knowledge of the cortical distribution of afferent inflow. Recent investigations have revealed a detailed organisation of the skin, joint and muscle afferent projections. This report shall be restricted to the projections of group I muscle afferents to the cat's sensorimotor cortex. Such projections are found in the cytoarchitectonic areas 3a and 3b, which form an interesting zone along the border of the motor cortex (4 γ).

The group I muscle afferents from the forelimb ascend in the dorsal columns, relay in the cuneate nucleus and in the contralateral nucleus ventralis posterolateralis of the thalamus and reach at least 4 separate cortical projection fields. The most well known of these is the one located near the postcruciate dimple (Pcd), which was originally described by Amassian and Berlin (J. Physiol. 1958. 143. 61P) and by Oscarsson and Rosén (J. Physiol. 1963. 169. 924). Later another projection area was found in the lower bank of the anterior suprasylvian sulcus. Still another group I area was localized in the ansate sulcus by Silfvenius (Acta physiol. scand. 1968. 74. 25A), who also described a separate locus in area 4 γ at the lateral end of the cruciate sulcus.

The group I path from the hindlimb ascends in the dorsolateral fascicle of the spinal cord. The location of the segmental relay is unknown, but evidence of a medullary synapse in nucleus Z of Brodal has been reported to this congress by Landgren and Silfvenius (Acta physiol. scand. 1969. in press). The location of the thalamic relay was so far not established, but cortical projections were found in two places, one rostromedial to Pcd and another near the cruiate sulcus on the medial surface of the hemisphere. Both these fields are located in areas 3a and 3b and overlap with the motor cortex (4 γ).

These cortical group I loci are consistently found in all investigated animals. Their size is of the order of 2-3 mm in diameter and thus slightly too large to match the size of a functional cortical column. They seem to depend on branching axons from the same thalamic relay neurone. The conduction time in the group I path to the cortex is short (forelimb: 5 msec, hindlimb: 10 msec) and comparable to that of the primary projections from the skin.

Many efferent paths take their origin in the region of the cortical group I loci. Some of the cortical cells, that are discharged by the group I afferents, send their axons into the pyramidal tract, but appart from this fact very little is known about the destinations of the efferent paths. Electrical stimulation of the motor cortex affects the motoneurone, spinal interneurones, primary afferents and neurones in ascending tracts. More experimental work is, however, required to elucidate the possible relations between the cortical group I neurones and these efferent channels.

41 Silfvenius, H. (Department of Physiology, University of Göteborg, Sweden): PROJECTIONS OF PACINIAN AFFERENTS TO THE CORTICAL GROUP I LOCI.

Muscle spindles and pacinian receptors are sensitive mechanoreceptors located in deep tissues. They respond to vibration and have rapidly conducting afferents. Their adaptation rate differs and therefore their response to continuous stimulation. The spindle records length of muscle and functions in motor control. The pacinian receptor subserves sensation of vibration. Both do, however, contribute to the dynamic component of the response to movements. The role of pacinian afferents in motor mechanisms should be considered.

The projections of pacinian afferents to the motor cortex and its border zones were investigated in cats anesthetized with chloralose. Electrical stimulation of the fore- and hindlimb interosseous nerves was used, as was natural stimulation of interosseous pacinian receptors with a mechanical pulse. Cortical surface- and depth potentials were recorded with ball- and microelectrodes.

The pacinian projections overlap with the Group I projections. Responses to activation of pacinian afferents were observed in all Group I loci. The pacinian projections differed, however, from those of the Group I afferents. The former were larger and extended into primary projections of skin afferents. Pacinian afferents also project to the motor cortex, 4γ. The pacinian receptors thus contribute to the information which is made available to the motor cortex and they may therefore influence the control of movements.

42 Körner, L. (Department of Physiology, University of Göteborg, Sweden): THE PROJECTIONS OF LOW THRESHOLD JOINT AFFERENTS TO THE CEREBRAL CORTEX OF THE CAT.

Low threshold joint afferents are considered to subserve kinesthesis. They take origin in the Golgi endings of the joint ligaments and in the Ruffini endings of the joint capsulae. Their discharge is slowly adapting and its adapted frequency is a function of the joint angle. The cortical projections of these afferents has recently been investigated in cats anaesthetized with chloralose (Körner, L. and S. Landgren, Acta physiol. scand. 1969, in press). Primary projection areas were found near the primary SI projection fields of the low threshold cutaneous afferents. In addition, however, other projection fields were localized to the cortical loci of the low threshold muscle afferents. These last mentioned projections should be functionally related to the projections of the group I muscle afferents and are therefore probably not involved in kinesthesis but in motor control.

Oscarsson, O., Rosén, I. and Sjölund, B. (Institute of Physiology, University of Lund, Sweden): INFORMATION CARRIED BY THE FORELIMB GROUP I PATH TO THE CEREBRAL CORTEX. 43

Stimulation of group I muscle afferents evokes activity in the sensorimotor cortex of the cat (see Landgren, S., this symposium) and monkey (Albe-Fessard, D., Liebeskind, J. and Lamarre, Y., C. R. Acad., Paris 1965. 261. 3891). However, stimulation of these afferents in the cat does not influence its general behaviour or EEG, neither will volleys evoked in these afferents serve as a discriminative stimulus (Giaquinto, S., Pompeiano, O. and Swett, J.E., Arch. ital. Biol. 1963. 101. 133; Swett, J.E. and Bourassa, C.M., J. Neurophysiol. 1967. 30. 530). Stretch of muscle in man does not result in conscious perception and cannot serve as a basis for "position sense" (see Gelfan, S. and Carter, S., Exp. Neurol. 1967. 18. 469). It has been suggested that the forelimb group I path to the cerebral cortex is a feedback system used for adjusting the motor output from the cortex (Oscarsson, O. and Rosén, I., J. Physiol., Lond., 1963. 169. 924).

The information carried by the forelimb group I path to the cat cerebral cortex has been investigated in some detail. Observations reported at this congress (Rosén, I. and Sjölund, B.) suggest that this path is activated from muscle spindle but not tendon organ afferents. The second order neurones in the cuneate nucleus are usually activated from one muscle only, wheras the third order neurones in the thalamus are usually activated from a group of synergistic muscles (Rosén, I., Acta physiol. scand. 1968. 73. Suppl. 310. 13A). Some neurones at both levels are also activated from group II muscle afferents suggesting a highly specific convergence of the two types of afferents from muscle spindles. No excitation is evoked by stimulation of group III muscle afferents (Rosén, I., in preparation).

The fourth order neurones in the cortex are usually activated not only from synergists but also from antagonists and muscles acting at different joints. These neurones receive also strong excitation from cutaneous afferents through unknown thalamocortical or corticocortical paths (Oscarsson, O., Rosén, I. and Sulg, I., J. Physiol., Lond., 1966. 183. 189).

Fifth order neurones have been identified in the cortex. They show the same convergence pattern as the fourth order neurones but are inhibited instead of excited (Grampp, W. and Oscarsson, O., In "Structure and Function of Inhibitory Neuronal Mechanisms", Ed. Euler, C. von, Skoglund, S. and Söderberg, U., Pergamon Press 1968, p.351). This suggests that many of the fourth order neurones are local inhibitory cells but some send their axons into the pyramidal tract and are presumably excitatory (Swett, J.E. and Bourassa, C.M., J. Physiol., Lond., 1967. 189. 101).

14 Laursen, A. Mosfeldt (Institute of Neurophysiology, University of Copenhagen, Denmark): QUANTITATIVE ANALYSIS OF THE BEHAVIORAL DEFICIT PRODUCED BY INTERRUPTION OF A PYRAMIDAL TRACT IN MONKEYS.

Although the pyramidal tract is the largest efferent tract in the brain stem of higher mammals lesions to it produce little lasting deficit. The pyramidal syndrome of clinical neurology is absent after surgical interruption of the tract in man and after seven months movements of the fingers were only slightly less well executed on the affected than on the normal side (Bucy, P.C., Keplinger, J.E., and Siqueira, E.B., J.Neurosurg. 1964. 21. 385). In cat pyramidotomy eliminates placing and flexion reflexes and contralateral extremities become weak; two to three weeks after the operation, however, neurological examination reveals nothing abnormal apart from some slowness of motion (Marshall, C. Quart.Rev.Biol. 1933. 31. 68). Cats trained to press a lever under the brighter of two lights could, after pyramidotomy, not be trained to respond rapidly though their ability to execute fast movements was unchanged (Laursen, A.M., and Wiesendanger, M., Brain Research 1967. 5. 207). In addition to this motor deficit a sensory impairment is to be expected. Pyramidal fibers end in sensory relay nuclei of the medulla oblongata and spinal cord and corticofugal inhibition of transmission through primary afferents in the spinal cord is exerted in part through the pyramidal tract (Lindblom, U.F., and Ottosson, J.O., Acta physiol.scand. 1957. 38. 309).

The functional significance of pyramidal control of afferent transmission is unknown. I have tested the hypothesis that two different sensory tasks are associated with two different "settings" of the neurons in the dorsal horn and that the pyramidal tract does the setting. That the modality of dorsal horn neurons may change was observed in acute experiments by Wall (J.Physiol.1967. 188. 403). We trained monkeys to reach into a dark box with the right hand, grasp a silver plated handle and turn it 30 degrees right or left depending on whether its temperature was 29 or 25°C; the two temperatures were presented in a random sequence. In this way two difficult discriminations were performed rapidly one after the other and the postulated switching of the modality of dorsal horn neurons should occur. In control trials a red or a green light indicated the required direction of turning.

The number of errors made in turning the handle 30 degrees (kinesthetic errors) and the time it took to turn it was compared in 1000 preoperative and 1000 trials about 3 months after the lesion. Control and experimental trials were similarly affected and thus the hypothesis was not confirmed. Decision time in warm trials was selectively increased indicating, possibly, a deficient "range setting" for temperature after the lesion. This new hypothesis is currently being investigated.

Kernell, D. (The Nobel Institute for Neurophysiology, Karolinska Institutet, 104 01 Stockholm 60, Sweden): 45 THE PYRAMIDAL PROJECTION TO THE SPINAL CORD.

The present communication will mainly deal with studies on primates, and it will largely be restricted to that portion of the motor cortex efferents which consists of large (i.e. fast conducting) fibres running in the pyramidal tract (PT). There is much to suggest that the function of these fast PT units is different from that of the more numerous PT units with slower axonal conduction velocities. Recordings from discharging PT units in conscious primates suggest that it is predominantly the fast PT units that are primarily engaged in the control of rapid and distinct movements (Evarts, E. V., J. Neurophysiol. 1966. 29. 1011). In the primate (but not in the cat) there is a monosynaptic excitatory connexion between fast PT units and spinal alpha motoneurones (Bernhard, C. G., Bohm, E. & Petersén, I., Acta physiol. scand., 1953. 29. suppl. 106. 79). These monosynaptic effects from fast PT units are more powerful in motoneurones of distal limb muscles (e.g. for hands and fingers) than in those of more proximal muscles (Phillips, C. G. & Porter, R., Progr. Brain Res., 1964. 12. 222; Clough, J. F. M., Kernell, D. & Phillips, C. G., J. Physiol. (Lond.) 1968. 198. 145). The direct connexion between fast PT units and alpha motoneurones would seem to be especially advantageous for the fine control of 'skilled' hand and finger movements. Among the alpha motoneurones of a given nerve, the cells which receive a strong monosynaptic input from fast PT units also tend to receive a comparatively powerful monosynaptic excitation from primary afferents of muscle spindles (Clough et al., 1968). This arrangement is of interest with respect to the possible role of gamma motoneurones and muscle spindles for the control of phasic movements initiated by fast PT units.

In addition to their monosynaptic excitatory effects, the fast PT units give rise to a disynaptically mediated inhibition in many motoneurones (Preston, J. B. & Whitlock, D. G., J. Neurophysiol. 1960. 23. 154). This disynaptic projection might perhaps serve to inhibit the antagonists to movements initiated by fast PT units. Such a reciprocal pattern of innervation is, however, probably difficult to establish with the aid of electrical stimulation to the cortex, because the cell bodies of PT units innervating motoneurones of different muscles are likely to be intermingled. PT cells which lie very close together in the motor cortex may show a reciprocal discharge pattern during voluntary movements (Evarts, E. V., J. Neurophysiol., 1965. 28. 216).

For an interpretation of experiments concerning the long-latency effects (excitatory and inhibitory) which may be elicited in motoneurones by cortical stimulation, it has to be taken into account that a strong cortical stimulus pulse will elicit a rapid repetitive discharge of fast PT units (Kernell, D. & Wu Chien-Ping, J. Physiol. (Lond.) 1967. 191. 653 & 673).

In the cat the PT fibres have functional connexions with interneurones of various spinal reflex arcs (Lundberg, A. & Voorhoeve, P., Acta physiol. scand., 1962. 56. 201). It would be surprising if comparable projections did not exist in primates as well.

Jansen, J.K.S. (Institute of Physiology, University of Oslo, Norway): TRANSFER OF SIGNALS FROM MUSCLE STRETCH RECEPTORS. 46

So far, two groups of second order neurones activated by primary afferents from muscle stretch receptors have been investigated in particular detail. The motoneurones are in many respects the commonly accepted model of synaptic activation of nerve cells. The second group is the cells of origin of the dorsal spino-cerebellar tract (DSCT). Our own studies concern the latter group. In several ways their behaviour is simpler and more completely understood.

The transfer of signals to DSCT neurones are characterized by:

1. A high degree of specificity.
2. A high degree of linearity within their functional range.
3. A high efficiency of signal transfer.
4. A characteristic firing pattern of the second order neurones.

These points are explained: 1. By the anatomical coupling. 2. By the properties of the neurones. 3. By the properties of the synapsis and 4 by the properties of the input activity, the synaptic and neuronal properties.

Simplified models have been formulated which account quantitatively for the behaviour of the DSCT neurones. The synaptic transfer to the DSCT neurones can be inhibited by inputs from neighbouring muscles or by descending activity through the pyramidel tract. Such inhibition may reduce the "gain" of synaptic transfer, and may affect the firing pattern of the second order neurones. These observations can be explained by the same model.

The properties of the motoneurones are matched to the mechanical properties of skeletal muscles. Their afferent input from muscle stretch receptors is differently organized and in most respects motoneuronal behaviour is more complex than that of the DSCT neurones.

47 Euler, C.v. (Nobel Institute for Neurophysiology, Karolinska Institutet, Stockholm, Sweden): NEURAL CONTROL MECHANISMS DETERMINING PACE AND DEPTH OF RESPIRATORY MOVEMENTS.

It is now almost generally accepted that rhythmic breathing represents a recurring inhibition of a 'drive' for ventilation which is continously computed on the basis of information from peripheral and central chemoreceptors and other central and peripheral neural inputs.

Two interacting mechanisms are known to operate the rhythmic inhibition: 1) the internal bulbo-pontine pacemaker mechanisms and 2) the mechanisms governed by the pulmonary mechanoreceptors. The latter feed back information on volume , rate of expansion and transmural pressure of the lungs. A quantitative knowledge has not been available about how this information is used centrally to modify inspiratory and expiratory times or the depth of the breaths. To what extent the volume dependent feed-back mechanism constitutets an oscillating mechanism in its own right or whether it operates primarily by modifying the internal pacemaker is the matter under investigation.

The internal bulbo-pontine pacemaker seems to be the dominating frequency governor in eupnoeic breating in man (Guz, A. et al. Resp. Physiol. 1966. 1. 206) and in thermal polypnoea and panting in animal. It has been shown both in intact and vagotomized cats (Euler, C.v., F. Herrero & I. Wexler. Resp. Physiol 1969 in press) that as in man (Hey, E.N. et al. Resp. Physiol 1966. 1. 193), increased body temperature is unique among respiratory stimuli in that it alone will change the slope constant of the linear relation between pulmonary ventilation and tidal volume thus affecting mainly the frequency parameter. After vagotomy an increase in hypercapnic or hypoxic 'drive' causes virtually no change in respiratory frequency. The vagal mechanisms alone are responsible for the increase in frequency when 'drive' is increased both in man and animal.

In our attempt to gain further insight in the mode of operation of the control mechanisms determining rate and depth of breathing the analysis has taken the form of a dialogue between animal experiments (on cat) on one hand and analog modeling of the respiratory control mechanisms on the other.

This work by F. Clark, C.v. Euler & B. Roos (unpublished) has so far given the following results. Respiratory timing is governed by neural activity during inspiration. The duration of this inspiratory activity sets the duration of the phase of expiration. Volume information during inspiration serves to shorten the duration of the inspiratory phase without changing the linear expiratory to inspiratory time relation. The responsiveness to volume pulses,, which is low at the onset of inspiration increases steeply with time during that phase This explains i. a. how , in case of increasing 'drive' tidal volume can increase as inspiratory time shortens and why the high rate of breathing in panting is but little influenced by the vagal input. Volume information during the expiratory phase lengthens the duration of that phase but has no apparent effect on the following inspiration. Thus under corditions of constant drive and temperature each cycle, beginning with inspiration is an independent event.

Larsson, S. (Physiological Institute, Odense University, Odense, Denmark): **48**
ETIOLOGICAL AND CURATIVE ASPECTS ON OBESITY.

In recent years more knowledge of the etiology of obesity has been gained. Thus, we can now divide in large obesity into the following groups:

1. Hereditary obesity. This type of obesity will express itself differently. On the whole, however, endocrinological disturbances are not so common in man in the etiology of obesity.

2. Inactivity obesity. This type of obesity is probably the most common in the present human society. In this case the etiology is quite clear. I will refer to the nomenclature given by Dr. Passmore, who calls the present average human being Homo Sedentarius, instead of Homo Sapiens.

3. Dietary obesity. This group we can divide into two subunits, namely:

   a. Overeating. Naturally this overeating is a specific overeating in relation to energy expenditure.

   b. Dietary imbalance. In this case the energy expenditure is low and the diet usually too high in fats and carbohydrates. At the same time the individual has an extremely low muscular activity.

As to the treatment of obesity it is in most cases completely wrong to suggest decrease in caloric consumption. Experiments have shown that on the whole the best effect to cure obesity is to increase muscular exercise. In this respect one has to take into consideration two aspects, namely weight reduction and fat decrease of the body. Generally in human patients the desire is to decrease in weight fairly rapidly. Many of the fundamental experiments have been made in rather small animals where the life span is short. Unfortunately many of the weight reducing programmes given in the weekly magazines particularly directed to ladies guarantee an extremely rapid weight reduction, usually without any scientific background.

For the majority of the obese people the cure ought to be based upon dietary anamnesis and correction of food intake supplemented with a program of increased muscular activity. The conclusion for this statement will be discussed by referring to experiments upon both animals and man.

49 Donner, K.O. (Department of Physiological Zoology, University of Helsinki, Finland): PHOTOCHEMICAL ASPECTS OF THE RECEPTOR PROCESS IN VISION.

The absorption of photons by the visual pigment situated in the lamellar structure of the outer limb of vertebrate photoreceptors, triggers excitation. Such quantum absorptions initiate a series of changes in the molecules, the first step being the isomerization of the chromophore from the 11-cis to the all-trans form. This is followed by a series of relatively long-lasting thermal reactions that are fairly well known for rhodopsin in solution (Wald, G., Nature 1968. 219. 800). With certain differences these reactions can also be shown to occur in situ, for instance, in the rods of the isolated frog retina. Here the rhodopsin molecules are highly oriented so that they mainly absorb light incident parallel to the long axis of the rods. This mode of orientation is preserved by the photoproducts until the separation between the chromophore and the specific protein, opsin, occurs. Much before this visual excitation takes place and must then be mediated by some molecular change not associated with any change in the orientation of the chromophore.

Evidence from parallel investigations of the thermal decay of the photoproducts and sensitivity measurements with electrophysiological methods on single ganglion cells (Donner, K.O. & Reuter, T., J. Physiol. 1968. 199. 59) suggests that these products have an important function in the adaptation process. It thus appears that small amounts of metarhodopsin II present in the rods exert a negative feed-back on the excitation produced and thus reduce the sensitivity of the receptors to an extent depending on the rhodopsin bleached by the prevailing illumination or by immediately preceding exposures of the retina to light. This effect is, however, not identical with the reduction of sensitivity directly caused by a background of light. The relation between the amounts of metarhodopsin II and threshold can be quantitatively measured. It closely agrees with the properties of the parametric feed-back postulated in studies of the Limulus photoreceptor (Fuortes, M.G.F. & Hodgkin, A.L., J. Physiol. 1964. 172. 239). A sufficient amount of metarhodopsin II finally saturates the rods and makes them practically inexcitable.

It further appears that the regeneration of rhodopsin in the rods also affects their sensitivity to light, but in a different way. The rate of synthesis of new molecules has a sensitivity-reducing effect similar to that produced by backgrounds of real light, and the relation between threshold and rate of regeneration thus follows Weber's law. However, this effect is much weaker than that produced by a background. Thus a background light of an intensity that gives an absorption of about 1 quantum per rod per sec has an effect equivalent to that produced by the synthesis of about 3000 molecules per rod per sec in darkness.

Skou, J. C. (Institute of Physiology, University of Aarhus, Denmark): THE MOLECULAR BASIS FOR THE ACTIVE TRANSPORT OF Na^+ AND K^+. 50

The main problem in relation to active transport of Na^+ and K^+ is how the energy from the hydrolysis of ATP is converted into a movement of ions against an electrochemical gradient. A step on the way to solve this problem is the isolation of an enzyme system from cell membranes which fulfil a number of requirements to a transport system: the substrate for the system is ATP; it is asymmetric in the sense that it has a site with an apparent affinity for Na^+ higher than for K^+ which when the system is located in the cell membrane is facing the inside of the membrane and another site which has an apparent affinity for K^+ higher than for Na^+ and which is facing the outside of the membrane; Na^+ is required at the "Na site" and K^+ at the "K site" for activation; the system has the same quantitative relationship to Na^+ and K^+ as the transport system in the intact cell membrane; it can be isolated from all cells which have been shown to have a K^+ coupled Na^+ transport; G-strophanthin which is a specific inhibitor of active transport of Na^+ and K^+ inhibits the enzyme system. This suggest that the system is part of or the transport system.

There is indirect evidence that the system under the influence of Mg^{++}, ATP, Na^+ and K^+ undergo a change in structural configuration and it is possible to distinguish between two different states of the system. The one is characterized by being insensitive to G-strophanthin while the other is sensitive to G-strophanthin. MgATP turn the system into the G-strophanthin sensitive state; this is due to an effect of ATP as such and not to a hydrolysis of ATP. With Na^+ at the "Na site" and K^+ at the "K site" the system in the G-strophanthin sensitive state has the catalytic activity and the hydrolysis of ATP turn the system into the G-strophanthin insensitive state. It seems likely that it is a cycling between these two states which leads to the transport of the cations.

On the basis of the ability of Mg^{++}, ATP, Na^+ and K^+ to influence the two different states of the enzyme system it is possible to set up a hypothetical model for the transport system which operates with four different forms of the system. One which gives a Na^+: Na^+ exchange, another which gives a K^+: K^+ exchange, a third which gives a K^+ coupled Na^+ transport and which due to the exchange of Na^+ for K^+ leads to the fourth of the forms. They all exist at the same time in the cell and the ratio between them is determined by the ratio of Na^+ and K^+ in the internal and in the external solutions.

51 Hartiala, K. (Department of Physiology, University of Turku, Turku, Finland): THE ROLE OF GASTROINTESTINAL WALL IN DRUG METABOLISM.

Whole animal, tissue slice and isolated subcellular particle experiments have demonstrated that the mucous membrane of the whole alimentary tract takes an active part in the so-called phase I (oxidation, reduction, hydrolysis) and phase II (synthetic) reactions. (Ann.Med.exp.Fenn.1955. 33.239; Biochem.Pharmac.1961.6.82; Nature 1964.201.1036 and 1965.205.809). Also the enzymes and the nucleotides responsible for these syntheses have been located in the mucosal specimens. The type and capacity of these reactions in the gastrointestinal tract are species dependent and are obviously the same as those carried by the liver of the same animal species. Substrates for these reactions involve compounds produced by the body itself (e.g. steroids) and foreign compounds widely used for therapeutic purposes. The amount of metabolites produced by these reactions during the absorption processes can be large. This has been demonstrated in dogs by using 4-C^{14} marked steroids (progesterone, androstenedione, testosterone or 17α-methyl-testosterone) and placing them into isolated intestinal segments. The first metabolites could be detected within few minutes after the application of the sample. After half an hour about 40 different polar metabolic products were found in the portal blood. Sterilization of the gut lumen does not alter these metabolic patterns. The sequence of these reactions can be followed and it possibly reflects the metabolic arrangements within the endoplasmic reticulum.

The upper part of the duodenum possesses the highest capacity for the synthetic reactions. The distribution patterns of the enzymes and nucleotides responsible e.g. for glucuronide conjugation as well as those involved in oxidative phosphorylation processes are not linear and reveal specific differences.

The fetal development of these functions in the liver, intestine or kidney reveals species differences. In general they are far from the adult level at the time of birth and practically absent during the first half of the fetal period.

The gastrointestinal mucous membrane thus, exerts a great variety of metabolic effects on foreign substances. These reactions change the chemical and biological nature of the absorbed substrates although they operate mainly on trace levels. Part of the nucleotide-bound energy sources of these reactions are the same necessary for the local synthesis of epithelial and connective tissue glycoproteins.

Bill, A. (The Wallenberg Laboratory and the Institute of Pharmacology, University of Uppsala, Sweden): 52 THE DYNAMICS OF AQUEOUS HUMOR.

The continuous formation and drainage of aqueous humor contributes to maintain the intraocular volume and pressure within narrow limits which is a prerequisite for precise function of the optical apparatus. A second task is to nutriate the avascular intraocular tissues.

Results from several laboratories indicate that in the ciliary processes there is active transport of Na^+, Cl^- and ascorbic acid into the posterior chamber and transport of I^- and some organic anions in the other direction. The movement of water into the posterior chamber seems to be secondary to the net transport of ions. Mechanisms for facilitated diffusion contribute to give rapid movement of amino acids into the aqueous humor of the posterior chamber. The low concentration of plasma proteins in the aqueous humor and the high concentration in the tissue fluid of the ciliary processes, about 60-70 percent of that in plasma, make it seem unlikely that ultrafiltration of tissue fluid contributes to the formation of the aqueous humor; a tendency to reabsorption of freshly secreted aqueous humor seems more likely.

The aqueous humor flows from the posterior chamber into the anterior chamber via the pupil. Exchange of matter with the vitreous humor and the lens changes the composition of the fluid and possibly also its volume. Recent experiments show that if there is normally any flow of aqueous humor into the vitreous humor it is not appreciably influenced by a moderate rise in the intraocular pressure.

In the anterior chamber there is exchange between the aqueous humor and the cornea, the chamber angle tissue, the iris and part of the lens. Experiments with myoglobin, albumin and gammaglobulin have shown that the three proteins leave the anterior chamber at almost exactly the same rates in spite of differences in size. The proteins thus seem to be drained by bulk flow. In vervet monkeys (Cercopithecus ethiops) part of the bulk flow, about 1.1 µl/min, occurs through a trabecular meshwork into the canal of Schlemm and from there into intra- and episcleral veins. The rate of flow is proportional to the driving pressure. Another part of the aqueous humor about 0.6 µl/min is drained into the interstitial tissue spaces of the ciliary muscle, where it mixes with the tissue fluid and is drained with such fluid into the space beneath the sclera and out through the sclera. Part of this fluid is resorbed into the conjunctival lymph vessels. This flow is very little influenced by moderate changes in eye pressure. Low molecular weight substances like diodrast and iodoantipyrine leave the anterior chamber both by bulk flow and diffusion. Data for iodoantipyrine elimination indicate that the blood flow through the iris vessels that exchange matter with the anterior chamber is very low, about 4 µl/min.

A rise in eye pressure tends to lower the rate of aqueous formation most probably by interfering with the circulation of blood in the ciliary processes. Adrenergic drugs and sympathetic stimulation tend to increase the rate of formation of aqueous humor, pilocarpine and acetazolamide reduce the secretion rate. There is no clearcut evidence indicating an extraocular center for regulation of intraocular pressure or rate of aqueous formation according to information derived from receptors sensing intraocular pressure or rate of flow of aqueous humor.

53 Fuxe, K. and Hökfelt, T. (Department of Histology, Karolinska Institutet, Stockholm, Sverige): ON THE ROLE OF CENTRAL MONOAMINE NEURONS IN NEUROENDOCRINE MECHANISMS.

Many areas of the hypothalamus, the preoptic area and the limbic system are known to receive a large number of 5-HT and NA nerve terminals, which arise from fibres mainly ascending in the lateral hypothalamic area and which in turn originate from cell bodies in the lower brain stem. Furthermore, there exists a tubero-infundibular DA neuron system which innervates the external layer of the median eminence. Here the DA nerve terminals probably establish axo-axonic contacts with "peptidergic" neurons involved in the central gonadotrophin secretion. Using amine synthesis inhibitors and labelled monoamines the turnover of the various monoamine nerve terminal systems have been studied under various endocrinological states by way of a histochemical and biochemical monoamine analysis. The turnover will give an indication of the state of activity in the monoamine nerve terminals and therefore an indication of their role in neuro-endocrine mechanisms can be obtained.
Tubero-infundibular DA neurons. The turnover is markedly increased in pregnancy, pseudopregnancy and lactation but not significantly changed after adrenalectomy, stress or thyroxine treatment as shown in the male rat. This strongly suggests that these DA neurons are involved in the control of gonadotrophin secretion. In agreement with this it has been found that the activity varies during the ovarian cycle, being somewhat lower in proestrus; that the activity is low after castration and very low after hypophysectomy and relatively high after androgen-sterilization and exposure to constant light; that estrogen and testosteron but not progesteron and hydrocortison cause a marked activation of the system particularly in the castrated state. Furthermore, prolactine in low amounts but not FSH, LH, ACTH or vasopressin is capable of causing a marked activation of the system. Certain antifertility steroids such as mestranol and ethynodiol diacetate also cause a marked activation of the DA neurons. Under these various conditions the turnover in the nigro-neostriatal DA neurons is not significantly changed, demonstrating the specificity in the response of the tubero-infundibular DA system. To explain the present results the working hypothesis is given that the DA released in the median eminence at least partly acts to inhibit the synthesis and/or release of LRF from the neurons storing this factor. A direct action on the anterior pituitary can be excluded, since DA even in high concentrations in the incubation medium does not influence the secretion of LH from the anterior pituitary. - The role of central NA and 5-HT neurons in neuroendocrine functions will also be discussed.

Waaler, B.A. (Institute of Physiology, University of Oslo): 54
PLASMA KININS IN PHYSIOLOGY AND PATHOPHYSIOLOGY.

A variety of enzymes can release peptide material with profound circulatory effects from proteins in mammalian plasma. Two linear peptides: bradykinin and kallidin (9 and 10 amino acids) have been identified as active substances - plasma kinins - resulting from such reactions. Interest in the plasma kinins stems mainly from their great potency as vasodilator and hypotensive agents. In addition they can cause increased permeability of small vessels, pain sensation, contraction of several types of smooth muscles, and possibly leucocyte emigration and accumulation.

Biochemical work has revealed interesting information about kinin structure and specificity, kinin formation and kinin inactivation. The biological activity of plasma kinins depends on specific characteristics of the amino acid chain, and further knowledge here may lead to a much wanted concept of the receptor mechanisms involved. - Plasma kinins can develop from several plasma protein precursors, kininogens. One or more of these have molecular weights in the region of 50 000, and will be relatively well represented in interstitial fluid. Another kininogen, with a molecular weight of about 197 000, will be much more confined to the intravascular space (Jacobsen and Kriz, Br.J.Pharmac. Chemother. 1967, 29, 25). Various kinin-forming enzymes act on one or both kininogens and kinin formation may occur intravascularly as well as extravascularly.

It has been claimed that plasma kinins play a dominant role in the functional vasodilatation in active glandular tissue (Hilton, S.M., Physiol.Rev.1962, 42, 265). The submandibulary gland of the cat is the best analyzed structure in this connection. Antagonists maintain that vasodilator nerves are responsible for such dilatation (Schachter, M. and Beilenson, S., Gastroenterology 1967,52,401). The intermediate view that both mechanisms play an important role has received support from investigations by Gautvik (personal communication), indicating that on chorda thympani stimulation there is an initial, marked decrease in vascular resistance in the cat submandibulary gland, probably mediated directly via vasodilator nerves. Formation of plasma kinins increased and prolonged the vasodilator effect.

Plasma kinins have been considered as mediators in the inflammatory response, and they have also been discussed as being involved in a variety of pathophysiological situations: shocks of various types, asthmatic attacks, foreign body granulomas and pneumoconioses, hereditary angioneurotic edema, carcinoid syndrome etc. In these and other connections the participation of plasma kinins have been difficult to prove or disprove. If participating, plasma kinins do probably act together with other agents or mechanisms. As long as there are no specific antikinins available, a more exact evaluation of the possible contribution of kinins seems difficult.

55 Enger, Per S. (Institute of Physiology, University of Oslo, Norway): RECEPTION OF SOUND AND VIBRATIONS BY THE FISH EAR.

The sound frequencies that can be perceived by the fish ear range from less than 30 Hz to above 5000 Hz in Ostariophysine species (teleosts which posess a chain of ossicles connecting the swimbladder directly to the inner ear). In most non-Ostariophysids the upper limit of pitch perception is less than 1000 Hz, but can be as high as 3000-4000 Hz in some species with air-filled cavities in close proximity to the labyrinth (Mormyridae, Clupeidae).

The physical stimulus for the auditory (as well as the lateral line) receptors appears to be particle displacement rather than acoustic pressure. An underwater sound source, however, produces both a progagated sound pressure wave and a local water displacement (vibration). The sound pressure amplitude decreases linearly with distance, whereas the local displacement (the socalled near-field effect) decreases with distance to the second or third power. The local water displacement can stimulate auditory receptors directly and will at close range interfere with determinations of sound pressure thresholds. Perception of propagated sound is accomplished by air bladders or vesicles in teleosts. A swimbladder will undergo volume changes in response to pressure changes. These volume changes produce local particle displacements which in turn stimulate the auditory receptors.

Sound pressure thresholds are known for a few species and range from above 1 μbar in the least sensitive non-Ostariophysids to -45 dB ref. 1 μbar in the goldfish (Ostariophysi). In order to compare these values with measurements of sound and noise in the sea, the frequency band width over which the acoustic pressure is integrated must be known. From recent experiments it appears that the response of auditory neurones is the same to pure tones as to noise of 1/3 and 1 octave bandwidths centered at the frequency of the pure tone.

Wiesel, T. (Department of Neurobiology, Harvard Medical School, U.S.A.) **56**

FUNCTIONAL ORGANIZATION OF THE MONKEY VISUAL CORTEX.

Classical anatomical and physiological studies have shown that the contralateral visual field projects onto the primary visual (striate) cortex in an orderly and topographical way. The precise topography is observed also on a cellular level; in microelectrode penetrations through the striate cortex, receptive fields of successively recorded cells are always close together and indeed overlap to a large extent. In a penetration perpendicular to the surface the fields stay in more or less the same position on the retina. In an oblique penetration there is a slight gradual shift of field position after a traverse of many mm.

Besides having practically the same field positions, neighboring cells are similar in a number of other ways. Two cells near each other usually have the same receptive field orientation and the same eye preference. As the cortex is traversed these two variables change not gradually, but in sudden jumps. Orientation, for example, remains constant during a vertical penetration from surface to white matter, but in oblique penetrations it stays constant for a small distance, and then shifts abruptly to a new value. Cells having the same receptive field orientation are aggregated into small discrete column-like regions extending through the cortex with walls perpendicular to the cortical surface. There are as many as 15-17 kinds of orientation columns. Regions defined by eye preference are similarly columnar in shape; these columns are of just two types, and they seem to be larger than the orientation columns. In these two systems the walls separating columns seem to be quite independent.

Cutting across these systems of vertical columns are the cortical layers. Cells in the striate cortex differ in the relative complexity of their stimulus requirements and can be subdivided into 'simple', 'complex', and 'hypercomplex' cells. Cells in the upper and lower layers are mostly complex and hypercomplex, cells in layer 4, roughly midway between surface and white matter, are most often simple. Some units (which may be afferent fibers from the lateral geniculate body) have concentric fields. Furthermore, cells in layer 4, unlike cells in other layers, are seldom driven by both eyes; instead some are driven by one eye, some by the other. The fourth layer receives direct geniculate input and the cells in this layer would therefore be expected to have the least elaborate properties of any cells in the striate cortex. It is therefore perhaps not surprising to find 'simple' cells driven monocularly in this layer.

In summary, the cortex can be looked upon as a system organized vertically and horizontally in entirely different ways. In the vertical system (in which cells encountered in a vertical penetration through the cortex have common features) stimulus dimensions such as line orientation and ocular dominance are mapped in sets of superimposed but independent mosaics. The horizontal system segregates cells in layers by hierarchical orders, the lowest orders located in layer IV, the higher orders in the upper and lower layers.

57 Crone, C. (Institute of Medical Physiology A, University of Copenhagen, Denmark). THE BLOOD-BRAIN BARRIER.

The ability of a molecule to pass from plasma into brain tissue is determined by the presence of lipophilic groups rather than by the size of the molecule. This was demonstrated in experiments where the uptake in brain of a homologous series: propanol, 1,2-propanediol and glycerol was determined during a single passage (Crone 1964). It was found that Collander's general findings with respect to cell permeability of non-electrolytes applies to the brain.

As is the case in many cells glucose constitutes an exception. It is rapidly transferred across the blood-brain barrier. The mechanism shows saturation kinetics and stereospecificity (Crone 1965). The transport can be inhibited by the presence of similar compounds (methyl-O-Glucose).

Anatomists have claimed that the transport of glucose into the brain takes place via the glial cells covering the cerebral capillaries. The view is probably wrong as demonstrated by Wolfe and Nicholls (1967) who could find no preferential uptake of glucose by glial cells using autoradiographic techniques.

It still remains unsettled what is the morphological substrate of the blood-brain barrier. Several proposals have been made: 1) a special structure of the capillary endothelium, 2) a virtual non-existing interstitial space in the brain, 3) glial cell covering. The concept of a vanishingly small interstitial space at least seems to be wrong (Rall, Oppelt & Patlak, 1962).

Morphological studies of the fate of protein markers injected either into the blood or into the cerebral ventricles show that the individual endothelial cells in the brain capillaries are connected by "tight junctions" through which protein cannot pass (Brightman and Reese, 1969).

Capillaries from various organs present different permeabilities due to widely different morphology of the endothelial cells, ranging from fenestrated capillaries with wide interendothelial gaps (liver and intestine) to brain with no fenestrations and tight junctions between the endothelial cells.

It is tempting to speculate that the blood-brain barrier is a reflection of a special morphology of the brain capillaries.

Brightman & Reese (1969). J. Cell Biol. 40:648-677.
Crone, C. (1965). Acta physiol. Scand. 64:407-417.
Crone, C. (1965). J. physiol. (Lond.). 181:103-113.
Rall, Oppelt & Patlak (1962). Life Sciences 2:43-48.
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Ehinger, B., Falck, B., Persson, H., Rosengren, E. and Sporrong, B. (The Institute of Anatomy and Histology and the Department of Pharmacology, University of Lund, and the Research Laboratories, AB Draco, Lund, Sweden): LOCALIZATION OF ACETYLCHOLINE IN THE FELINE IRIS. **58**

Acetylcholine was bioassayed in the normal cat iris, and also after selective sympathetic or parasympathetic denervations. Sympathetic denervation caused no significant change in the acetylcholine content of the cat iris, whereas selective parasympathetic denervation reduced the acetylcholine content below the level of detectability, which on the average was at about 5 per cent of the acetylcholine content of the normal iris. It is concluded that if adrenergic terminals contain any acetylcholine, it is less than what is detectable with the methods available at present, most certainly less than 6 per cent of the acetylcholine content of cholinergic neurons. On the basis of these and other recently obtained observations the hypothesis of Burn and Rand of a cholinergic link in the adrenergic transmission is discussed. It is proposed that it is more reasonable to suppose an interaction between peripheral adrenergic and cholinergic terminals than to presume a cholinergic mechanism within adrenergic nerves.

Klinge, E., O. Penttilä and P. Pohto (Department of Pharmacology, University of Helsinki, Finland): CELLS EXHIBITING MONOAMINE FLUORESCENCE IN URETHRAL TISSUE OF BULL AND RABBIT. **59**

Pieces of tissue were freeze dried in vacuo and exposed to formaldehyde vapour. Numerous non-neuronal cells exhibiting intense greenish-yellow or yellow fluorescence were observed in or in close vicinity to the urethral mucosa of bulls and rabbits. In bulls the surrounding cavernous tissue was practically devoid of adrenergic nerve fibres, whereas they were readily detectable in this tissue of rabbits. Using a modified thiocholine method no acetylcholinesterase activity was detected in the monoamine cells. No nerve fibres entered the urethral mucosa of either animal. It is likely that the fluorescent amine in the cells of both animals is 5-hydroxytryptamine, but in the bulls there also might be some dopamine. This is indicated by the monoamine contents observed in the respective tissues (Penttilä, O. and A. Vartiainen, Acta pharmacol. (Kbh.) 1964, 21, 145). In the urethral mucosa of the bull the 5-hydroxytryptamine content is about three times as high as in the surrounding cavernous tissue. Incubation experiments also showed that the 5-hydroxytryptophan decarboxylase activity in the bull urethral tissue exceeds by far that of the surrounding cavernous tissue, whereas the monoamine oxidase activity is about equal. The functional role of the monoamine cells is undefined.

60 Ericson, Lars E. (Dept. of Anatomy, University of Göteborg, Sweden): SUBCELLULAR LOCALIZATION OF 5-HYDROXYTRYPTAMINE IN MOUSE THYROID PARAFOLLICULAR CELLS.

It is now well established that the thyroid parafollicular cells (P.F. cells) secrete calcitonin. Like several other cell types elaborating polypeptide hormones the P.F. cells have monoaminergic mechanisms. Light microscopic studies have demonstrated that 5-HT is a naturally occurring compound in these cells in goat and sheep. In most other species, however the presence of monoamines in P.F. cells can only be demonstrated after injection of monoamine precursors (5-HTP and DOPA) which are decarboxylated within the cells to the corresponding amines. These are stored in the cytoplasm by a mechanism sensitive to reserpine.

In the present study, which was performed to elucidate the intracellular localization of the monoamines, mice were injected with tritiated 5-HTP. The thyroids were fixed by perfusion with glutaraldehyde and prepared for light and electron microscopic autoradiography.

In light microscopic sections autoradiographic grains were recovered exclusively over P.F. cells. In electron microscopic autoradiographs the close association between silver grains and the specific granules of the P.F. cells, confirmed by quantitative analysis, was very evident. Grains were seldom encountered over cytoplasmic parts devoid of granules.

Thus the present study clearly demonstrates that a monoamine is localized to the granules which are the most distinctive cytoplasmic components of the P.F. cells (Ekholm and Ericson, J. Ultrastruct. Res. 23, 253, 1968) and which in all probality also contain calcitonin (Ericson, J. Ultrastruct. Res. 24, 145, 1968).

61 Bygdeman, S. and Stjärne, L. (Department of Physiology, Karolinska Institutet, Stockholm, Sweden): THE ROLE OF CALCIUM UPTAKE IN COLLAGEN INDUCED PLATELET AGGREGATION.

Platelet aggregation induced by a collagen fibril suspension is calcium dependent and involves a release of adenine nucleotides, 5-hydroxy-tryptamine and other compounds stored in granules within the platelets. Stormorken (Acta physiol. scand. 1969. 75. 38) has recently argued that the platelets should be regarded as secretory cells and has put forward the hypothesis that the release reactions, involved in platelet aggregation was similar or identical with that observed in some other cell types i. e. adrenal medullary cells. To test this hypothesis and since it is known that uptake of calcium is a necessary step in the release of catecholamines adrenal medullary cells we have thought it to be of interest to study if an uptake of calcium occurred also in the platelets during the aggregation reaction.

In a first series of experiment it was found that guanethidin, which is reported to inhibit calcium uptake, in concentrations from 10^{-6} to 10^{-4} M did not significantly influence the aggregation response to a subsequent addition of collagen in human platelet rich plasma. In a second series of experiments human platelet rich plasma was incubated with tracer amounts of Ca^{45} and the uptake of the isotope after aggregation was determined. No difference in uptake was observed between these experiments and control experiments in which no aggregation was induced.

The results do not support the view that the release reaction involved in platelet aggregation is of the same nature as that observed in adrenal medullary cells.

Håkanson, R., Lindstrand, K., Nordgren, L. and Owman, Ch. (Departments of Pharmacology, Biochemistry and Histology, University of Lund, Sweden): **62**
GASTRIC ARGYROPHIL (ENTEROCHROMAFFIN-LIKE) CELLS: A POSSIBLE STORAGE SITE FOR THE ANTIPERNICIOUS PRINCIPLE (INTRINSIC FACTOR).

Two systems of cells with a possibly endocrine function occur in gastric mucosa. One is the enterochromaffin cell system, which is the major storage site for gastrointestinal 5-hydroxytryptamine. These cells are argyrophil as well as argentaffin. The other system consists of cells which are morphologically similar to the enterochromaffin cells but devoid of 5-hydroxytryptamine (Håkanson, Lilja and Owman, European J. Pharmacol. 1967.1.188). These enterochromaffin-like cells stain argyrophil but not argentaffin. In some species these cells contain histamine (Håkanson and Owman, Life Sci. 1967.6.759). It has been suggested that these two cell systems are active in the formation of gastrointestinal polypeptide hormones (Aures and Håkanson, European J. Pharmacol. 1968.3.316; Pearse, Nature 1969.221.1210). Recently, the system of enterochromaffin-like cells has been implicated as the source of the antipernicious principle (intrinsic factor) (Håkanson et al. in press). This assumption is based on the excellent correlation between the distribution of this cell system and of the intrinsic factor in a number of species. It is also supported by evidence indicating a close parallelism between the development of this cell system and of the intrinsic factor in neonatal animals.

Serck-Hanssen, Guldborg.(Dept. of Biochem., University of Bergen, Norway): **63**
CHROMOGRANIN, A CONSTITUENT OF THE CHROMAFFIN GRANULE MEMBRANE.

The catecholamines, CA, of the chromaffin cells of the adrenal medulla are released on stimulation together with ATP and the soluble protein (Banks, P. & Helle, K., Biochem. J.1965. 97. 40c). The mechanism of release is probably by exocytosis (Schneider, F.H. et al., Br. J. Pharmacol. 1967. 31.94). The granule membrane is not incorporated in the plasma membrane after release of CA but exists as discrete particles within the cell (Poisner, A.M. et al., Biochem. Pharmacol. 1967. 16. 2101, Malamed, S. et al., Ibid. 1968. 17. 241). It has been shown that chromogranin, the main soluble protein of the chromaffin granule, also is a major component of the granule membrane obtained after lysis of isolated granules by osmotic shock or ultrasonic vibration (Helle, K.B. & Serck-Hanssen, G., Pharmacol. Res. Comm. 1969. 1).

The chromogranin of the membrane has now been studied in adrenals perfused in vitro and stimulated by acetylcholine. The results show that the large granule fraction from stimulated and control glands contain 15-20 % chromogranin based on mg protein. On further resolution of the large granule fraction on sucrose gradient, chromogranin can be recovered in two fractions corresponding to that of free membranes and intact granules. In stimulated glands the amount of CA found in the large granule fraction and the membrane fraction from the gradient is only 1/2-1/3 of that obtained for control glands. The higher loss of CA to that of chromogranin indicates that on stimulation a major part of the chromogranin is retained within the cell. This is in agreement with it being bound to the granule membrane.

64 Sachs, Ch. (Department of Histology, Karolinska Institutet, Stockholm, Sweden). EFFECTS OF 6-HYDROXYDOPAMINE ON SYMPATHETIC ADRENERGIC NEURONS.

6-hydroxydopamine (6-OH-DA) has been shown to produce a long lasting depletion of noradrenaline (NA) from various peripheral organs (Porter et al., J. Pharmacol. 1963. 140.308; Laverty et al., Brit. J. Pharmacol. 1965. 24. 549; Thoenen and Tranzer, N-S's Arch. exp. Path. Pharmak., 1968. 261. 271). Electron microscopic and fluorescence histochemical studies have revealed that 6-OH-DA induces a selective destruction of adrenergic nerve terminals (Tranzer and Thoenen, N-S's Arch. exp. Path. Pharmak., 1967. 257. 342; Malmfors and Sachs, Europ.J.Pharmacol. 1968. 3. 89). The present investigation is concearned with the time-course of 6-OH-DA induced disappearance of endogenous NA, its reappearance and changes in uptake-storage properties of NA in adrenergic nerves. 6-OH-DA (20 mg/kg i.v.) causes a general reduction of formaldehyde-induced fluorescence of NA in the adrenergic nerves of mouse atria within 15-20 minutes whereas after 1 hr only a few nerve terminals generally can be observed. The NA content as found by chemical assay, are at these time-intervals 60-80% and 20% respectively of untreated. A restoration of the adrenergic ground-plexus can be observed histochemically after 3-4 weeks, while the NA content is not restored completely until 8 weeks after the 6-OH-DA injection. Desmethylimipramine or NA in high concentrations can partially prevent the depleting action of 6-OH-DA. The uptake of ^{3}H-6-OH-DA is considerably reduced after sympathetic denervation. These data speak in favour of the view that 6-OH-DA or some metabolite has to be taken up by the adrenergic nerve endings to produce its effect.

65 Malmfors, T., and Olson, L. (Dept. of Histology, Karolinska Institutet, Stockholm, Sweden): FUNCTIONAL REINNERVATION BY ADRENERGIC NERVE TERMINALS IN TRANSPLANTED INTESTINAL SMOOTH MUSCLE.

Sympathetic adrenergic neurons have been shown to possess great regenerative capacity both as regards their non-terminal axons (Olson, L., Histochemie 1969. 17. 349) and their terminal parts (Malmfors, T. and L. Olson, Acta Physiol. Scand. 1967. 71. 401). The present investigation concerns the return of neuromuscular transmission after regeneration of adrenergic nerves. Longitudinal strips were prepared from the muscle layer of the rat ileum, thus interrupting the normal supply of adrenergic nerves to the muscle layer without any damage to the nerve cells of the plexus of Auerbach. The strips were homologeously transplanted to the anterior chamber of the eye. Transplants survived well and could be readily observed through the cornea. Vascular contacts were established between the transplant and host iris and spontaneous rythmic contractions were soon observed in the transplants (typically 1 per 2 sec. often in bursts). These spontaneous contractions could be promptly and completely inhibited by electrical stimulation of the cervical sympathetic trunc (10/sec. 6v., dur. 1 msec). The inhibition could be repeated after periods of rest during which the spontaneous contractions reappeared. Fluorescence histochemical investigation revealed a growth of adrenergic nerves from the normally present plexus in the iris of the host eye to the transplant, where varicose nerve terminals were found to reinnervate the plexus of Auerbach surrounding the non-fluorescent cell bodies to an almost normal extent.

Lundberg,D.(Institute of Pharmacology,University of Uppsala,Sweden): 66
EFFECTS OF SOME DRUGS ON THE BRETYLIUM-INDUCED DELAY OF THE DEGENERATION RELEASE OF SYMPATHETIC TRANSMITTER.

It is known that bretylium delays the depletion of the noradrenaline stores which follows division of sympathetic axons (Benmiloud,M. and U.S.v.Euler,Acta physiol.scand.1963.59.34) and that bretylium among four potent neurone-blockers studied causes the greatest delay of a degeneration contraction in the rat (Lundberg,D.,Acta physiol.scand.1969.75.415).To study the delaying effect of bretylium further some drugs active at the adrenergic nerve terminals were tested for bretylium-antagonizing action.

The degeneration contraction of periorbital smooth muscles produced by high cervical ganglionectomy was measured intermittently in conscious rats.Drugs were given s.c. within 1-3 hrs before the expected start of the contraction.- Injected after bretylium Lu 3-010, desmethylimipramine (DMI) and protriptyline at 10 mg/kg and metaraminol and dexamphetamine at 2 and 4 mg/kg,respectively,significantly shortened the bretylium-induced delay,whereas trimipramine and cocaine at 10 and 10-20 mg/kg,respectively,had no reversing effect. Given before bretylium DMI and dexamphetamine but not metaraminol or cocaine reduced the delay.In fact cocaine significantly prolonged the delay.

The results are still hard to interpret. Interference with the amine pump of the axon membrane does not seem to be sufficient to explain the antagonism to bretylium in the situation studied.

Palmer, B., and K.-A. Norberg. (Department of Histology, Karolinska Institutet, and Research Laboratory of the Thorax Clinic, Karolinska Hospital, Stockholm, Sweden): IMPROVEMENT OF BLOOD FLOW IN EXPERIMENTAL SKIN FLAPS BY PHENTOLAMINE. 67

Cutaneous blood vessels in a three-sided skin flap become sympathetically denervated within 24-48 hours (Jurell, G., K.-A. Norberg and B. Palmer, Acta physiol. scand. 1968. 74. 511). Within this period the uptake mechanism in the neuronal cell membrane is destroyed (Malmfors,T., and Ch. Sachs, Acta physiol. scand. 1965. 64. 211). This probably can explain the early supersensitivity for catecholamines. A supersensitivity of the blood vessels in the apex of a flap would create conditions for vasoconstriction leading to tissue necrosis. With the aim to elucidate some of the reasons for failure of the flap surgery technique, the blood flow in experimental flaps in rats was analyzed during ether anesthesia by means of the ^{133}Xe clearance method, according to Lassen et al.(Lancet 1964. 1.686). The flow was expressed as the quotient between the clearance values for the flap and contralateral control side, expressed in per cent. The flow in the apex raised from about 15 per cent initially to about 65 per cent on the 7th day. Blocking of the adrenergic α-receptors by phentolamine (Regitine® 1 mg/kg b.w. i.m.) caused an improvement in blood flow in the apex up to about 70 per cent already on the 3rd day. The possibility thus arises that denervation supersensitivity may be of major importance for the blood flow in flaps during the first critical days postoperatively.

68 Johansson, B., B. Ljung, T. Malmfors and L. Olson (Dept. of Physiology, Univ. of Göteborg and Dept. of Histology, Karolinska Inst., Stockholm, Sweden): DENERVATION SUPERSENSITIVITY IN THE SPARSELY INNERVATED SMOOTH MUSCLE OF THE PORTAL VEIN.

The propagating smooth muscle of the rat portal vein was shown by the fluorescence technique to receive a sparse adrenergic nerve supply organized as a continuous two-dimensional plexus between the heavy longitudinal and the inner circular muscle layers. Scattered nerve terminals were found at the adventitio-medial border. In view of this sparse innervation it was considered of interest to study the degree of "supersensitivity after denervation". Mechanical responses of isolated portal veins from sham-operated and ganglionectomized (coeliac +mesenteric) rats were studied. NA dose-response curves for six successfully denervated preparations, examined 3 to 17 days after operation, were parallelly displaced to the left, corresponding to a mean change of ED_{50} from 1.0×10^{-6}M to 1.3×10^{-7}M. After blockade of neural NA uptake by cocaine the ED_{50} of innervated preparations was 3.7×10^{-8}. Excitatory responses to Ach were not affected by chronic denervation. The results indicate that the supersensitivity to NA after denervation is due to elimination of the neural NA uptake. It appears that the "innervated" smooth muscle cells play a major role not only in the neural response but also in the response to exogenous NA.

69 Anggård, E. and R. Sjöström (Department of Pharmacology, Karolinska Institutet, Stockholm, Sweden): SEPARATION OF ACIDIC AND NEUTRAL CATECHOLAMINE METABOLITES ON LIPOPHILIC SEPHADEX.

Methylated and hydroxypropionylated Sephadex has recently become available for chromatography of lipophilic substances such as steroids and bile acids (Nyström and Sjövall 1964, 1965). In the present study vanillylmandelic acid, homovanillic acid, 3,4-dihydroxymandelic acid, 3,4-dihydroxyphenylacetic acid, 3-methoxy-4-hydroxyphenylethylene glycol and 3,4-dihydroxyphenylethylene glycol were separated on hydroxypropionylated Sephadex (Sephadex LH-20), Pharmacia, Uppsala, Sweden). Ethylene chloride and methanol were used as solvents. The acids were run as the methyl esters.

Separation of the abovementioned compounds was obtained in a single analysis. The method is fast (4-6 hours), efficient (600-800 theoretical plates/foot) and the column can be used repeatedly without repackning. It appears to be useful for purification prior to gaschromatographic analysis.

Farnebo, L.-O. (Department of Histology, Karolinska institutet, Stockholm, Sweden): RELEASE OF ^{3}H-NORADRENALINE FROM ISOLATED RAT IRIS BY FIELD STIMULATION. 70

Field stimulation, which is known to cause depolarization of nerve membranes, markedly increase the efflux of ^{3}H-noradrenaline (^{3}H-NA) from nervous tissues previously incubated in ^{3}H-NA (Baldessarini and Kopin, J. Pharmacol. exp. Ther. 1967. 156. 31-38).

Isolated rat irides were incubated in ^{3}H-NA and then stimulated by an electrical field (biphasic pulses, 10/sec, 2 msec, 5 V/cm). Stimulation caused a marked increase in the efflux of total ^{3}H. The stimulus induced release was the same in irides from untreated rats and rats pretreated with a tyrosine hydroxylase inhibitor (H 44/68, 1 h). The efflux of ^{3}H per minute was high during the first 15 min of stimulation but then declined. In irides from untreated rats, stimulation for 60 min caused a slight reduction in fluorescence intensity of the adrenergic nerves, while after H 44/68 pretreatment the reduction was more pronounced. No stimulation induced ^{3}H efflux was found in irides from rats pretreated with reserpine (16 hrs) and nialamid (1 h). Irides (from untreated rats) which were incubated in reserpine phosphate 10^{-7} M after incubation in ^{3}H-NA showed a high spontaneous ^{3}H efflux. This efflux was further increased by stimulation. The stimulus induced release was considerably higher than that from irides not incubated in reserpine.

The technique used presents new possibilities for the study of release mechanisms in adrenergic nerves.

Hedqvist, P. (Department of Physiology, Karolinska Institutet, Stockholm, Sweden): PROSTAGLANDIN E AS A MODULATOR OF NORADRENALINE RELEASE FROM SYMPATHETIC NERVES. 71

Prostaglandin E (PGE), which is known to be released by sympathetic nerve stimulation, lowers the arterial blood pressure and counteracts the increase of blood pressure caused by noradrenaline (NA) (c.f. Bergström, Carlson and Weeks, Pharmacol. Rev. 1968. 20. 1). This indicates that PGE may play a modulatory role on the sympathetic neuro-effector system. The present experiments were performed to study wether PGE may modify the release of NA from sympathetic nerves.

Isolated cat spleens were perfused at a constant rate with salinic medium. Close arterial infusion of PGE_1, 10^{-6} - 4 10^{-6} M, was found occasionally to reduce the outflow of NA in response to stimulation of the splenic nerves. When PGE_2, within the same concentration range, was infused instead, the NA overflow response consistently fell, often to less than 50 per cent of that of the preceding control stimulation. Addition to the perfusion medium of phenoxybenzamine (PBA), which has been shown to inhibit the release of PGE, resulted in a several-fold increase of the NA overflow response to nerve stimulation. Simultaneous infusion of PGE_2 strongly counteracted this effect of PBA, leading to NA output figures close to those from the preceding control stimulation.

It is suggested that PGE, locally mobilized by sympathetic nerve stimulation, may counteract further release of NA from the nerves by a negative feed back mechanism.

2 Sjöstrand, N.O. and G.Swedin. (Dept. of Physiology, Karolinska Institutet, Stockholm, Sweden): POTENTIATION BY SMOOTH MUSCLE STIMULANTS OF AN HYPOGASTRIC-NERVE-SEMINAL VESICLE PREPARATION FROM THE GUINEA-PIG.

Potentiation by smooth muscle stimulants of the hypogastric nerve-vas deferans preparation has previously been described (Sjöstrand, N.O., Nature 1961. 192.1190; c.f. Sjöstrand, N.O. and G. Swedin, Acta physiol scand. 1968. 74. 472).

The contractions of the seminal vesicle were recorded with a balloon inserted in the lumen of the seminal vesicle.

Adrenaline, noradrenaline, acetylcholine, histamine, serotonine, bradykinin, prostaglandin E_1 and bariumchloride were found to potentiate the responses of the seminal vesicle to hypogastric nerve stimulation. This potentiation occurred with doses much smaller than those giving a direct contraction of the organ.

It is suggested that in vivo such a potentiation can occur and be of importance for the emission.

73 Lundborg, P.G. (Department of Pharmacology, University of Göteborg, Sweden): EFFECT OF (+)-AMPHETAMINE ON THE SUBCELLULAR DISTRIBUTION OF α-METHYLATED AMINES.

In in vivo experiments on mouse hearts the releasing effect of (+)amphetamine on previously administered racemic ^{3}H-metaraminol or ^{3}H-α-methyl-noradrenaline was studied. Also the effect of previously administered (+)amphetamine on the uptake and subcellular distribution of ^{3}H-metaraminol and ^{3}H-α-methyl-noradrenaline was investigated.

The dose used of (+)amphetamine was of importance for the subcellular site of attack of the drug.

30 min after the administration of ^{3}H-metaraminol 5 mg/kg of (+)amphetamine caused a release of the labelled amine predominantly from the particulate (granular) fraction, 0.2 mg/kg of (+)amphetamine, however, caused a release exclusively from the supernatant fraction.

24 hrs after the administration of ^{3}H-metaraminol (+)amphetamine caused a release of ^{3}H-metaraminol only from the supernatant fraction.

If the animals were pretreated with reserpine before administration of ^{3}H-metaraminol (+)amphetamine caused a pronounced release of the amine from all fractions.

The affinity of ^{3}H-α-methyl-noradrenaline for granular binding sites is higher than that of ^{3}H-metaraminol. Thus the extragranular pool of ^{3}H-α-methyl-noradrenaline is small. Therefore it was hard to demonstrate a release of this amine from the supernatant fraction. After reserpine treatment, however, (+)amphetamine caused considerable release of ^{3}H-α-methyl-noradrenaline and this release was more pronounced from the supernatant than from the particulate fraction.

Peterson, C. and Diamant, B. Dept. of Pharmacology, Karolinska institutet, Stockholm 60, Sweden): THE EFFECT OF SOME HEXOSES AND PENTOSES ON HISTAMINE RELEASE FROM ISOLATED RAT MAST CELLS INDUCED BY ATP. 74

Histamine release from isolated rat mast cells is induced by ATP in the presence of Ca^{++} (Diamant, B. and Krüger, P-G., Acta physiol. scand. 1967. 71. 291). Peritoneal rat mast cells were isolated in the absence of glucose. Incubation of the cells with ATP (4.5×10^{-5}M) at 37° for 15 min released about 20 % of their histamine content. Preincubation with glucose and mannose (5.6×10^{-4}M) at 37°for 10 min increased the histamine liberation to about 50 % while the sugars alone had no releasing activity. Other hexoses investigated, such as fructose, galactose, L(-) glucose and the pentoses ribose and 2-deoxyribose had no enhancing effect on the histamine release. Incubations of mast cells with glucose and mannose at 37° for 15 min induced a significantly higher production of lactate as compared to controls or incubations with the other sugars.

It is concluded that glucose and mannose potentiate histamine release by ATP and that these sugars give rise to the production of lactate in the mast cells.

Dahlquist, R. and Diamant, B. (Dept. of Pharmacology, Karolinska institutet, Stockholm 60, Sweden): INFLUENCE OF ATP UPON SODIUM FLUXES ACROSS THE MAST CELL MEMBRANE. 75

Searching for a mechanism by which ATP induces histamine release from isolated rat mast cells, $^{22}NaCl$ was used to investigate whether ATP would induce sodium uptake into the cells from the medium. Histamine might then be released by intracellular ion exchange.

The presence of ATP (0.5mM) in the incubation medium increased sodium uptake 2.5 times, compared to controls without ATP. This effect was independent of the presence of Ca^{++} (1mM). In the presence of 1 mM of Mg^{++} the stimulatory effect of ATP on sodium uptake was 7 times that in controls without ATP. In contrast, ATP-induced histamine release does not occur in the absence of Ca^{++}, and Mg^{++} exerts an inhibitory effect (Diamant and Krüger, Acta physiol. scand. 1967. 71. 291). ITP, CTP, GTP, TTP and UTP could not be substituted for ATP.

The mast cells accumulated sodium during the first 20 min of incubation. Calculations indicate that within this time ATP had disappeared due to hydrolysis by an ATP-splitting enzyme in the cell membrane. The ATP-Mg-stimulated sodium uptake was completely inhibited by oligomycin (10^{-8}M), while ouabain (1mM) was without inhibitory effects. On prolonged incubation, the sodium content of the cells decreased. This decrease was counteracted by ouabain (1mM) and glucose (5.6mM) when added to cells which had accumulated sodium for 20 min.

It is concluded that the mechanism by which ATP induces histamine release from rat mast cells is not through an exchange of sodium taken up from the extracellular medium for intracellular histamine.

76 Fredholm, B.[1] and Hägermark, Ö. (Departments of Pharmacology and Dermatology, Karolinska Institutet, Stockholm, Sweden): STUDIES ON HEAT-INDUCED HISTAMINE RELEASE FROM SKIN AND FROM PERITONEAL MAST CELLS OF THE RAT.

Thermal injury evokes an inflammatory reaction in the exposed tissue. There are several indications that mast cells and substances released from these cells are involved in the reaction. In the present work the degranulating and histamine releasing effects of moderate heat were studied on rat skin and peritoneal mast cells.

The cutaneous mast cells were degranulated in vivo by immersing the animals in water at 60-80°C for 20 sec. In vitro, histamine was released from minced skin and from peritoneal mast cells exposed to temperatures above 52°C. The rate of release was markedly increased by raising the temperature from 56°C to 60°C. The metabolic inhibitors 2,4-dinitrophenol and potassium cyanide did not inhibit the release process. The kinetics of the heat-induced release differed from that caused by compound 48/80. It is concluded that the histamine release induced by heat does not involve the energy-requiring mechanisms concerned in the release caused by e.g. antigen and compound 48/80.

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77 Strandberg, K. (Department of Pharmacology, Karolinska Institutet, Stockholm, Sweden): PURIFICATION AND PROPERTIES OF A LIPID-SOLUBLE SMOOTH-MUSCLE STIMULATING PRINCIPLE, SRS, APPEARING ON HISTAMINE RELEASE IN CAT PAWS.

Lipid-soluble smooth-muscle stimulating material appears in the effluents from cat paws perfused with the histamine releasing agent compound 48/80 (Chakravarty et al. Acta.physiol. scand. 1959. 45. 255). This material has been designated "slow reacting substance", SRS, since it elicits slow, sustained, non-tachyphylactic contractions of the isolated guinea-pig ileum. It has been shown, however, that this SRS actually consists of several principles with different biological and chemical properties (Änggård et al. Acta physiol. scand. 1963. 59. 97).

In the present investigation cat paws were perfused for 90 min with a modified Tyrode solution (pH 7) containing compound 48/80 (1 µg/ml) at 28-32°C. The lyophilized effluents were extracted with 80 per cent aqueous ethanol and the principle producing the slow contraction of the guinea-pig ileum was purified by solvent partition (water/ether), silicic acid and anion exchange chromatography. The overall recovery was 10-20 per cent, calculated from the non-histamine smooth-muscle activity of the ethanolic extract. The purified material was dialysable, soluble in polar solvents, acid-labile, heat-stable and it behaved homogenously on thin layer chromatography in different systems. Studies of the influence of chemicals reactive with certain functional groups on the biological activity of the material indicated that the principle was probably an unsaturated hydroxy-carboxylic acid.

Bergendorff, A. (Department of Pharmacology, Karolinska Institutet, Stockholm, Sweden): UPTAKE OF HISTAMINE AND OTHER BIOGENIC AMINES BY MAST CELL GRANULES AND "ARTIFICIAL GRANULES". 78

Histamine and serotonin are localized to the granule fraction of rat mast cells. The granules consist of a heparin-protein complex.

Histamine containing granules have been isolated from rat mast cells lysed in deionized water. The endogenous histamine is removed by suspending the granules in sodium chloride solution, which results in an exchange of histamine for sodium. By repeated suspension in deionized water the granules are then depleted of sodium.

The uptake and storage of histamine in granules prepared as described above have been shown to be dependent on pH and histamine concentration indicating a linkage of the amine to weakly acid groups in the granule heparin protein complex. The storage of histamine is also influenced by the presence of other cations. The granules can take up and store other biogenic amines than histamine.

"Artificial granules" have been prepared by reacting sodium heparinate with a basic protein having a molecular weight about the same as the granular protein. The cation (various amines and sodium) uptake and storage properties of "artificial granules" are similar to those of granules isolated from mast cells.

Frisk-Holmberg,M. (Department of Pharmacology, Karolinska Institutet Stockholm, Sweden): STUDIES IN THE MECHANISM OF CHLORPROMAZINE INDUCED HISTAMINE RELEASE FROM ISOLATED RAT PERITONEAL MAST CELLS. 79

Mast cells incubated with chlorpromazine in concentrations 3.10^{-5} M to 6.10^{-2} M release 10-70% of their histamine. The release is not accompanied by degranulation.The release is completed within 5 to 10 min., is dependent on ph and increases with decreasing ionization (increased ph) and is considerably reduced at 0°C. After washing, the mast cells respond on a second exposure to chlorpromazine. Metabolic inhibitors slightly stimulate, tetrodotoxin 10 µg/ml reduces release.Chlorpromazine 3.10^{-5}M to 6.10^{-2}M (CPZ-S^{35}) probably enters the mast cells. The cellular content of ^{22}Na is slightly increased after exposure to chlorpromazine of cells, suspended in ^{22}Na containing medium.

It is concluded that the release of histamine occurs intracellullary but that the concentration of chlorpromazine in the cells is too low to explain the release of histamine as due to an ionic exchange between chlorpromazine and histamine in the granules.

80 Lindström, L. (Department of Pharmacology, University of Uppsala, Sweden): INTERACTION BETWEEN CHOLINERGIC AND MONOAMINERGIC MECHANISMS WITH REGARD TO OESTROUS BEHAVIOUR IN FEMALE RATS.

It has previously been demonstrated that psychopharmacological agents that increase the central monoamine level and muscarinic alkaloids inhibit the hormone activated oestrous behaviour in the female rat (Meyerson, B.J., Acta Physiol. Scand. 1964. 63. suppl. 241; Lindström L. and Meyerson B.J., Psychopharmacologia 1967.11.405). The present experiments were performed to study a possible relationship between heat inhibitory monoaminergic and cholinergic mechanisms.

Increased monoaminergic tone in the central nervous system brought about by pargyline (25 mg/kg) or desmethylimipramine (2 mg/kg) prolonged the inhibitory effect of pilocarpine (25 mg/kg), whereas amine depletion by reserpine (1 mg/kg) counteracted the inhibitory effect of pilocarpine (25 mg/kg) and oxotremorine (1 mg/kg). Pilocarpine also lacked an effect after p-chlorophenylalanine (400 mg/kg), a serotonine synthesis inhibitor.

A relationship between cholinergic and monoaminergic central nervous mechanisms inhibiting the oestrous behaviour in the female rat is suggested. It is assumed that the inhibitory effect of pilocarpine is dependent on the monoamine level. The effect of p-chlorophenylalanine indicate that serotonine is important.

81 Meyerson, B.J. and E-B.Nordström (Department of Pharmacology, University of Uppsala, Sweden): SEXUAL MOTIVATION IN THE FEMALE RAT: (I) THE EFFECT OF OESTROGEN AND TESTOSTERONE MEASURED BY AN INCREASING BARRIER METHOD.

Different techniques are employed in this laboratory to study the role of gonadal hormones in the production of sexual motivation. One method is designed to measure the willingness of an animal to pay a painful stimulus to reach a sexually active partner. The subject (S) has to pass from one cage via an electric grid to a second cage which holds the incentive animal. The intensity of the current is stepwise increased every second time S passes. By an electromechanical devise we record the time spent before S passes the grid and the amount of barrier shock (i.e. the number of crossings over the grid) the animal is willing to bear.

S were adult sexually experienced ovariectomized Sprague-Dawley rats. The sexual contact was restricted i.e. contact with stimulus animal (vigorous male) was allowed but the animals separated by a net. The experimentswere performed one run a day for 12 consecutive days, starting one day (day 0) before the hormone treatment. The number of passages over the grid S was willing to perform was significantly increased by oestradiol benzoate 25 and 100 µg/kg s.c. and testosterone proprionate 0.5 and 1.0 mg/kg s.c. at the lower dose levels the maximal response was obtained at day 4. After the higher doses the maximal effect was seen one or two days later.

It is concluded that both oestradiol and testosterone induce in the ovariectomized rat a desire to seek contact with a sexually active male.

Lindström, L. and Meyerson, B. (Department of Pharmacology, University of Uppsala, Sweden): SEXUAL MOTIVATION IN THE FEMALE RAT: (II) THE EFFECT OF OESTROGEN MEASURED BY A T- MAZE CHOICE METHOD. 82

A T-maze choice method has been developed to study sexual motivation in the rat. The subject runs through a runway which brings it to a chamber, the end of which has two exits. One exit leads into a cage with an incentive animal, the other exit to an indifferent animal. We record automatically the running time, the time the animal spends to make up its choice and the specific choise. In the present experiments the contact with the stimuli animals was restricted, i.e. contact was allowed but the animals separated by a net.

Ovariectomized Sprague-Dawley rats were tested during a 12 day period with start on day 0. Oestradiol benzoate 25 or 100 µg/kg s.c. was given at day 1. A sexually active male and a castrated male respectively was used as stimuli animals. Oestrogen treated subjects displayed a clearcut preference to choose the sexually active male. This was not seen in oil treated controls. The maximal response was obtained at day 4-5. A slightly longer latency was seen when the higher dose level of the hormone was used. The time relationship between injection of the oestrogen and the maximal response is in agreement with the data obtained by the increasing barrier method employed in this laboratory.

It seems as if oestrogen induces a goal directed urge to seek contact with a sexually active male.

Kjellberg, B. and Randrup, A. (AB Ferrosan, Malmö, Sweden and St.Hans Hospital, Roskilde, Denmark): VARIOUS FORMS OF STEREOTYPED, ABNORMAL BEHAVIOUR INDUCED BY AMPHETAMINE IN NON-HUMAN PRIMATES. 83

There are to our knowledge relatively few reports dealing with the effects of amphetamine upon the behaviour of non-human primates. We have studied the behavioural effects of one single dose of d-amphetaminesulphate (4 mg/kg s.c.) upon 4 primate monkeys (Cercopithecus aethiops).

As in rats we found selective stimulation of certain behavioural elements and suppression of others resulting in a behaviour with strongly stereotyped character. One monkey raised its right forepaw and looked at it continously for several hours. Another monkey made continous locomotion repeating a certain route covering only a part of the cage and two were sitting at the front wall of the cage seizing continously the bars of the wall while repeating certain movements of the limbs and the head. For periods, movements of the mouth and the tongue were also seen. Grooming was absent in all the monkeys during the period of stereotyped behaviour and then gradually returned. In contrast to rats, however, the behaviour was also abnormal the next day 24-32 hours after amphetamine. The monkey looking at its hand did this much more than normally but not continously, grooming and other normal activities were interspersed. Another monkey performed on the second day new forms of abnormal behaviour, which were not seen during the first day.

It is concluded that when compared to e.g. rats, the behaviour displayed by these primate monkeys after one single dose of amphetamine is highly individual though rather constant for each individual.

84 Jonas, W. (Sct. Hans Hospital, dept. E, Roskilde, Denmark): TETRABENAZINE INDUCED CATACHOLAMINE RELEASE AS IT AFFECTS THE EXCITATORY BEHAVIOUR AF RATS, PRETEATED WITH AMPHETAMINE. The amphetamine (AMPH) excitatory response including stereotyped behaviour seems to depend on the interaction with dopamine. (Randrup, A. and Jonas, W., J. Pham. Pharmac., 19, 483, (1969) and Jonas, W. and Scheel-Krüger, J., Arch. Int. Pharmacodyn. (1969) *in press*). In this paper the mechanism underlying the reappearence of the AMPH excitation induced by tetrabenazine (TBZ) was investigated in rats. All rats recieved AMPH (5 mg/kg). 4 hours later when the excitation had attenuated the rats recieved scopolamine (SCP) (10 mg/kg), aceperone(ACP) (10 mg/kg), spiramide (SPI) (2 mg/kg) or saline. 30 min. later the excitation was reinduced by TBZ (50 mg/kg). A quantitative evaluation of the behaviour features: locomotion, sniffing, gnawing, rearing, eating and drinking was preformed.

SCP significantly prolonged the TBZ reversal effect, ACP, a potent noradrenaline blocking agent, did not have any effect on the reversal, while SPI, a potent and specific dopamine blocking agent, completely antagonized the TBZ reversal.

It is concluded that this AMPH-TBZ excitatory effect depends not on the noradrenaline but on the dopamine release.

85 Scheel-Krüger, J. (Sct. Hans Hospital, Dept. E., Roskilde, Denmark): PHARMACOLOGICAL STUDIES ON A COUNTERBALANCING ADRENERGIC-CHOLINERGIC SYSTEM IN THE BRAIN.

Previous papers have described the behavioural excitation with increased locomotion and stereotyped behaviour induced by amphetamine or apomorphine in rats and mice. Particularly, the stereotyped behaviour seems produced by activating a dopaminergic mechanism in corpus striatum.

The results of this paper show that a cholinergic system exists, which shows antagonism against this adrenergically provoked behaviour, since the stereotypy is potentiated and prolonged by anticholinergica (atropine, scopolamine) and weakly antagonized by cholinergica(physostigmine,oxotremorine) Among the characteristic actions for neuroleptica are the antagonism of amphetamine or apomorphine induced stereotyped behaviour and development of catalepsy, which actions are probably produced by blockade of adrenergic (dopaminergic?) mechanisms in brain. Results of combined drug treatments of neuroleptica and anticholinergica or cholinergica show that the inhibitory activity of a cholinergic system also contributes to the development of these characteristic actions of neuroleptica.

It is concluded that a counterbalancing adrenergic-cholinergic system exists in brain, which involves behavioral features as locomotor activity and stereotyped behavior.

Ahtee, L. & Shillito, E. (A.R.C. Institute of Animal Physiology, Babraham, Cambridge, England): THE BEHAVIOURAL EFFECTS OF BENZODIAZEPINES AND ATROPINE. **86**

The atropine-like drugs are often combined with benzodiazepines in the treatment of gastric ulcer and related disorders. It is possible that the combination of these drugs may produce interesting effects on behaviour and we have investigated this in mice.

Male albino mice were observed in reversed daylight on a tunnel board to test exploration (Shillito, E.,J. Physiol.(Lond.) 1968, 200, 35P) for 5 min ½ hr after i.p. injection of drugs or saline. Following this their motor activity was assessed in an open cage for 3 min. This procedure was repeated on the following day without any injections. The drugs used were atropine 5 or 10 mg/kg, methylatropine 5 or 10 mg/kg, chlordiazepoxide 25 or 50 mg/kg and diazepam 10 or 20 mg/kg.

Atropine or methylatropine did not alter the motor activity of mice, while chlordiazepoxide and diazepam increased the activity especially in the lower doses used. All the compounds used except methylatropine adversely affected the exploratory behaviour. When 10 mg/kg of atropine was given with the benzodiazepines activity of the mice was reduced below normal and exploratory behaviour was further impeded, but this did not occur when methylatropine was used with the benzodiazepines.

The benzodiazepines and atropine may impair exploration by affecting kinaesthesis. It seems that it is a central effect of atropine which modifies the behavioural effects of the benzodiazepines, since methylatropine does not have this effect, although some peripheral anticholinergic effects might be antagonized by these drugs (Ahtee, L. & Pohto, P., Scand.J.clin.Lab.Invest. 1968, 21 Suppl.101, 86).

Lycke, E., Modigh, K. and Roos, B.-E. (Departments of Virology and Pharmacology, University of Göteborg, Sweden): SOME ASPECTS OF MONOAMINE METABOLISM IN MOUSE BRAIN AND AGGRESSIVENESS. **87**

Herpes simplex virus (HSV) encephalitis in mice produces an increased synthesis rate of the monoamines dopamine (DA) and 5-hydroxytryptamine (5-HT) in brain tissue (Lycke, E. and Roos, B.-E., Experientia 1968. 24, 687). In the present investigation HSV inoculated mice were treated with an effective inhibitor of 5-HT synthesis, H 69/17 (the methyl ester hydrochloride of d,l,-p-chlorphenylalanine) in a daily dose of 400 mg/kg i.p. After 4 days the animals showed a marked excitation and an aggressive behavior. In control mice (only inoculated with HSV) a marked excitation but no aggressiveness was observed. If normal mice were treated with H 69/17 followed by L-DOPA (500 mg/kg i.p.) at different time intervals, they developed an immensely aggressive behavior when L-DOPA was given 7 hours or later after the administration of the inhibitor. The same aggressiveness was observed after another effective inhibitor of 5-HT synthesis (H 22/54, α-propyldopacetamide) followed after 1 hour by L-DOPA in a dose of 200 mg/kg or more.

The present study indicates a correlation between the observed aggressive behavior and a low degree of 5-HT receptor stimulation in combination with a high degree of DA receptor stimulation.

88 Malmnäs, C-O. and Meyerson, B.J. (Department of Pharmacology, University of Uppsala, Sweden): THE EFFECT OF ACTH ON COPULATORY BEHAVIOUR IN THE MALE RAT.

The present experiments were conducted to study the effect of adrenocortical stimulation on copulatory behaviour in male rats. The mating behaviour of adult Sprague-Dawley rats, given copulatory experience before castration, was restored by injection of testosterone proprionat once weekly (0,3; 0,5 or 0,7 mg/kg s.c.). The testosterone treatment was chosen to permit a stimulatory or inhibitory effect of further treatments to be discovered. To achiev a sustained adrenocortical stimulatory effect an ACTH zinc chlorid preparation was used (Cortophin prolongat, Pharmacia, Uppsala). ACTH 10 I.E. i.m. was given either (A) at the same time as the testosterone treatment or (B) 6 hours before the tests for mating behaviour. Tests for copulatory behaviour were performed either 3, 5 or 9 days after the testosterone treatment.

No increase of the sexual activity was found in the ACTH treated animals. However, at day 3 (experiment A) the number of animals which ejaculated was decreased and the time from onset of the test to first intromission was increased.

At the concentration and for the length of treatment here employed ACTH could not augment any of the indices of sexual activity tested for. It is concluded, however, that some components of the copulatory behaviour was inhibited either by a direct effect of ACTH or presumably through the liberation of adrenocortical steroids.

89 Rasmussen, Folke (Department of Pharmacology and Toxicology, The Royal Veterinary and Agricultural University, Copenhagen, Denmark): ACTIVE MAMMARY EXCRETION OF N^4-ACETYLATED p-AMINOHIPPURIC ACID.

Different excretion patterns were observed for p-aminohippuric acid and its acetylated metabolite in mammary excretion experiments in goats and cows. The excretion of p-aminohippuric acid conformed to passive diffusion of the non-protein bound and un-ionized fraction. Thus, the p-aminohippuric acid (pK_a 3.8) appeared in ultrafiltrates of milk in concentrations 1/4-1/10 of the concentrations in ultrafiltrates of blood plasma. The concentrations of acetylated p-aminohippuric acid (pK_a 3.9) in ultrafiltrates of milk were on the other hand equal to or higher than in ultrafiltrates of blood plasma.

Further experiments were conducted by intravenous infusion of acetylated p-aminohippuric acid. After acid hydrolysis the concentrations of acetylated p-aminohippuric acid in milk and blood plasma were estimated according to the method described by Bratton & Marshall, J.biol. Chem. 1939, 128, 537. It was found that the concentrations of acetylated p-aminohippuric acid in milk and in ultrafiltrates of milk are equal to or higher than in plasma (ratio M/P) and ultrafiltrates of plasma (ratio M.Ultr./P.Ultr), respectively. It was also found that the milk-to-plasma ratios decrease with increasing concentrations of acetylated p-aminohippuric acid in blood plasma.

It is concluded that an active saturable excretory process is involved in the mammary excretion of acetylated p-aminohippuric acid.

90 Holm, J. & Broen Christensen, C. (Department of Pharmacology, University of Copenhagen, Denmark): DECAMETHONIUH UPTAKE BY SLICES OF MOUSE KIDNEY AND THE INHIBITORY EFFECT OF D-TUBOCURARINE.

Decamethonium is concentrated in the liver and kidneys of mice in vivo (Broen Christensen, C. & Holm, J., Acta Pharmacol. et toxicol. 1969, 27, 17). Slices of mouse liver accumulate decamethonium and the uptake is inhibited by d-tubocurarine (Broen Christensen, C., Acta Pharmacol. et Toxicol. 1967, 25, suppl. 4, 46). The present study was made to investigate the uptake of decamethonium by mouse kidney slices with particular regard to d-tubocurarine inhibition. - Kidney slices were incubated in a medium containing ^{14}C-decamethonium (2×10^{-6} M) and the slice-to-medium concentration ratio (S/M ratio) was measured. - In oxygen atmosphere the S/M ratio, which approximated 9 after 1 hr., continued to increase within 3 hrs. incubation. The S/M ratio decreased when raising the decamethonium concentration. In nitrogen atmosphere the S/M ratio reached a constant value of about 1 within 1/2 hr. When the decamethonium concentration was 2×10^{-6} M, d-tubocurarine (2×10^{-6} M) reduced the S/M ratio by 9%, ten- and hundredfold higher concentrations of d-tubocurarine reduced the S/M ratio by 50 and 80% respectively. - Mouse kidney slices accumulate decamethonium against an apparent concentration gradient and the process, partly depending upon oxygen, shows saturation. The uptake consists of a d-tubocurarine-sensitive part and a part much less sensitive to the inhibitor.

91 Pelkonen,O., Vorne,M., Kärki,N.T. (Department of Pharmacology, University of Oulu, Finland): DRUG-METABOLIZING ACTIVITY IN THE LIVER, INTESTINE AND KIDNEY OF HUMAN FOETUS.

In general foetal and newborn animals show a low level of activity to metabolize drugs. In this study we have determined the capability of human foetal tissues to oxidise (pethidine, hexobarbitone, chlorpromazine), reduce (p-nitrobenzoic acid) and conjugate (p-nitrophenol) drugs.

The age of the foetuses varied from 8 to 22 weeks and the weight ranged from 19 to 280 gr. After homogenisation the tissue preparations were centrifuged at 12000 g and the supernatant was used for enzymatic studies.

No conjugative or pethidine metabolizing activity was present in studied tissues. Low level of hexobarbital metabolizing activity was found in some trials. We were not able with the method used to find cytochrome P-450 in any tissue. Weak but significant enzymatic degradation of chlorpromazine and p-nitrobenzoic acid was found in foetal tissues. The mean metabolic activity for chlorpromazine was in liver 0.09, in kidney 0.04 and in intestine 0.03 µM/g tissue/hr. The Michaelis constant for liver preparation was $6.25\cdot10^{-5}$M. The rate of metabolism for p-nitrobenzoic acid was in liver 0.07 in intestine 0.03 and in kidney 0 µM/g tissue/hr. The drug metabolizing activity of the liver preparation was more stable than that of kidney or intestine.

92 Harri, M.-P. and Nienstedt, W. (Department of Physiology, University of Turku,Finland): THE METABOLISM OF TESTOSTERONE IN THE CANINE INTESTINAL MUCOSA *IN VITRO*.

The metabolism of steroids during absorption from the ileum of dogs has been studied earlier *in vivo* (Nienstedt,W. and Hartiala,K. Acta physiol.scand.1966.Suppl.277.68). The results have shown that the metabolism is chiefly reductive and hydroxylative. In order to investigate the *in vitro* metabolism of the same steroids three different fractions from the mucosal cells of ileum were prepared: homogenate, microsomes and high speed supernatant, each representing 1 g of fresh tissue. After incubating with 4-^{14}C-testosterone and a $NADPH_2$-regenerating system the resulting metabolites were analyzed with different chromatographic systems. The metabolism of testosterone varied greatly in different fractions. The homogenate yielded 5α-androstan-3-ol-17-ones, the microsomes 5α-androstan-17β-ol-3-one, and both yielded diols, mostly 5α-androstan-3β,17β-diol. The supernatant yielded 5β-androstan-3β,17β-diol, possibly *via* androst-4-en-3β,17β-diol. The supernatant metabolites could not be detected among the homogenate metabolites. The findings indicate that the testosterone metabolizing enzyme systems may be located in different parts of the cell. The tentative results obtained with progesterone have revealed similar patterns.

93 Karlog, O. (Department of Pharmacology and Toxicology, The Royal Veterinary and Agricultural University, Copenhagen, Denmark): RATE OF FORMATION OF UNREACTIVATABLE CHOLINESTERASES IN BLOOD OF PIGS INTOXICATED BY DIMETHYL- AND DIETHYLTHIOPHOSPHATES.

Pigs protected by atropine were given intramuscular injections of parathion, methylparathion, malathion or dimethoate resp.. In blood samples the activities of cholinesterases of plasma and erythrocytes were measured before and after reactivation with pralidoxime.

After inhibition with the diethylcompound (parathion) 50% reactivation of the erythrocytcholinesterase was still possible after 40 hours, and for plasma cholinesterase after 16 hours. For methylparathion and the two other dimethylcompounds the corresponding figures were 6 hours and about one hour.

The faster transformation of the dimethyl phosphorylated cholinesterases to the unreactivatable form means that oxime therapy might not be as successful as in intoxications with diethylcompounds.

Wahlström, G. (Department of Pharmacology; University of Uppsala, Sweden): THE EFFECTS OF REPEATED LONG-TERM TREATMENTS WITH BARBITAL OR ALCOHOL ON A HEXOBARBITAL ANAESTHESIA THRESHOLD IN MALE RATS. 94

To determine the threshold dose hexobarbital was infused in a tail vein with a constant rate (0.25 mg/kg/sec) and as threshold was taken the dose needed to obtain a burst suppression of 1 sec or more in the EEG. After treatment with barbital (200 mg/kg/sec) for 6 weeks there is an increase in the threshold dose which has disappeared within a week (Wahlström, G., Acta Pharmacol. Toxicol. 1968, 26, 64).

In the present experiments a first barbital treatment for 6 weeks was followed by a second similar treatment 3 months later. The treshold dose was recorded at weekly intervals after the treatments. In one experiment the second barbital treatment was substituted by alcohol (10 % w/v) administration in the drinking water for 16 weeks. The rats had access to alcohol only for two one-hour periods each day during the activity.

Compared with the first treatment there was still an increase in the hexobarbital thresholds one week after the end of the second treatment. A new increase was seen three weeks after the second treatment. After the first treatment there was only a trace of a similar increase. If alcohol was substituted for barbital an increase three weeks after the end of the alcohol treatment was also seen. The first barbital treatment thus seems to induce a change which makes the increase in threshold dose after the second treatment more marked. An abstinence period of three months has not restituted this change.

This study has been supported by the Tri-Centennial Fund of the Bank of Sweden.

Åberg, G. and B. Wahlström (Department of Zoophysiology, University of Göteborg, Sweden): VASCULAR EFFECTS OF MEPIVACAINE. 95

Isolated vena porta preparations from rats were contracted when mepivacaine (Carbocaine®) was added to the bath fluid. The frequency of action potentials was increased, but there was no depolarization of the membranes. The spasmogenic effect of mepivacaine was not blocked by α-adrenergic blocking drugs. Tetracaine relaxed vena porta preparations and could even relax vessels that were contracted with mepivacaine The vasoconstrictor potency of L(+)-mepivacaine was much stronger than that of D(-)-mepivacaine.

The vascular effects were also studied in vivo using an electromagnetic flow meter and a thermographic method (IR-camera). The vascular tone in the anaesthetized area might be explained as the result of a balance where the dilating effect of a vasomotor nerve block and the constricting action of the local anaesthetic compound are important.

The vascular effects of local anaesthetics are of great importance for the duration of local anaesthesia. Thus there were statistically significant differences between the duration of the local anaesthetic effects of the optial isomers of mepivacaine in vivo although there were no differences between the nerve blocking effects of these compounds in vitro.

96 Elwin, C-E.(Department of Pharmacology,Karolinska institutet,Sweden): ISOLATION OF GASTRIC SECRETORY STIMULANTS IN PEPTONE FRACTIONATED ACCORDING TO MOLECULAR SIZE.

Bathing the pyloric mucosa with protein hydrolysates causes acid secretion from the parietal cells, probably by release of the antral hormone. This was believed to be due to the large molecules of the hydrolysates (Woodward et al., Gastroenterology 1954. 27. 766). However, no evidence of the specific nature of such molecules has been presented. On the other hand, amino acids and peptides may release the antral hormone (Elwin, C-E. & B. Uvnäs, Gastrin, UCLA Forum Med. Sci.1966.No.5.69)

Peptone (Bacto-peptone, Difco) was separated into two fractions by ultrafiltration through a membrane with the cutoff at 1000 mol.w.(Diaflo, UM-2). Tests of stimulatory power on gastric acid secretion were performed by perfusion of the isolated, innervated antral pouch of Pavlov-pouch dogs. Dose-response curves were done at pH 7 with 4, 8 and 16 % solutions of peptone and corresponding proportions of the fractions with molecules larger or less than 1000 mol.w. Antrum perfusion with solutions of the small molecular fraction in amounts corresponding to 4 and 8 % peptone caused an acid secretory response from the fundic pouch equal to 90-100 % of that of peptone alone, whereas solutions of the larger molecules at these concentrations showed almost no effect. Solutions of the large molecular fraction equal to 16 % peptone showed a clear but much less pronounced effect.

Results indicate that molecules of small size in small amounts of proteins are potent gastric secretory stimulants. The small molecular fraction will be further separated.

97 Svensson, S.-O. and Emås, S. (University Clinic of Surgery, Allmänna Sjukhuset, Malmö, Sweden): EFFECT OF SECRETIN ON HISTAMINE AND PENTAGASTRIN STIMULATED ACID SECRETION IN CONSCIOUS CATS.

Intravenous injection of secretin or acidification of the duodenum inhibits gastrin but does not alter histamine stimulated acid secretion in dogs. It has been suggested that the inhibition of acid secretion by duodenal acidification is mediated by released secretin in dogs. The effect of secretin on histamine and pentagastrin stimulated acid secretion has not been studied in conscious cats. Such studies were therefore carried out.

Cats provided with gastric fistulas of the vagally innervated stomach were continuously infused intravenously with histamine or pentagastrin for 7 hours in doses that produced 50 to 75 per cent of the maximal acid response. After 2 hours' infusion 5 or 20 units of natural secretin per kg body weight was given as a rapid intravenous injection; in control experiments secretin was exchanged for saline. Five and 20 units of secretin per kg did not significantly alter histamine but reduced pentagastrin stimulated acid secretion by 30 and 40 per cent, respectively, in the first post-injection 15-minute period. Secretion remained significantly depressed only for an additional 15-minute period. Regurgitation of duodenal contents might have contributed to but does not explain the entire reduction of acid secretion. It is concluded that secretin is a less potent inhibitor of acid secretion in cats than in dogs. While in progress similar results were reported on gastrin stimulated acid secretion in conscious cats with vagally denervated fundic pouches (Stening et al., Personal communication).

This study was supported by Swedish Medical Research Council (Grant no. B68-17X-2324-01).

Jørgen Gliemann and Henrik Sørensen. (Institute of Medical Physiology C, University of Copenhagen, Denmark): 98 THE BIOLOGICAL ACTIVITY OF PROINSULIN.

Shaw and Chance (Diabetes 17, 737, 1968) showed that pancreatic trypsin inhibitor depressed the biological activity of proinsulin on rat epididymal fat tissue. It was suggested that proinsulin exerts its biological effect because it is converted to an active molecule by proteolytic enzymes present in adipose tissue.

We have investigated the biological activity on isolated rat fat cells of beef proinsulin, prepared and purified by Steiner et al. (Diabetes 17, 725, 1968). The findings were: 1: The dose-response relationships for proinsulin and insulin were parallel. 2: The time course of the action of proinsulin and insulin were identical. 3: The biological activity of proinsulin was about 1 per cent of that of insulin. 4: The activity of proinsulin on fat tissue was not significantly different from that on fat cells. 5: Pancreatic trypsin inhibitor failed to exert a specific depression on the activity of proinsulin on either fat cells or fat tissue.

It is concluded that the biological effect of proinsulin is about 1 per cent (or less) of that of insulin, and that there is no evidence for a conversion of proinsulin to a more active molecule by fat tissue in vitro.

Lybeck, H., Leppäluoto, J. and Virkkunen, P. (Institute of Physiology, University of Helsinki, Finland): 99 EXTRATHYROIDAL THYROXINE FORMATION BY RAT TISSUES IN VITRO.

Several approaches have been made to resolve the problem of whether thyroxine is formed outside the thyroid and, although there is an impressive array of indirect evidence to support this idea, the question remains open. The aim of this investigation is to present direct evidence of thyroxine formation by different rat tissues in vitro. In a series of in vitro and vivo experiments with whole rat thyroid lobes, it was shown that when the lobes were preincubated with I-131 or labelled with this isotope during electrophoresis, they released thyroxine on the paper strip after a suitable running time (Lybeck et al. in press). With the same technique a weighed portion of rat thyroid, salivary gland, stomach, liver or abdominal muscle was placed on the paper strip and 15 mm cathodally of them 1 microC of I-131 was pipetted. The tissue was then submitted to electrophoresis for 2 hrs (veronal buffer, 300 V) and thereafter the radioactive proteins, which the tissue had released onto the paper strip were eluted with NH_3 and chromatographed in BAcW and BAmW. Autoradiography of the chromatograms revealed that the thyroid had released I-131 and thyroxine, but that the radioactive proteins from all the different tissue contained some thyroxine although in lesser degree together with origin material and I-131. These results demonstrate that at least when these tissues were offered I-131 they could form radioactive "extrathyroidal" thyroxine.

100 Carstensen,H.,L.Wide,N.Terner and L.Thorén (Department of Physiology,Biological Institute, Umeå University, Umeå; and the Departments of Clinical Chemistry and Surgery, Academic Hospital, University of Uppsala, Uppsala, Sweden): THE INFLUENCE OF SURGICAL STRESS ON PLASMA TESTOSTERONE AND LH IN THE HUMAN MALE.

It was shown previously that gastric resection in human male subjects was followed by decreased levels of testosterone in plasma during the first 3-4 days and by highly increased levels 8-10 days after the operation (Carstensen,H.,N.Terner,L.Thorén and L.Wide, Acta physiol. Scand. 1968.73.24A). These observations were confirmed in new experiments, except that the late increase was not a regular finding. It was speculated whether the early decrease was related to increased cortisol levels, known to occur during the first days after a major operation. This is unlikely since only slight depression of plasma testosterone was observed during voluntary i-m intake of a high dose of cortisone acetate for 3 days in a healthy subject with high normal plasma testosterone values.

After gastrectomy, plasma LH concentrations showed a steady increase to values above normal 8-10 days after the operation, and did not parallel the urinary excretion, which was earlier reported by us to be high during the first days after the operation and low at the time of the late increase in plasma testosterone concentration.

It is concluded that surgical stress activates LH and testosterone secretion considerably slower than it does ACTH and cortisol secretion.

101 Fredholm, B, and Lindskog, M. (Department of Pharmacology, Research Laboratories, AB Leo, Hälsingborg, Sweden): SOME EFFECTS OF A LONG-ACTING ESTRIOL POLYMER, POLYESTRIOL PHOSPHATE.

It has earlier been shown that polymeric phosphates of estradiol and diethylstilbestrol exert prolonged estrogenic activity. This is assumed to be due to the slow release of the monomer in the body (Diczfalusy, E. et al., Acta Chem. Scand. 1958. 12. 1675; ibid 1959. 13. 1011).

During recent years there has been an increasing interest in estriol, since certain evidence suggests that this compound stimulates the cervix, vagina and vulva more efficiently than estradiol or estrone, while the latter are more effective on the corpus uteri (cf Merrill, R., Physiol. Rev. 1958. 38. 463). Recently a polymer of estriol, polyestriol phosphate (SEP) was described (Könyves, I., Tidskr. Kjemi, Bergvesen og Metallurgi 1965. 12. 288).

In the present work the uterine weight and the morphological changes (proliferation) of the vaginal epithelium of mice was followed during 40 days after a single injection of SEP. Other groups of mice received single or repeated injections of estriol. After SEP, 15 mg/kg, the uterine weight increased from 15 to about 100 mg and remained so for 15-20 days. The duration of the cytological changes in the vaginal epithelium was of the same order.

It is concluded that SEP exerts a prolonged estrogenic activity in the mouse.

Ahrén, K. and L. Hamberger (Department of Physiology, University of Göteborg, Sweden): EFFECT OF CYCLIC 3′, 5′-AMP ON OVARIAN AMINO ACID TRANSPORT. **102**

It has recently been found that adenosine 3′, 5′-(cyclic)-monophosphate (cyclic 3′, 5′-AMP) increases the rate of ovarian glycolysis when added to isolated ovaries from prepubertal rats, and it is possible that this nucleotide mediates at least some of the reported effects of gonadotrophins on ovarian carbohydrate metabolism (Ahrén et al., In "The Gonads". Ed. K. McKerns, New York, 1969).

Studies have now been started to analyse whether cyclic 3′, 5′-AMP might be a factor of importance also for the regulation of amino acid transport and protein synthesis in the ovary. Prepubertal rat ovaries were incubated in Krebs bicarbonate buffer containing the non-utilizable amino acid α-aminoisobutyric acid (AIB-^{14}C) or leucine-^{3}H. Distribution ratios between the intracellular compartment and the incubation medium for the two amino acids were determined after 2 hr of incubation. Addition of cyclic 3′, 5′-AMP as a sodium-dibutyryl salt in a concentration of 10 mM increased the distribution ratio for AIB-^{14}C from 13.7 ± 0.8 to 26.3 ± 0.9 and for leucine-^{3}H from 1.52 ± 0.06 to 2.96 ± 0.12, thus indicating a pronounced stimulatory effect of cyclic 3′, 5′-AMP on ovarian amino acid transport. – Studies are now in progress in order to elucidate whether this effect of cyclic 3′, 5′-AMP has a physiological significance particularly in relation to previously reported effects of follicle stimulating hormone (FSH) on ovarian amino acid transport and protein synthesis (e.g. Ahrén et al., Acta physiol. scand. 1967. 71. 211).

Larsen, J. A. (Institute of Physiology, University of Aarhus, Denmark): THE EFFECT OF HYPOTHERMIA ON LIVER FUNCTION. **103**

It was previously observed that slight cooling of cats anesthetized with chloralose caused a reversible decrease in the elimination rate of glycerol of about 40 per cent. The change appeared to be a all or none reaction which, once evoked, was uneffected by a further decrease in temperature.

In the present experiments the elimination rate of glycerol was followed when the body temperature was changed in steps. Each temperature level was kept constant for at least 45 minutes. The temperature was recorded continously by means of a thermistor placed in the right atrium. Furthermore the oxygen uptake was followed.

It was found that the decrease in elimination rate was initiated between temperatures of 36.0 to 37.5 degrees. The oxygen uptake decreased immediatly upon cooling and was reduced by 6-8 per cent for each degree fall in temperature, corresponding to a Q_{10}-value of about 2. The oxygen uptake was often reduced without any change in the elimination rate of glycerol. In accordance with previous experiments there was no change in liver blood flow and plasma free fatty acids.

The results may be explained by assuming that cooling elicits vasoconstriction within the liver accompanied by redistribution of liver blood flow.

104 Karlsson, B.W. & Carlsson, E.I. (Institute of Zoophysiology, University of Lund, Sweden) RELEASE OF LACTIC DEHYDROGENASE (LDH) AND MALIC DEHYDROGENASE (MDH) FROM SLICES OF RAT MAMMARY GLAND, SKELETAL MUSCLE AND LIVER.

The activities of the isoenzymes of LDH (EC 1.1.1.27) in rat mammary gland and mammary gland secretions change in parallel as lactation proceeds, while those of MDH (EC 1.1.1.38) do not (Karlsson B.W. & Carlsson, E.I. Comp. Biochem. Physiol. 1968.25.949) In an attempt to identify factors which may regulate this secretion of enzymes, the release of LDH and MDH from rat mammary gland, skeletal muscle and liver slices has been comparatively studied.

Incubation for 10-180 minutes at 37°C in protein and/or salt solutions revealed successively increasing activities in the media. One factor responsible for this release might be the ratio between intracellular and extracellular concentrations of the enzyme molecules in the original organ, as higher rates of release were noted in slices from organs with higher initial enzyme activities (cf Schmidt, E. et al Enzym. Biol. Chem. 1966. 7. 185) Another factor is the intracellular localization, because enzyme electrophoresis of the incubation media revealed only negligible activities of M-MDH (the MDH form localized in the mitochondria) while activities of S-MDH (the soluble MDH form) and all the five LD isoenzymes were registrable and found to increase during incubation.

Provided that inhibiting or activating processes did not interfere with the enzyme activities, it is concluded that LDH and MDH may be released from mammary gland in vitro in all essentials in the same way as from liver and skeletal muscle. It should be considered whether or not a similar release may occur in vivo.

105 Aitio,A.and Hänninen,O.(Department of Physiology.University of Turku,Finland):STUDIES ON THE FLUX RATE IN THE GLUCURONIC ACID PATHWAY IN NORMAL AND PHENOBARBITAL TREATED RATS.

Phenobarbital administration causes an induction of some enzymes in the glucuronic acid pathway (Hänninen,O.,Ann.Acad. Sci.Fenn.1968.A2. No 142). In order to study the significance in vivo of the enzyme induction, normal and drug treated rats were loaded with pathway metabolites.- D-glucuronic, L-gulonic and D-glucaric acid and their lactones were injected intraperitoneally in different doses before and during the phenobarbital treatment (80mg/kg daily). The urinary excretion of D-glucaric and L-ascorbic acids was determined daily.

The responses in D-glucaric acid excretion were similar in the normal and treated rats with the exception of D-glucuronic acid load: no increase in the normal rats, but a 2-3 - fold increase in the 3 or 4 days treated rats. L-ascorbic acid excretion was insensitive in both states to D-glucaric acid and lactone. With other metabolites a lower response was found in the treated than in the normal rats.

The 2-4-fold increase of liver uronolactonase and glucuronolactonedehydrogenase can explain the D-glucuronic acid stimulated D-glucaric acid excretion in the treated rats. The inability of D-glucaric acid and lactone to reduce urinary L-ascorbic acid excretion speaks against the role of β-glucuronidase in the biosynthesis of L-ascor.ic acid.

Fredholm, B.B. (Department of Pharmacology, Karolinska Institutet, Stockholm, Sweden): INHIBITION OF FATTY ACID RELEASE FROM ADIPOSE TISSUE BY HIGH ARTERIAL LACTATE CONCENTRATIONS. 106

In various conditions following e.g. administration of catecholamines, during exercise and in shock blood lactate levels can increase from a normal of about 1 mM to 10 mM or more.

The possibility that such changes in arterial lactate concentration might interfere with the release of free fatty acids (FFA) and glycerol from adipose tissue (Issekutz, B., Jr. and H.I. Miller, Proc.Soc. exptl. Biol.Med. 1962. 110. 237) was tested in isolated perfused canine subcutaneous adipose tissue (Rosell, S., Acta physiol.scand. 1966. 67. 343). When the arterial lactate concentration was increased by more than 5 mM the FFA release following sympathetic nerve stimulation was significantly reduced. The lipolytic rate, as estimated by the release of glycerol, was not significantly altered, however. Na-L(+)-lactate thus increased the re-esterification of FFA in adipose tissue. On the other hand, Na-pyruvate significantly reduced the re-esterification.

The evidence indicates that high arterial lactate levels, such that might occur e.g. during exercise, can decrease the release of FFA by increasing the re-esterification.

Fredholm, B.B. and Frisk-Holmberg, M. (Department of Pharmacology Karolinska Institutet, Stockholm, Sweden): RELEASE OF HISTAMINE AND PRODUCTS OF LIPOLYSIS FOLLOWING THE ADMINISTRATION OF COMPOUND 48/80 IN CANINE SUBCUTANEOUS ADIPOSE TISSUE. 107

The release of products of lipolysis in subcutaneous adipose tissue, i.e. glycerol and free fatty acids (FFA), following compound 48/80 was reported recently (Fredholm, B.B., H.C. Meng and S. Rosell, Life Sci. 1968. 7. 1209). Since histamine was shown to affect lipolysis similarly it was hypothesisized that compound 48/80 acted indirectly by releasing adipose tissue histamine, and that this was the lipolytic agent proper. The present study lent further credence to this assumption by showing that histamine is indeed released from adipose tissue by compound 48/80. The amounts of histamine recovered in the venous blood following lipolytic amounts of compound 48/80 were found to be 1-20 times higher the minimal effective dose of injected histamine (0.5μg).

Moreover, the threshold dose of compound 48/80 was the same (5-25μg) for vascular, lipolytic and histamine releasing effects. Upon repeated administration of compound 48/80 the vascular and metabolic responses declined in parallell to a diminished release of histamine. In conclusion subcutaneous adipose tissue mast cells may play a role in fatty acid mobilization via the release of its histamine content.

108 Hänninen, O. and Hietanen, E.(Department of Physiology,University of Turku, Finland): EFFECT OF GASTROINTESTINAL SURGERY ON THE METABOLIC GRADIENT IN THE SMALL INTESTINE OF THE RAT.

The metabolic activity decreases greatly from the oral to the aboral end in the small intestine.This is reflected e.g. in the activity of alkaline phosphatase and ATP hydrolyzing enzyme system (Hänninen et al.,Acta Chem. Scand. 1964.18.937; 1965.19.817). In order to study the role of food and gastric chyme in the formation of this gradient the normal anatomy was disturbed by partial resection (20 cm) of jejunum or by pyloric ligation with gastrojejunostomy. - The activity of the enzymes mentioned above was determined as described earlier from the uncentrifuged aqueous extracts of the mucosal samples.

The activity of alkaline phosphatase remained unchanged at least one month in the distal gut after partial jejunal resection. Also the gastrojejunostomy was without an effect on the enzyme activity in the jejunum. But the activity of ATP hydrolyzing enzyme system increased markedly in this area. On the other hand the jejunal resection did not affect on the enzyme activity in the distal small intestine.

The gradient of alkaline phosphatase appears thus to be independent from the normal passage of the chyme, but the response of ATP hydrolyzing enzyme system indicates that the chyme probably contributes to its control.

109 Mollegaard, K.E. (Veterinary Mollegaard Hansens Avlslaboratorier A/S Ejby, pr. 4623 Ll. Skensved, Danmark): SPF BREEDING AND GNOTOBIOTECHNIQUE.

It has proved possible to effect breeding of SPF animals (Special Patogene Free) in a barrier stable without occurence of bacterial infections. SPF animals are derived from gnotobiotians at the Zentralinstitut für Versuchstierzucht in Hannover.

For the SPF breeding is used a barrier stable of 635 sq.metres, built as a house in a larger house. The barrier stable with its passage facilities, autoclave, formalin lock, immerse tank, supply cylinder, staff lock, and technical aids will be described. As the autoclave treatment causes a destruction of protein, indicated by a fall in biological value (B.V.) of the feed from 85,4 to 82,5 (at 110° C for 10 minutes) and 71 (at 133° C for 10 minutes) the necessity of a feed with sufficient content of protein is mentioned. Especially the loss of vitamins A and B_1 is stated. The breeding method, especially the experience with permanent monogamous breeding is stated.

After mention of bacteriological status of the animals as of July 1, 1968 and May the 1., 1969, the necessary microbiological control arrangements are described. Practical advice is given allowing the investigator to use SPF animals without barrier.

The conclusions: 1) It is possible to breed SPF animals indicated by their bacteriological status by use of barrier stable.

2) Use of SPF animals gives the investigator better results even without use of barriere arrangements.

Pruett, E.D.R. (Institute of Work Physiology, Oslo, Norway): INSULIN, FFA AND GLUCOSE DURING AND AFTER LONG-TERM WORK IN MEN. **110**

In order to investigate the relationship between plasma immunoreactive insulin (IRI), blood glucose and plasma FFA during and after heavy muscular exercise, long-term work experiments were carried out at work loads of 20%, 50 % and 70% of the subjects' maximal aerobic work capacity (max V_{O2}) in healthy young men. The subjects, fasting, exercised alternately on a bicycle ergometer and a treadmill for six hours or until exhausted, whichever occurred first. Venous blood samples were drawn while the subject exercised, and an intravenous glucose tolerance test was administered 15 min after the final bout of work.

Blood glucose fell to an average 60% of the pre-exercise value during work at 50% and 70% of max V_{O2}. Plasma IRI fell during all levels of work, and FFA, inorganic phosphorus and potassium levels increased indicating that insulin activity probably had decreased. Thus, the fall in blood glucose concentration during exercise is due to some factor other than an increased plasma insulin.

Glucose tolerance was found to increase 2-2 1/2 times after the exhausting work which had reduced the body's carbohydrate stores. The increase in plasma IRI levels upon glucose infusion was immediate and independent of the preceeding work load, and the IRI levels followed the glucose disappearance curves closely after heavy work. FFA mobilized during the work disappeared rapidly from the blood after the infusion of glucose and the rise in plasma IRI, but the level rose again after the glucose and IRI had returned to the pre-exercise levels.

N.B. IRI assays by Dr. S. Oseid, University Hospital of Oslo, Norway.

Hermansen, L., E.D.R. Pruett, J.-B. Osnes[1] and F.A. Giere[2] (Institute of Work Physiology, Oslo, Norway): CHANGES IN INSULIN AND BLOOD SUGAR CONCENTRATION IN RESPONSE TO MAXIMAL EXERCISE OF SHORT DURATION. **111**

The adrenal medullary hormones are known to increase the blood sugar concentration and to assist in counteracting the hypoglycemic action of insulin. Maximal exercise is known to elicit an increased release of these amines from the adrenals and/or the sympathetic nerves. The aim of this investigation was to study the effect of intermittent maximal exercise on the insulin and blood sugar levels.

5 healthy subjects, 2 female and 3 male, ran on the treadmill at their greatest possible speed for 5-one minute bouts. Each exercise period was followed by a four minute rest period. Blood samples were withdrawn during and after each work bout via indwelling catheters and were analyzed for glucose and plasma immunoreactive insulin (IRI).

The mean blood glucose increased from 90 mg/100 ml at rest to 170 mg/100 ml immediately following the fifth work bout, while the mean plasma IRI increased from 13 μU/ml at rest to 43 μU/ ml after the fifth work bout. The blood glucose concentration began to increase earlier than the plasma IRI indicating that the insulin rise is due to the increase in glucose.

[1] Norwegian Army Medical Service

[2] On leave from Lake Forest College, Lake Forest, Illinois

116 Forster, H. and K. Klausen. (Pulmonary Function Laboratory, University Hospital, Madison, Wis., and Laboratory for the Theory of Gymnastics, University of Copenhagen, Denmark): VENTILATION DURING EXERCISE AND DURING CO_2 STIMULATION AT SEA LEVEL AND AT ALTITUDE.

The effect of chronic exposure to hypoxia on ventilatory control was investigated in two independent field studies (Leadville, Colo., 3100 m, 10 subjects, and Barcroft Laboratory, Calif., 3800 m, 12 subjects). Ventilation was measured during exercise at sea level (pre and post altitude) and at intervals during acclimatization to these altitudes. It was found that at all intensities of work the ventilation (BTPS) was greater at altitude than at sea level, when the partial pressure of O_2 in the inspired air was the same at the two altitudes (PIO_2 = 105, 145, 400 mmHg). In addition the ventilatory response to CO_2 stimulation, determined on three subjects at sea level and again periodically at 3100 m altitude, was also found to be markedly elevated at altitude. Of the total increases, a large portion was already present after only 18-24 hours of acclimatization, while the remaining increase occurred more gradually over the first two weeks of acclimatization. It is suggested that part of the increase in ventilation at altitude can be attributed to a reduced bicarbonate concentration and that the rate at which the increase in ventilation occurred is indicative of the rate at which the re-adjustments in buffering capacity are made.

117 Sørensen, Sören C. (Cardiovascular Research Institute and Dept. of Physiology, Univ. of California, San Francisco Medical Center, San Francisco, California, U.S.A.): VENTILATORY ACCLIMATIZATION TO CHRONIC HYPOXIA AFTER TOTAL DENERVATION OF THE PERIPHERAL CHEMORECEPTORS.

We earlier demonstrated that man born at high altitude and goats deprived of their carotid chemoreceptors acclimatized to chronic hypoxia (lowered their $Paco_2$) even though hypoxia caused initially either no ventilatory stimulation or ventilatory depression. We wanted to examine if acclimatization would occur with total absence of peripheral chemoreceptors. Chronic denervation of both carotid and aortic chemoreceptors was performed in rabbits. Completeness of denervation was ascertained by testing the ventilatory response to I.V. NaCN. Ventilatory acclimatization to hypoxia was measured as the decrease in $[HCO_3^-]$ in cerebrospinal fluid after the awake rabbit had been exposed to hypoxia (P_B = 470 mm Hg) for 24 hours. Cerebrospinal fluid (csf) was sampled from the cisterna magna immediately after a lethal dose of barbiturate.

	Denervated rabbits		Intact rabbits	
	$[HCO_3^-]_{csf}$	$[lactate]_{csf}$	$[HCO_3^-]_{csf}$	$[lactate]_{csf}$
control	25.4 ± 1.3 (6)		24.1 ± .9 (6)	3.23 ± .33 (6)
24 hr. hypoxia	20.4 ± 1.1 (6)	4.91 ± 1.90 (6)	19.1 ± .9 (7)	3.64 ± .86 (7)

Concentrations are expressed in meq/L ± standard deviation. ( ) = number of animals. The presence of a peripheral chemoreceptor drive is not essential for acclimatization to occur. We propose that lactic and pyruvic acid efflux from hypoxic cells through brain extracellular fluid (ecf) to blood exceeds HCO_3^- influx to ecf, thereby reducing $[HCO_3^-]$ without imposing a stoichiometric relation between decrease in $[HCO_3^-]$ and increase in [lactate] . Supported by grants HE 06285 and GM 09262 from the National Institutes of Health.

Wilkoff, W.G., B. Rasmussen, and S. Kozlowski. (Laboratory for the Theory of Gymnastics, University of Copenhagen, Denmark): THE EFFECT OF HIGH OXYGEN TENSIONS ON VENTILATION DURING SEVERE EXERCISE. 118

The purpose of this investigation was to study the time course of the ventilatory transients caused by changing the arterial oxygen tension during severe exercise in man. This time course was compared with the changes in Pa_{CO_2}, pH and blood lactate concentration during and after the transient period to see if these parameters could explain the changes in ventilation or whether it was due to altered Pa_{O_2} per se.

The full depressing effect on ventilation was obtained in 10 - 20 sec after the shift from a lower to a higher oxygen tension for all gas mixtures used. When shifting from a higher to a lower tension the rise in ventilation took longer and was of a different character. The changes in lactate concentration were too slow to explain the changes in ventilation. The shifts in Pa_{CO_2} and pH were found to be opposite in direction to those one would expect if these factors were causing the ventilatory changes. It is discussed whether the changes in the ventilation can be explained by: 1) an effect on aortic and carotid chemoreceptors, 2) an interaction with anaerobic processes of the muscles, 3) direct effect on the respiratory center, or by 4) some action on the airways and lungs.

119

Balldin, U. and C. Lundgren (Laboratory of Aviation and Naval Medicine, University of Lund, Sweden): THE EFFECT OF IMMERSION ON INERT GAS ELIMINATION.

The greater risk of decompression sickness in wet than in dry dives induced this work. The effects of submersion itself on nitrogen elimination were studied. Nitrogen elimination from the tissues was measured continuously with an electronic nitrogenmeter during oxygen breathing. In the wet experiments the subjects sat immersed to the chin in water with temperatures between 32° and 37°C. During submersion there was a significantly higher rate of nitrogen elimination than under dry conditions (temperature about 29°C). For instance, the amount of nitrogen eliminated was 15 to 70% larger in a 60 minute period of oxygen breathing sitting in water of 35°C as compared to dry conditions. Apart from the effects of temperature an important factor increasing the nitrogen elimination appears to be the submersion itself.

Submersion might influence the ventilation-perfusion ratio in the lungs, cardiac output and its distribution and the rate of transfer of gas between tissue and blood.

Preliminary experiments performed in collaboration with S. Mellander and J. Lundvall showed that intramuscular deposits of ^{133}Xe were cleared at a faster rate from dependent regions during submersion than in dry conditions. This might at least partly be related to the opening up of capillaries increasing the capillary exchange surface. The submersion leads to decreased vascular transmural pressure which, in turn, may reduce the myogenic precapillary sphincter tone normally prevailing in dependent regions.

120 Rusko, H. (Institute of Physiology of Exercise, University of Jyväskylä, Finland): INDIRECT METHODS IN THE ESTIMATION OF MAXIMAL OXYGEN UPTAKE.

Three extrapolation methods (Andersen, K. and Smith-Sivertsen, J., in Yoshimura, H. and Weiner, J.: Human adaptability and its Methodology, Tokio 1966) were compared with other indirect methods (Åstrand's nomogram and the deltaRQ-method). The subjects were 14 male and 18 female students of physical education and 16 conscripts in military service (mean max. O_2-uptake 3,95, 2,55 and 3,59 l/min respectively).

It was found that the three extrapolation methods underestimated the max. O_2-uptake by 1,5-4,3% (±7,3-8,9%). There were no significant differences between the three methods but each of them tended to be the more accurate the higher the max. O_2-uptake. The correlations between these and the direct method varied between .62-.82. Åstrand's nomogram overestimated the max. O_2-uptake by 8,1% (±12,7%). The maximal heart rate was however only 185,7; when this was taken into account the nomogram underestimated the max. O_2-uptake by 3,3%. The correlation between the nomogram and the direct method was .67. The deltaRQ-method overestimated the max. O_2-uptake by 14,7% (±10,1%), and the method tended to be the more accurate the higher the max. O_2-uptake. The correlation with the direct method was .54.

It is concluded that all the extrapolation methods underestimate the max. O_2-uptake, evidently because of the asymptotic relationship between heart rate and oxygen uptake (work load). This asymptotic error tends to be the smaller the higher the max. O_2-uptake. All the extrapolation methods were found to be more accurate than Åstrand's nomogram or the deltaRQ -method.

121 Areskog, N.-H. (Children's Nutrition Unit, Addis Ababa, Ethiopia and Dept. of Clinical Physiology, University of Uppsala, Sweden): PHYSICAL FITNESS IN ETHIOPIAN YOUNG ADULTS RELATED TO NUTRITIONAL STATUS.

The long-range unfavourable effects of poor nutrition - widespread in developing countries - on the functional performance of the body are still unclear. In the present study from Ethiopia 42 low salary workers (W), age 18-25 years, were compared with an optimally nutriated group of 50 cadets (C), age 18-22 years. Nutritional interviews, anthropometric measurements, bicycle ergometry, muscle dynamometry and spirometry with ventilation tests were performed.

The mean weights and heights were for W 54.0 kg and 1.69 m and for C 58.6 kg and 1.70 m. The cadets had gained on an average 5 kg after one year in the air force, indicating the order of magnitude of the difference in body weight between poorly nourished and well-nourished male Ethiopians. Physical work capacity measured as W_{170} was 717.0 ± 117.8 kpm/min for W and 825.0 ± 153.3 kpm/min for C. Also the muscle force tests showed lower values for W than for C. Vital capacity was 4.3 ± 0.6 l for both groups. Maximal voluntary ventilation (MVV) was 166 ± 39 l/min for W and 178 ± 34 l/min for C.

Compared to Swedish age mates the physical fitness is throughout lower for the two Ethiopian groups also as calculated per kilo body weight.

In spite of often very inadequate diets the achievements were on the whole reasonably good which might be due to adaptational mechanisms.

Thomson, D. (Institute of Physiology, University of Lund, Sweden): CARDIAC OUTPUT DURING POSITIVE AND NEGATIVE WORK. 122

Muscle work in everyday life includes both positive (concentric) and negative (eccentric) muscular movements. On an ordinary bicycle ergometer only positive musclework can be done and thus circulatory parameters has almost exclusively been studied during this kind of work. Earlier investigations by Asmussen (Acta Physiol.Scand. 28:364-382,1953) has shown the oxygen consumption to be much greater during positive leg work than during negative work of the same magnitude.

A study of the central circulation has been done on normal healthy young men doing both positive and negative arm or leg work on a special bicycleergometer. Cardiac output was determined with a CO_2-rebreathing method earlier developed in the laboratory. (Recent investigation by Ferguson, et al. J.Appl.Physiol. 25:450-454,1968 has shown an excellent agreement between this method and the dye-dilution method during work conditions).

The oxygen consumption was 3-4 times greater during positive than during negative work. Positive and negative work with the same oxygen consumption were termed equivalent. Cardiac output and heart rate (with the exception of positive arm work) were greater during negative work compared with an equivalent positive work. Stroke volume was greater during positive work. The results show that during negative work heart rate and cardiac output is not governed solely by the demand for oxygen in the tissues. Other factors such as heat removal may control the central circulation in negative work.

Stensvold, I.[1], and L. Hermansen, (Laboratory of Physiology, Norges Idrettshøgskole, Oslo, Norway): THE RATE OF LACTIC ACID REMOVAL DURING RECOVERY WHILE WORKING AND RESTING. 123

It has been known that lactic acid removal is facilitated during recovery by the performance of light exercise. The purpose of this investagation was to study the relationship between the rate of lactate removal and the metabolic rate during the recovery period.

5 trained subjects (3 female and 2 male) were subjected to 3 bouts of exhausting exercise of 1 min duration with a short rest period (4 min) after each bout. Blood lactic acid concentration was found to increase to 145 ± 7 mg% (mean ± S.E.) in the female, and to 205 ± 4 mg% in the male subjects.

After this severe exercise, lactic acid removal was followed during recovery both at rest and during work. The rate of removal was found to be 3.1 ± 0.1 mg%/min (mean ± S.E.) at rest and 5.6 ± 0.4, 7.8 ± 0.8, 7.5 ± 0.6, and 6.3 ± 0.3 mg%/min during the work at 30, 60, 70 and 80% of the maximal oxygen uptake respectively.

On the basis of these results it appears that the maximal rate of lactic acid removal occurs during recovery exercise representing 60 - 70% of the maximal oxygen uptake.

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124 Karlsson J, Diamant B and Saltin B. (Department of Physiology, GIH and department of Pharmacology, Karolinska Institutet, Stockholm, Sweden): PYRUVATE AND LACTATE CONCENTRATIONS IN MUSCLE TISSUE AND BLOOD DURING EXHAUSTIVE EXERCISE:

Excess lactate (XL) was proposed by Huckabee as being a better indication of anaerobic conditions in the muscle than blood lactate determinations since lactate (HLa) accumulated in the blood, should be due not only to an oxygen deficit but also to an increased lactate production from accumulated pyruvate. To study the changes induced by exercise on the relationships between HPy and HLa in muscle tissue and blood, HPy and HLa were determined in muscle biopsy specimens (M. Quadriceps femoris,L) and blood in 4 subjects before and after heavy bicycle exercise, exhausting the subject within 2-3 min. Biopsies were taken within 5 sec after exhaustion.

In the resting muscle HPy was 0.06 mmoles x kg^{-1} wet muscle and increased only to 0.14 mmoles x kg^{-1} after the exercise, whereas HLa increased from 2.4 to 14.0 mmoles x kg^{-1} wet muscle. In the blood the corresponding values for HPy were 0.14 and 0.53 mmoles x l^{-1} and for HLa 1.4 and 10.2 mmoles x l^{-1} respectively. Thus the HLa/HPy ratio increased from 40 to 100 in the muscle and from 12 to 19 in the blood due to the exercise. The results support that the dominant fraction of the HLa in the muscle tissue after exercise is due to an oxygen deficit. A good correlation has previously been demonstrated between the HLa in the muscle and the oxygen deficit.

125 Greenleaf, J,K-E Olsson and B. Saltin.(Department of Physiology, GIH, Stockholm, Sweden): MUSCLE GLYCOGEN CONTENT AND ITS SIGNIFICANCE FOR THE WATER CONTENT OF THE BODY.

Water is bound to glycogen in the liver of different specimen and values of 2-4 g water x g^{-1} glycogen is given. If the same amount of water is bound to glycogen stored in human muscles a significant change in total body water would occur. In one series of experiments the mean value for the muscle glycogen contents (tigh and shoulder) was increased with 15 g x kg^{-1} wet muscle in 19 subjects which may represent approximately 500 g glycogen. With this storage of glycogen total body water (T_2O-space) increased from 45.8 to 48.0 l ($P<0.001$) and body weight from 73.8 to 76.2 kg.

The results seems to demonstrate that a change in the muscle glycogen content of the body causes the body weight to increase significantly more than the amount of stored glycogen and this "extra" increase in weight is due to an enhanced deposition of water. To test wether this glycogen water may have a role in covering parts of sweat losses 8 subjects twice performed prolonged exercise with no water intake. The first time with a low glycogen content in muscles and the second time with filled glycogen stores. Sweat losses were the same but plasma volume was more reduced when the initial glycogen content was low. It is concluded that the deposition of water with glycogen in the muscle cells is of importance during prolonged exercise to reduce the negative effects of sweating.

Kaijser, L. (Department of Clinical Physiology, Karolinska Hospital, Stockholm, Sweden): THE INFLUENCE OF OXYGEN UNDER HYPERBARIC PRESSURE ON THE CAPACITY FOR DYNAMIC FOREARM WORK. 126

The capacity of the circulatory system to transport oxygen is considered one of the main limiting factors for muscle work. During oxygen breathing at increased ambient pressure, the oxygen content of the arterial blood is increased considerably, and hence the oxygen transport capacity is supposed to be augmented.

Six healthy male volunteers were studied during dynamic forearm work breathing oxygen at 3 atmospheres (3 ata) and as comparison breathing air at 1 ata. Blood was sampled from percutaneous teflon catheters in the brachial artery and a deep vein of the active forearm.

At a low work intensity, the a-v O_2 difference was on the average 19.3 ml/l higher during oxygen than air breathing ($p < 0.01$), suggesting about 15 per cent smaller blood flow at corresponding work intensities. When the work was increased the a-v O_2 difference increased during air but not during oxygen breathing. At exhaustion at the highest work intensity there was no significant difference between oxygen and air breathing ($p > 0.05$). In agreement with this the average performance time to exhaustion was not significantly increased ($p > 0.05$) by oxygen breathing.

It is concluded that under normal circumstances the oxygen transport to the muscle is not the limiting factor for aerobic muscle work.

Banister, E.W., J.E. Taunton, T. Patrick, P. Oforsagd and W.R. Duncan. (Hyperbaric Research Unit, Vancouver, Canada and the Laboratories of Aviation and Naval Medicine, Karolinska Institutet, Stockholm). PHYSIOLOGICAL ADJUSTMENTS TO EXHAUSTIVE EXERCISE AFTER PROLONGED EXPOSURE TO OXYGEN AT HIGH PRESSURE (OHP). 127

The physiological adjustments to severe exercise, before during and after prolonged exposure to hyperbaric oxygen have been studied in two subjects. Standard bicycle ergometer work (1500 kpm/min at 100 rpm) was performed either at 1 ATA (air) or 2 ATA (100% O_2). After a two hour period during which oxygen was breathed at 2 ATA the exercise was repeated under the reversed environmental conditions of the first task. Preoxygenation of at least 1/2 hour preceded any exercise breathing oxygen. Lactic acid accumulation, acid-base balance and respiratory gas exchange were measured.

Lactic acid accumulation was significantly reduced as a result of hyperbaric oxygenation. This reduction appeared time dependent being greater after 2 hours oxygen breathing than after a 1/2 hour. Evidence for the potentiation of exercise tolerance in normoxia after prior, long continued hyperbaric oxygenation was equivocal being observed in one subject but not in the other. Arterial P_{aO_2} values decreased progressively from an initial high value throughout the period of oxygenation while P_{aCO_2} was slightly elevated during exercise in OHP over values for equivalent exercise in normoxia. Ambient ventilation and $\dot{V}_{CO_2}$ STPD were significantly depressed in exercise in OHP and $\dot{V}_{O_2}$ STPD and $\dot{V}_{CO_2}$ during exercise in normoxia after oxygenation also reduced.

128 Iisalo,E., Kanto,J. and Pihlajamäki,K.(Department of Pharmacology,University of Turku,Finland): EFFECTS OF FINNISH SAUNA ON CIRCULATION DURING TREATMENT WITH PROPRANOLOL AND GUANETHIDINE

The effects of Finnish sauna on circulation was studied in healthy young students receiving placebo,propranolol (40 mg one hour before bathing) or guanethidine (20 mg twice a day for 4 days).The sauna temperature ranged between 80-93°C.No differences were observed in the rise of sublingual temperature during the treatments.During a 20 minutes'bathing there was no significant change in systolic blood pressure;after the bath there was an immediate fall in all groups,most markedly in the guanethidine group.All groups recorded a slight fall in diastolic blood pressure which in the guanethidine group continued after the bath. Pulse rate in this group was slightly higher than in the placebo group while being much lower in the propranolol group.

In a cold pressor test immediately after the bathing no significant differences were recorded between the groups.

In a work test with a bicycle ergometer after the bath, the increase in systolic blood pressure was smaller both after guanethidine and propranolol as compared to the placebo. The diastolic blood pressure was lower after guanethidine as compared to the other groups.The increase in pulse rate after propranolol was clearly lower as compared to the other groups.

It is concluded that especially hypertensive patients treated with propranolol or guanethidine should be careful in taking sauna baths.

129 Arvela, P., Huikko, M. (Department of Pharmacology, University of Oulu, Finland): EFFECT OF PROPRANOLOL ON PLASMA FFA-LEVELS AND URINARY EXCRETION OF CATECHOLAMINES DURING FINNISH SAUNA BATH.

Exposing the organism to cold or hot environment causes changes in hormonal balance which aim to maintain homeostasis. It is evident that the sympathetic system is activated by hot environment. (Huikko, M. et al: Acta Physiol. Scand., 68 (1966), 316). In the present study 8 medical students served as test subjects. They were subjected twice to the sauna bath and received either 10 mg of propranolol or placebo. Propranolol was effective in preventing tachycardia produced by staying in sauna, but did not clearly affect the blood pressure. Sauna bath induced an increase in the plasma FFA-level and propranolol prevented this increase effectively. In the present study we were able to confirm our earlier finding, that sauna bath increases the urinary excretion of noradrenaline significantly. This effect was more pronounced after propranolol. There was no change in adrenaline excretion after propranolol.

The increased demand for calories is often attributable to the conditions with sympathetic overactivity. However in the case of hot stress this increase seems unlogical and is apparently to be interpreted as an unavoidable consequence of the sympathetic stimulation.

Hermansen, L. and Osnes, J.-B.[1], (Institute of Work Physiology, Oslo, Norway): MUSCLE AND BLOOD pH BEFORE AND AFTER MAXIMAL WORK OF SHORT DURATION. **130**

The aim of this investigation was to study the relationship between muscle and blood pH before and after exhausting exercise of short duration.

Five healthy young men were subjected to maximal bicycle exercise with a work load which would exhaust the subject within approximately 2 minutes. Needle biopsies of the lateral part of the quadriceps muscle were taken at rest, immediately after work, and during the recovery period. The muscle samples were frozen rapidly in liquid nitrogen, homogenized in a cold isotonic KCl/NaCl solution, and the pH of the homogenates measured with a conventional pH meter (Radiometer). Capillary blood samples were taken simultaneously with the biopsies.

Resting pH values of muscle and blood were 6.98 ± 0.04 (mean ± S.E.) and 7.40 ± 0.01 respectively. After the maximal exercise the muscle pH was 6.46 ± 0.05 and the pH of the blood had decreased to 7.18 ± 0.02.

These observations show that the maximal exercise lowered the pH of the muscle by 0.52 units, while the corresponding reduction in the blood pH was 0.22 units. Measurements during the recovery period (40 min) revealed a more rapid increase in the pH of the muscle than of the blood.

[1]Norwegian Army Medical Service

Høvde, R.[1], and L. Hermansen, (Institute of Work Physiology, Oslo, Norway): BODY TEMPERATURE DURING EXERCISE IN CHILDREN. **131**

It is well established that body temperature increases more rapidly in children than in adults in response to infection. This observation might indicate that there is a difference between the body temperature regulation of children and that of adults. The aim of this investigation was to compare the body temperature response to exercise in children to that previously found in adults.

8 boys, aged 10 - 11 years, worked on the bicycle ergometer at three different work intensities demanding an oxygen uptake of 0.58, 0.97, and 1.36 l/min (approximately 30, 50 and 70% of their maximal oxygen uptake) Rectal temperature increased within 45 min from the resting base level to a new plateau, which level was directly proportional to the work load. Rectal temperature at the end of one hour of work at each of the three work loads was found to be 37.4, 37.9 and 38.4°C respectively. On the basis of these results it appears that there is no difference between children (10 - 11 years) and adults as far as body temperature regulation during exercise is concerned.

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132 *Oseid, S., R. Høvde*[1], *J.-B. Osnes*[2], *and L. Hermansen,* (Pediatric Research Institute, University Hospital, and Institute of Work Physiology Oslo, Norway): CIRCULATORY RESPONSE TO PROLONGED EXERCISE IN PREPUBERTAL BOYS.

Very few data have been published concerning the capacity for prolonged work in prepubertal children. Recently it has become common practice for children to take part in rather heavy and prolonged physical exercise. On the other hand, children at school are not especially trained for prolonged work in their physical fitness programs. The aim of this study was to investigate the circulatory response to prolonged severe exercise in 11 year old boys.

23 boys, members of one school class in Oslo, were tested on the treadmill. They ran for one hour at a constant speed which demanded an oxygen uptake of 1.45 ± 0.04 l/min (mean ± S.E.), representing on the average 67% of their maximal oxygen uptake. The heart rate increased from 166 beats/min after 5 min of running to 180 beats/min after 25 min and reached 185 beats/min after 60 min. In separate tests, maximal heart rate was found to be 204 ± 1.2 beats/min (mean ± S:E.) The subjects were not exhausted and completed the work in good clinical condition. On the basis of these results it appears that the circulatory adjustments to heavy prolonged work in children do not differ significantly from that previously found in adults.

[1]Institute of Zoophysiology, University of Oslo

[2]Norwegian Army Medical Service

133 Arfors,K.E., P. Malmberg and K.Pavek (Burn Center, University of Uppsala and Dept. of Experimental Medicine, Pharmacia AB, Uppsala): EVALUATION OF FISHER COMPUTER FOR CARDIAC OUTPUT ESTIMATED BY THERMODILUTION.

Fisher computer allows to obtain direct digital values of cardiac output after injection of thermal indicator into the right atrium or pulmonary artery and detecting the dilution process in the aorta (Slama and Piiper, Z.Kreisl. forsch. 1964. 53. 322). This principle was not, however, verified for very low output values and for left atrial injections of the indicator. Therefore the computer was checked with simultaneous measurements using the Fick O_2 principle and independent recordings of the thermodilution curves, which were integrated and output calculated manually. The experiments were done in anesthetized closed-chest dogs. The values given by computer correlated well with the Fick values in a broad range of flow (1.0 to 6.0 l/min) and, in the mean, there was no significant difference. In the individual dogs, however, the successively recorded values were often systematically different, showing either higher or lower values with one of the methods. Similarly, with manually evaluated thermodilution curves, there was very high correlation (r=0.96). Finally, using the computer after alternative injections into the PA and LA showed no systematic difference and high correlation (r=0.86). The computer may be used with indicator injections both into pulmonary artery and left atrium in the given flow range, however, systematic differences in individual animals have to be expected.

134

Arfors, K.E., P.Malmberg and K.Pavek (Burn Center, University of Uppsala and Dept. of Experimental Medicine, Pharmacia AB, Uppsala): THERMODILUTION TECHNIQUE: LOSS OF THE INDICATOR IN THE LUNG CIRCULATION.

The substantial part of the criticism of the cardiac output estimation by thermodilution method is the possibility of an irreversible exchange of "cold" between the site of injection and detection. This has been studied many times in animals including man by comparing outputs after left and right heart injections. No, or slight difference has been found within the normal range of the cardiac output. However, it seems more probable, that the loss might occur only with low values of the output. To investigate this phenomenon limited to the segment between the pulmonary artery (PA) and left atrium (LA), the indicator has been injected alternatively into these sites in dogs and the dilution was detected in the descending aorta. The paired values showed very high correlation (r=0.978) within the flow range of 1.0-5.0 l/min. The mean output (LA) was 14% higher when compared to the output (PA). One S.D. of differences between the pairs was 8.9% of the mean value. Thus, no loss was observed. The regression line showed that with lower values of output the right site was giving progressively lower values. This might be caused by an increase of the area by unrecognized recirculation. With the flow of approximately 0.7 l/min the pulmonary injections resulted in very prolonged downslope, making the estimation of the area impossible. On the other hand, the curves after LA injections showed a reasonable downslope.

135

Pavek,K. and K.Karovic (Pharmacia AB, Uppsala and the Inst. for Theory of Measurement, Academy of Sciences, Bratislava, Czechoslovakia): EFFECT OF INDICATOR VOLUME ON ESTIMATION OF CARDIAC OUTPUT BY THERMODILUTION.

The injection of thermal indicator either diminishes the inflowing blood flow at the site of injection by the volume of the injectate and we observe that the total flow rate is unchanged downstream, or, in the second extreme case, the volume of the injectate is added to the inflowing blood and downstream we find unchanged blood flow plus the volume of the indicator. In the first case it is appropriate to calculate the output/min - $Q_b = 60\ V_i \cdot (T_b - T_i)/0\int^{\infty} (T_b - T)(t)\,dt$; in the second case the mathematical solution can not be used for the calculation (Pavek, K. et al., Arch Kreisl. forsch. 1969,58. 228), but the situation may be approximated by $Q_b \approx [60\ V_i \cdot (T_b - T_i)/0\int^{\infty} (T_b - T)(t)\,dt] + 60\ V_i\ (\beta - 1)$, where $\beta = 0$. However, in real situation β may be ≥ 1. To estimate this, the thermal indicator was repeatedly injected into the left atrium and dilution detected in descending aorta in dogs with closed and opened chest. The volume of indicator was changed in steps of 4, 8 and 15 ml and was injected in two seconds. The resulting output was calculated using the first equation. The results showed slight increase with increased volume of indicator; thus the value of empirically estimated beta is approximately 0.6 to 0.9.

136 Öberg, P.Å. and U. Sjöstrand (Institute of Physiology and Medical Biophysics, Biomedical Centre, University of Uppsala, Sweden): CONTROL OF SYSTEMIC BLOOD PRESSURE BY CAROTID-SINUS NERVE STIMULATION.

Chronic carotid sinus nerve stimulation as a clinical method for the treatment of essential hypertension has successfully been used in a limited number of patients by Schwartz et al. (Am.J.Surgery 1967.114.5) and also by Bilgutay and Lillehei (J.A.M.A.1965.191.649). However, the studies referred were not based upon studies of the fundamental properties of the regulatory system interfered with.

A method has been worked out by which one of the closed control circuits in the blood-pressure-regulating system of the dog can be reversible opened and closed again with the aid of an on-line analog computer. This makes it possible to study the regulatory properties of the carotid-sinus reflex.

By studies in both time and frequency domains (Öberg, P.Å. and U. Sjöstrand, Acta physiol. scand. 1969.75.287) it is concluded that the electronic analog can be given optimal characteristics producing specified stability and systemic pressure level conditions. These results can be used as direct guidance in both the technical and clinical development of anti-hypertensive therapy by means of electronic pressure-regulating devices.

137 Akre, S. and H. Aars (Institute for Experimental Medical Research, University of Oslo, Norway): SYMPATHETIC RESPONSES TO CHANGES IN SYSTEMIC BLOOD PRESSURE.

Over a wide pressure range arterial baroreceptor activity is linearly related to blood pressure. The associated changes in efferent sympathetic nerve activity have been only incompletely studied. We have quantitatively recorded the activities in left aortic and left renal nerves in anesthetized rabbits. Control diastolic blood pressure ranged from 80 to 100 mmHg. Increasing the systemic blood pressure by noradrenaline infusion (1-6 μg/kg·min) or by inflation of a balloon in the descending aorta sharply reduced the sympathetic activity. Above 120 mm Hg the activity was usually completely abolished. In contrast, sympathetic activity showed only a small rise or remained unchanged in response to a reduction in blood pressure by bleeding. Aortic nerve activity was linearly related to pressure above 60 mm Hg. The small sympathetic response to reduced blood pressures was not due to a high activity at control pressure because sympathetic activity was capable of increasing 100-200 % during hypoxia. It is concluded that the baroreceptor reflex offers a better protection against a rise than a fall in blood pressure.

Hirvonen, L., J. Klossner and E. Länsimies (Cardiorespiratory Research Unit, Department of Physiology, University of Turku, Finland): CIRCULATORY RESPONSE TO HYPOXIA OF NEWBORN LAMB TREATED WITH ATROPINE OR ALPRENOLOL. 138

Nervous regulation of the blood circulation may be of importance for the survival of a newborn in hypoxia. A partial block of the autonomic system was caused by atropine or alprenolol (beta-blocking agent) in anesthetized newborn lambs. The animals were curarized and ventilated with a constant minute volume. Anoxic hypoxia was produced by the use of a low-pressure chamber.

Systemic arterial pressure varied within narrow limits in all groups. Pulmonary arterial pressure increased in control and atropine series. Only insignificant changes were observed in alprenolol-treated animals. Pulse pressure decreased in extreme conditions. Cardiac output remained practically unchanged in all groups. In these experimental conditions the acid-base balance of the blood did not correlate with the cardiac output. Acetylcholine decreased temporarily the systemic arterial pressure in all lamb groups equally at all stages of hypoxia. It decreased the pulmonary arterial pressure if elevated by hypoxia. Pressure changes caused by acetylcholine were not correlated with acid-base balance. The fact that the differences between the various lamb groups were small suggests that the autonomic nervous system has a secondary role in the adaptation of the newborn lamb to hypoxia.

Bolme, P. and J.Novotný (Department of Pharmacology, Karolinska Institutet, Stockholm, Sweden): ACTIVATION IN THE CONSCIOUS DOG OF THE SYMPATHETIC CHOLINERGIC VASODILATOR NERVES AND A PROPOSAL OF THEIR PHYSIOLOGICAL SIGNIFICANCE: 139

In conscious dogs hind limb blood flow was measured by an electromagnetic flowmeter with a probe fitted on the external iliac artery. Arterial pressure and heart rate were also recorded. Blood flow increase was produced in the hind limb as a conditional reflex response to either electrical skin stimulation or exercise on a treadmill. The blood flow increase was abolished or reduced by atropine ia. In addition a tachycardia, a slight increase in arterial pressure and an alerting reaction were elicited. The response to the conditional stimuli was similar to that observed in conscious dogs following electrical stimulation of sympathetic cholinergic vasodilator pathways in the brain (Bolme et al.Acta physiol.scand. 1967.70.334-346). No habituation to the conditional stimuli was observed.

The results indicate that the sympathetic cholinergic vasodilator nerves are activated in anticipation of situations in which increased skeletal muscle blood flow is suddenly required It is proposed that the role of the vasodilator nerves is not to regulate the local muscular blood flow and the supply of oxygen or substrates to the muscle, but rather to take part in a general homeostatic mechanism working to adjust the arterial pressure when blood is suddenly redistributed from the skin and viscera to the muscles.

140 Karppanen, H., Vapaatalo, H., Lahovaara, S., and Paasonen, M.K. (Department of Pharmacology, University of Helsinki, Finland): PINEALECTOMY-INDUCED HYPERTENSION IN RATS.

It has been demonstrated that pinealectomy (PE) causes hypertension in rats (Zanoboni, A. and Zanoboni-Muciaccia, W., Life Sci. 1967.6.2327). In this study the mechanism of PE induced hypertension and other phenomena were examined. A simplified PE method was developed by using electrocauterization through the skull. The body weight of PE animals increased slower than that of the sham-operated or control animals. The motility of PE rats was higher than that of the controls both in light and dark and their diurnal rhythm was disturbed. The blood pressure of PE rats was increased 1 week after PE and remained elevated up to 7 weeks. There was no consistent correlation between the plasma renin activity and the blood pressure increase. Na^+ and K^+ contents in serum were slightly lowered in PE rats, but their excretion in urine was normal. Angiotensin (20 ng/kg ip) and noradrenaline (20 µg/kg ip) caused smaller pressor effect in PE rats than in the controls. The brain noradrenaline concentration was decreased in PE rats 3 but no more 5 months after operation. The catecholamine contents of heart, spleen, kidney and adrenal glands were not changed. - The possible correlation between the changes observed and the hypertension is not known. (Supported by the P.I. Ahvenainen Foundation, the Heart Association and Sigrid Jusélius Foundation.)

141 Folkow, B., M. Hallbäck, Y. Lundgren and L. Weiss (Dept. of Physiology, University of Göteborg, Göteborg, Sweden): STRUCTURALLY BASED INCREASE OF FLOW RESISTANCE IN SPONTANEOUSLY HYPERTENSIVE RATS.

In essential hypertension in man, flow resistance in forearm or hand is raised largely in proportion to resting arterial pressure, even when the vascular smooth muscles are completely relaxed (e.g. Folkow, B. and R. Sivertsson, Life Sci. 1968. 7. 1283-1289); also the responses to noradrenaline suggest the presence of a morphological vascular adaptation in the form of an increased wall/lumen ratio (Sivertsson, R. and R. Olander, Life Sci. 1968. 7. 1291-1297). - In the present study, the entire systemic circuit (except for the coronaries) of normotensive and spontaneously hypertensive Wistar rats (SH/N strain[x]) was perfused with plasma substitutes. Pressure-flow curves for the maximally dilated vessels were plotted and PRU_{100} for identical pressure levels was calculated. - In the hypertensive rats, systemic flow resistance was increased largely in proportion to the raised arterial pressure even during maximal vasodilatation, the difference to the normotensive rats being highly significant. This suggests that the entire systemic vascular bed is structurally adapted in this type of chronic hypertension to such an extent that the morphological change of the resistance vessels might entirely account for the raised pressure level during rest.

[x]Generous gift from the Genetics Unit, National Institute of Health, Bethesda, USA.

142

A.Pekkarinen,K.Kätkä and T.Suramo (Department of Pharmacology, Univeraity of Turku,Finland): THE EFFECT OF ANTIHYPERTENSIVE DRUGS AND DIURETICS ON THE DEVELOPMENT OF EXPERIMENTAL COMBINED DESOXYCORTICOSTERONE AND NACL HYPERTENSION OF RATS

Experimental hypertension was developed in rats by daily injections (s.c.) of desoxycorticosterone acetate (DOCA)(Schering) (5 mg/kg) and 2% NaCl in drinking water.The mean blood pressure increased in 6 control groups from 111.0+2.1-116.5+1.3 mmHg in mmHg in 63-118 days with +66-+69 mmHg.(I)This increase was during the combined treatment with furosemide (Hoechst) and phentolamine (Ciba) p.o.(+13 and +28 mmHg),dibenzyline (SKF) and furosemide (+28 mmHg);phentolamine and hydrochlorothiazide (+34,5 mmHg),and with dibenzyline and hydrochlorothiazide(+32) highly significantly less than that of the control group.The combined treatment with hydrochlorothiazide and reserpine,or with furosemide and reserpine prevented the increase of BP (+33 and +31 mmHg) very highly significantly as well as that with phentolamine and α-methyldopa or guanethidine (+34 and +35 mmHg),furosemide with α-methyldopa(+38.5)hydrochlorothiazide with guanethidine or α-methyldopa(+40.5 and +44.5 mmHg).(III) A single diuretic or antihypertensive drugs were not as effective as their combined treatments(hydrochlorothiazide +47,furosemide +47 and +55,Hygroton® +55,dibenzyline +44,phentolamine +47,reserpine +37 add +51,guanethidine +51,guanoxane +45,bethanidine +50,phentolamine +47, α-methyldopa +43,hydralazine +57,Catapresan® +58 and chlorprothixene +58 mmHg.)

143

A.Pekkarinen,K.Kätkä and T.Suramo (Department of Pharmacology University of Turku,Finland):THE EFFECT OF ANTIHYPERTENSIVE DRUGS AND DIURETICS ON THE DEVELOPMENT OF EXPERIMENTAL COMBINED NORADRENALINE AND NACL HYPERTENSION OF RATS.

The experimental hypertension was developed in rats by daily adsorbtion of noradrenaline bitartrate (Winthrop 0.5 mg as base/ml glycerol)percutaneously from the tail and 2% NaCl in drinking water. Mean blood pressure increased in 5 NaCl and noradrenaline control groups from 110+1.8-117.5-1.7 mmHg to the maximum +35.0 and +40.0 mmHg above the initial basal level of BP in 110-192 days.With simultaneous daily treatment with hydrochlorothiazide (+13 and +14 mmHg),furosemide (+10, +10,0 and +14.5 mmHg) or reserpine (+9.0,+9.0 and+13.0 mmHg) in drinking water, the mean BP was highly significantly lower, approximately only 1/3 of that of noradrenaline NaCl control groups as well as during the combined treatment with hydrochlorothiazide and reserpine (+10.6 mmHg), or furosemide and reserpine (+10.0 mmHg).

Among other antihypertensives guanethidine (+10.0,+17.0, +22.0 mmHg),bethanidine (+16.0 mmHg),guanoxane (+17.0 mmHg), and α-methyldopa (+13.0, +20.0 and +26.3 mmHg),hydralazine (+21.0 mmHg) a diuretic Hygroton® (+24.0 mmHg), and chlorprothixene (+22 and +24.0 mmHg) showed a smaller final increase of BP than in the control group with hypertension,except in one group of guanethidine, α-methyldopa,phentolamine and chlorprothixene and the Catapresan® group (+27.0 mmHg).

144 A.Pekkarinen,K.Kätkä and T.Suramo (Department of Pharmacology, University of Turku,Finland): THE EFFECT OF ANTIHYPERTENSIVE DRUGS AND DIURETICS ON THE DEVELOPMENT OF EXPERIMENTAL COMBINED ADRENALINE AND NACL HYPERTENSION OF RATS.

Experimental hypertension was developed in rats by daily adsorbtion of adrenaline (0.5 mg/ml glycerol) percutaneously from the tail and 2.0% NaCl in drinking water.Mean blood pressure increased in the two control groups of 10 rats from 116.1±1.4 and 117.2±1.5 to 140.5±1.5 mmHg in 140 days with a slight tendency to decrease later.(I) During simultaneous daily treatment of peroral antihypertensive drugs or diuretics in drinking water,the mean maximum BP was slightly,but very highly significantly lower than in the adrenaline and NaCl control group in the phentolamine (+9.8 mmHg),furosemide(+14.0 mmHg),reserpine (+15.0 mmHg),bethanidine (+14.5 mmHg) groups(= above the initial level), and generally also very highly significantly lower in the guanethidine (+13.5 mmHg) and guanoxane (+13.5 mmHg) groups, except at the one point of maximum (II) BP was only very slightly, but significantly less during the simultaneous hydrochlorothiazide (+18.5 mmHg),α-methyldopa (+19.5 mmHg), and chlorprothixene treatment (+19.5 mmHg). (III) The mean maximum BP was nearly the same as in the control group, in hydralazine (+20.5 mmHg), Hygroton® (+21.8 mmHg), Aptin® (+21.5 mmHg),Aldactone® (+20.5 mmHg) and Cataprese® -groups (+25.0 mmHg).

145 Folkow,B., B.Löfving and B.Öberg (Dept. of Physiology, University of Göteborg, Göteborg, Sweden): FACTORS INFLUENCING BLOOD VISCOSITY IN VIVO AT VERY LOW FLOW RATES.

Recent studies on cats show that blood viscosity in vivo, being throughout much lower than in vitro, increases only moderately with flow reductions and even decreases at the very lowest flow range (Ojosugito et al. Acta physiol. scand. 1969. In press), in contrast to the marked increase of viscosity in vitro at low shear rates. - The mechanism responsible for the decrease of viscosity in vivo at low flows was analyzed in cats, using the maximally dilated calf muscle vessels as biological viscometer. Pressure-flow curves for blood and a Newtonian fluid (dextran-Tyrode) were compared in the lowest flow range, both when vascular passive-elastic recoil occurred with reductions of pressure head and transmural pressure, and when the vessels were maintained distended by keeping transmural pressure high throughout. - While blood viscosity decreased at very low flows when pressure-passive reductions of vascular lumina took place, prevention of such luminal reductions led to marked increases in blood viscosity at corresponding flows, the difference at a given low flow being up to threefold. - The earlier reported viscosity decrease at very low flows therefore seems to be a consequence of the vascular narrowing favouring the appearance of the Fåhraeus-Lindqvist phenomenon and bolus flow. This dependency of blood viscosity on vascular dimensions also at very low flows may have important implications. Thus, some reduction of apparent viscosity may be expected with intense vasoconstriction, while an increase would occur e.g. with severe venous stasis or in dependent limbs with low pressure head due to arterial obstruction.

Håkansson, B.A.S., Östling, S.G. and Zade-Oppen, A.M.M. (Institute of Physiology and Medical Biophysics, University of Uppsala, and Department of Clinical Chemistry, University Hospital, Uppsala, Sweden): THE NON-AFFINITY OF THE RED CELL SURFACE FOR DEXTRAN. 146

The effect of dextran on agglutination of red cells and platelets has been much studied. The associated problem of dextran adsorption by the cells has, however, been somewhat neglected.

A coating material, rendering the cells sticky has been described. (for references see: Swank, R.L., Appl. Physiol. 1958.12.125; Lee, W.H., in: Conference on Evaluation of Low Molecular Weight Dextran in Shock. Nat. Acad. Sci.-Nat. Res. Council, Washington, 1963, p.40) It has been suggested that the material is a dextran-protein complex. An adsorption of 2400 to 8900 dextran molecules per cell, using end-group labelled C-14-dextran, has been reported (Rothman, S. et al., Vox Sanguis. 1957.2.104).

We have measured the recovery of generally labelled C-14-dextran from the supernatant fluid of red cell suspensions in isotonic saline and plasma. Two dextran fractions with molecular weight distributions comparable with clinical dextrans were used. Dextran was added at concentrations ranging between 0.002 and 0.050 g/l. After correction to a standard hematocrit and for the interstitial volume the corrected concentrations after incubation were a linear function of the initial concentrations with a slope greater than 0.93, and the line passed through the origin.

From our results we conclude that dextran adsorption is improbable.

Bø.G. and A. Hauge (Institute of Physiology, University of Oslo, Norway): HYPEROSMOLALITY AND PULMONARY VASCULAR RESISTANCE. 147

It has been demonstrated that venous blood from exercising muscles has an elevated plasma osmolality (Mellander, S. & al. Angiologica 1967.4.310), and several investigators have reported that blood hyperosmolality is a potent pulmonary vasoconstrictor stimulus (Aviado, D.M. The Lung Circulation, Oxford 1965 p.302). This last effect appears to be teleologically disadvantageous. We therefore decided to examine the effect of graded rises in pulmonary arterial osmolality in a lung lobe preparation. In 15 anesthetized openthorax cats ventilated by positive pressure we recorded pulmonary arterial, left atrial and femoral arterial pressures. Left lower lobe arterial blood flow was measured by electromagnetic flowmeter. 2 M solutions of glucose and urea, and 1 M of mannitol and NaCl were infused at varying speed into the lobar artery over periods of 1 min. Knowing pre- and postlobe vascular pressures, lobe flow and hematocrit we calculated lobar vascular resistance (LVR) and changes in plasma osmolality. In all cats the hyperosmolar solutions reversibly reduced LVR (flow increased). The opposite effect was not seen. Reproducible dose-response curves were found. +20 mOsm/l plasma gave a mean LVR fall of 5% and +50 mOsm/l gave a 10% fall. A maximal fall of 30% could be obtained by elevating plasma osmolality by 100 mOsm/l. Bolus injections caused a rapid rise in LVR. This type of response, which may have been caused by sludging of blood, is possibly the one studied by previous investigators.

148 Lundvall, J. (Institute of Physiology, University of Lund, Sweden): FURTHER STUDIES ON REGIONAL HYPEROSMOLALITY IN RELATION TO EXERCISE HYPEREMIA.

The hypothesis that increased osmolality in the interstitial space of exercising muscle contributes to exercise hyperemia (Mellander et al. Angiologica 1967. 4. 310) was further tested in cat and man. I.a. hypertonic infusions to resting cat muscle caused progressive increases of blood flow. Maximal dilatation was reached when venous osmolality, reflecting interstitial tonicity, was raised by 40-100 mOsm/kg.

Similar experiments on the intact human forearm in which venous osmolality could be raised by 15-20 mOsm/kg led to a 3-to 5-fold increase of flow. Since exercise in cat and man results in regional hypertonicity of up to 30-40 mOsm/kg the data indicate a quantitatively important role of hyperosmolality in exercise hyperemia.

During hypertonic infusion arterial osmolality exceeded venous osmolality. Therefore possible transintimal effects on arteriolar smooth muscle would lead to overestimation of the role of hyperosmolality since flow has been related to venous osmolality. To test this possibility the vascular bed of the gut which also dilates during hypertonic infusion, was perfused in retrograde direction. In situations when control resistance was the same with forward and retrograde perfusion, hypertonic infusion gave about equally large dilatations at comparable hypertonicity in the outflows, despite intraluminal osmolality on the arterial side was higher with normal than retrograde perfusion. This seems to refute any significant transintimal effects of hyperosmolality, a conclusion which might be relevant also with regard to the site of action of other blood-borne vasoactive agents.

149 Eriksson, E. and B. Lisander (Depts of Physiology and Anatomy, University of Göteborg, Sweden): INTRAVITAL MICROSCOPY OF MUSCLE BLOOD FLOW IN CATS.

Only few microcirculatory studies of nervous and metabolic blood flow changes in skeletal muscle have been reported. Therefore, a method for intravital study of the tensor muscle of the crural fascia was developed, volume blood flow being judged by simultaneous recordings in adjacent muscles. The total muscle region was exposed to graded constrictor fibre stimulations or intraarterial vasodilator drug administrations during observations of internal arterial diameters, linear flow rates and capillary flow distribution. - Dilatations giving maximal flow increases caused some 50% diameter increases mainly in arteries of 15-100μ with less change in larger ones. No downstream vascular widening, or opening of "sphincters", was seen. - During "rest" the denervated vessels displayed a fairly steady tone; precapillary rhythmicity was not seen though capillary flow patterns changed perpetually, contents being stagnant or flow reversed, sometimes apparently due to cells passing the loops. - Intense vasoconstrictor fibre activations, which lowered flow 6-8fold, reduced diameters in arteries of 15-300μ, with a clear maximum at the 50μ size, where closure could occur; luminal reductions were absent in immediate precapillary sections. However, capillary flow ceased at such stimulations to return after 30 sec at prolonged stimulation, often in a rhythmic pattern. - These microcirculatory events seem to agree well with "macro circulatory" findings, although the sites of precapillary adjustments, leading to changes in resistance and capillary flow distribution, may not be distinctly separated morphologically.

Biber,B., O.Lundgren and J.Svanvik (Dept. of Physiology, University of Göteborg, Sweden): AN INDICATOR-DILUTION TECHNIQUE FOR STUDYING MEAN TRANSIT TIME AND BLOOD FLOW IN THE MUCOSA-SUBMUCOSA OF THE SMALL INTESTINE **150**

The study of blood flow in the mucosa-submucosa of the small intestine by means of diffusible tracers (e.g. krypton, xenon) is complicated by the existence of a counter-current exchange in the intestinal mucosa (Lundgren, O., Acta physiol. scand. 1967.suppl.303). An indicator-dilution technique using nondiffusible (intravascular) tracers has therefore been developed. - A cylindrical Geiger-Müller tube is placed in the intestinal lumen in close contact with the mucosa. The radioactive tracer is administered in a small volume by an "instantaneous", close intra-arterial injection. Mean transit time is calculated from the registered indicator-dilution curve. Regional blood volume is estimated as described by Wolgast (Wolgast, M., Acta physiol. scand. 1968. suppl. 313) from the radioactivity registered by the detector when the tracer has become uniformly distributed in the total blood volume. Total intestinal blood flow is continuously recorded by a drop recorder unit. - Experiments have been performed in which ^{32}P-labelled red blood cells have been used as the intravascular tracer. A 2-3 mm thick tissue layer around the detector is monitored. When total intestinal blood flow amounted to approximately 50 ml/min x 100 g, mean transit time (area under curve divided by height of curve) was estimated to 3-4 sec, relative blood volume to 10-20 % and blood flow to 200-400 ml/min x 100 g. During intense vasodilatation (total intestinal blood flow around 200 ml/min x 100 g) mean transit time was calculated to 1-2 sec and blood flow to 800-1200 ml/min x 100g.

Lundborg, G. (Lab.of exp.biology, Dept.of Anatomy, University of Gothenburg) **151**
STUDIES ON MICROVASCULAR REACTIONS OF RABBIT'S TIBIAL NERVE AFTER PROLONGED ISCHEMIA.

Using vital microscopic observation techniques (Brånemark, Med.Biol.Ill. XVI:2,100,1966; Lundborg and Brånemark, Adv.Microcirc. 1, 66,1968) the microvascular bed of rabbit's Tibial nerve has been studied in vivo under various experimental conditions. By inflating a tourniquet around the thigh one leg was kept ischemic for certain periods of time, and after release the restitution of intrafascicular, nutritive capillary circulation was studied. By stimulating the nerve at the ankle and registrating the elicited compound action potential at knee level the nerve function could be tested. Permeability disturbances were estimated by studying the endoneural distribution of a fluorescing "tracer", intravenously infused. Ultrastructural analyses were performed on endothelial cells and on structures involved in impulse transmission.

After ischemic periods of 6-8 hours there was a complete restitution of the endoneural microcirculation within the first minutes after release. Even after 12 hours ischemia the intraneural circulation was rapidly restored to a considerable extend.

The first visible signs of an endoneural oedema occurred after ischemic periods of 8-10 hours. In case of 2, 4 or 6 hours ischemia the maximum conduction velocity of the nerve was restored to original values within 5, 10 and 30 minutes resp. After 8 hours ischemia nerve activity could be registered only in exceptional cases. Long term studies have been performed in order to analyse the restitution capacity of neuromuscular structures considering the survival and function of the limb.

152 Delin, N.A. (Thoracic Surgery Research Laboratory, Karolinska Sjukhuset, Stockholm, Sweden): INFLUENCE OF EXPERIMENTAL VALVULAR AND SUPRAVALVULAR AORTIC STENOSIS ON INSTANTANEOUS CORONARY ARTERIAL BLOOD FLOW.

Acute supravalvular aortic stenosis (SVAST) was produced in dogs by constriction of the ascending aorta. Valvular aortic stenosis (VAST) was simulated by constriction of the ascending aorta and perfusion of the left coronary artery from a point distal to the constriction. Instantaneous flow was measured by an electromagnetic flowmeter on the circumflex branch of the left coronary artery ($\dot{Q}$ cor). During SVAST or VAST mean $\dot{Q}$ cor and myocardial O_2 consumption were increased while coronary arterio-venous O_2 difference remained unchanged. In VAST the increase in mean $\dot{Q}$ cor consisted in an increase in diastolic $\dot{Q}$ cor, systolic $\dot{Q}$ cor remaining low as in the control state. In SVAST there was a marked increase in systolic $\dot{Q}$ cor, accounting for the whole mean $\dot{Q}$ cor increase, and systolic and diastolic $\dot{Q}$ cor were equal Acute circulatory block by coronary infusion of Lycopodium spores reduced systolic and diastolic $\dot{Q}$ cor equally, leaving the pressure oscillations in the coronary artery unaffected. Thus in SVAST there is a large flow into the microcirculation during systole The low systolic $\dot{Q}$ cor in VAST is expected from the high myocardial tension and the low coronary pressure. The high systolic $\dot{Q}$ cor in SVAST is unexpected, for although the coronary arterial pressure is increased the myocardial tension is equally increased. It is concluded that myocardial contraction may constrict vessel lumina less than commonly believed.

153 HÄggendal, E. and Inger Winsö (Departments of Clinical Physiology and Anesthesiology II, University of Göteborg, Sweden): THE INFLUENCE OF THE ARTERIAL CARBON DIOXIDE TENSION ON THE CEREBROVASCULAR RESPONSE TO HYPOXIA.

It has been shown that cerebral vasoconstriction induced by hypocapnia is counteracted by the tissue hypoxia which exists when the cerebral venous oxygen tension has reached around 20 mm Hg - The purpose of this study was to investigate if the arterial carbon dioxide influences the "threshold" of the hypoxic dilation of the cerebral vessels.

The study was performed in anesthetized dogs. When the animals were normoventilated and the arterial oxygen saturation was lowered cerebral vasodilation occurred when the cerebral venous oxygen tension was about 30 - 35 mm Hg. During pronounced hyperventilation vasodilation consistently occurred at a lower venous oxygen tension (around 20 mm Hg).

It is concluded that the effect of hypoxia on the cerebral vessels is influenced by the carbon dioxide tension. During excessive hyperventilation the oxygen tension will reach significantly lower values until the hypoxic vasodilation appears than during normocapnia. A hypothetical explanation would be that carbon dioxide and oxygen act by similar mechanisms on the vessels (e.g. hydrogen ion concentration of the extracellular fluid around the smooth muscle cells). During respiratory alkalosis there will be needed more metabolic (hypoxic) acidosis in order to reach the same hydrogen ion concentration as in normocapnia and affect vasodilation.

Ekström-Jodal, B., Häggendal, E., Nilsson, N. J., and Norbäck, B. (Departments of Clinical Physiology I and II, Anesthesiology II and Surgery III, University of Göteborg, Sweden): INFLUENCE OF METABOLIC FACTORS ON THE CEREBRAL PRESSURE-FLOW RELATION IN HYPOTENSIVE STATES. **154**

Experimental evidence indicates that the autoregulation of cerebral blood flow is normally effected by non-metabolic factors. On the other hand the cerebral vascular bed is known to be sensitive also to metabolic influences. In the range of low perfusion pressures, below the limit of flow autoregulation, the conditions are less extensively investigated; we have previously found that the vascular resistance can be influenced by changes in oxygen and carbon dioxide tensions to about the same degree here as in the autoregulatory range.

In the present study, performed on anesthetized, artificially ventilated dogs, cerebral vasodilatation was first induced, usually by a continuous infusion of papaverine. By lowering the arterial pressure the flow was then reduced stepwise to normal or slightly subnormal values. In these conditions the pressure-flow relation tended to be curvilinear, convex towards the pressure axis, as seen in pressure-passive vascular beds. In a few animals, where the initial flow increase was brought about by carbon dioxide inhalation, however, the relation had an almost linear course, as demonstrated previously (Häggendal, E., and Johansson, B., Acta Physiol. Scand. 1965. 66. Suppl. 258.27) in hypoxic animals. Our impression is, therefore, that at perfusion pressures below the range of autoregulation a basically pressure-passive relation can be modified by metabolic factors, if these are strong enough.

Ballard, K., C.A. Cobb and S. Rosell. (Department of Pharmacology, Karolinska Institutet, Stockholm, Sweden): THE RELATIONSHIP BETWEEN LIPOLYTIC AND VASOACTIVE EFFECTS OF ADRENALINE AND NORADRENALINE ON CANINE SUBCUTANEOUS ADIPOSE TISSUE. **155**

Adrenaline and noradrenaline are potent lipolytic agents. Since they also have vasoactive properties which may influence the release of free fatty acids, it would be of interest to examine the relationship between the vascular and metabolic effects on adipose tissue.

Adrenaline, or noradrenaline, was added to the arterial blood perfusing canine subcutaneous adipose tissue in situ (Rosell, S., Acta physiol.scand. 1966. 67. 343). Either flow or, alternatively, pressure was constant during the perfusion. Concentrations ranged from .001 to 10µg/ml plasma ($6X10^{-9}$M to $6X10^{-5}$M). The lower concentrations were within ranges reported for resting dogs (Valk, A. de T. and Price, H.L., J.Clin.Invest. 1956. 35. 837). Glycerol release, indicating lipolytic rate, was determined. Adrenaline at concentrations above .01µg/ml plasma caused a net release of glycerol, concomitant with vasoconstriction. Noradrenaline enhanced glycerol release at .005µg/ml plasma but vascular responses were not observed until .01µg/ml plasma was exceeded. Both catecholamines produced only vasoconstriction.

The results indicate that catecholamine plasma levels present in dogs are sufficient to produce lipolysis in subcutaneous adipose tissue. Furthermore, lipolysis may occur without accompanying vasoconstriction.

156 Kovách, A.G.B., S. Rosell, P. Sándor, E. Koltay and N. Tomka. (Dept. of Exp.Res., Univ.Med.School, Budapest, Hungary and Dept. of Pharmacology, Karolinska Institutet, Stockholm, Sweden): BLOOD FLOW AND FREE FATTY ACID (FFA) RELEASE IN CANINE SUBCUTANEOUS ADIPOSE TISSUE IN HEMORRHAGIC SHOCK.

Little is known about vascular and metabolic reactions in adipose tissue in hemorrhagic shock. Therefore, during a procedure, known to cause irreversible shock, blood flow and release of FFA was measured in the subcutaneous adipose tissue (Rosell, S. Acta physiol. scand. 1966. 67. 343), of chloralose anaesthetized dogs. The animals were bled to 55mmHg for 90 min (BI) followed by bleeding to 33 mmHg for an additional 90 min (BII). The shed blood was then reinfused. In BI blood flow diminished from 6 to 0.6 ml/min/100g. In BII flow remained at 10% of control, or ceased. Following reinfusion normal blood flow could not be restored indicating damage to the vascular bed. Arterial FFA conc. fell in BI and BII. There was a small release of FFA.

In phenoxybenzamine pretreated animals blood flow was reduced to about 70% and 50% in BI and BII respectively. Following reinfusion pronounced hyperemia occurred. Arterial FFA increased during BI and BII and there was a definite rise in FFA release.

It is concluded that the vascular bed of canine subcutaneous adipose tissue is especially sensitive to hemorrhage. A pronounced restriction of blood flow results, which will have metabolic consequences. Phenoxybenzamine pretreatment may limit local damage by ensuring adequate blood flow.

157 Nicolaysen, G. (Institute of Physiology, University of Oslo, Norway): Ca^{++}, Mg^{++} AND CAPILLARY PERMEABILITY IN THE LUNG.

Ca^{++} is of importance for various characteristics of cell membranes, and probably also for cell adhesiveness. The exact effect of Ca^{++} in the capillary wall is insufficiently known, but observations indicate a role related to capillary permeability. - An isolated rabbit lung was perfused with plasma at 28°C and changes in preparation weight continuously followed. Addition of EDTA, leaving the calculated sum of ionized Ca and Mg in the perfusate at about 10 µmoles/l or less caused, within 10 to 20 min, an accellerating weight increase, reflecting edema formation. Such a weight increase, was stopped or reduced by addition of $CaCl_2$ or $MgSO_4$. Reduction of ionized Ca plus Mg to a calculated level of about 0.15 mmole/l did not result in edema. With 0.1 mmole/l ionized Ca and 0.4 mmole/l ionized Mg, the capillary filtration coefficient was reversibly increased. A situation with about 1 mmole/l of ionized Ca combined with about 0.1 mmole/l of ionized Mg, as achieved by EGTA-addition, did not result in edema formation, whereas edema developed on further reduction of ionized Mg to micromolar levels. - In order to produce edema in this preparation ionized Ca as well as Mg must be reduced then to very low levels in the perfusate, whereas the water permeability of the capillary wall is reversibly increased during less severe reductions of these ions.

Jonsson, J., K.-E. Arfors and H.H. Hint (Department of Experimental Medicine, Pharmacia AB and Institute of Zoophysiology, University of Uppsala, Sweden): STUDIES ON MICROCIRCULATORY COMMUNICATIONS BETWEEN THE BLOOD AND LYMPHATIC SYSTEMS. 158

Clark and Clark (J.Am.Anatomy, 60, 253, 1937) have by microcirculatory studies described the existence of fenestrations covered by a thin membrane between the postcapillary veins and lymphatic capillaries in rabbit ear chambers. They also observed haemorrhage through such a membrane. We have tried to confirm Clark and Clark's results and have found that the anatomical relationship between postcapillary veins and lymphatic capillaries in rabbit ear chamber are favourable to the existence of direct communications, but we have up to now not been able to demonstrate such communications anatomically. Functionally, we have found indirect evidence for free communication between vascular and lymphatic systems at highly increased central venous pressure after massive dextran infusions to rabbits. - Microcirculatory studies in the rat mesentery showed massive appearance of red cells in lymphatic microcirculatory vessels after reinfusion of blood in the state of haemorrhagic shock. In some cases flowing blood was seen in lymphatics in the same amount as in veins in the same area. These findings are earlier described by Baez (J.Appl.Physiol.21, 299, 1966; S.Baez, Flow prop of blood and other biol. syst., ed. by Copley & Stainsby, 1960, p.398) and can only be explained by haemorrhages directly from blood vessels into lymphatics. The common walls between the postcapillary veins and microcirculatory lymphatic vessels are probably weakened by haemorrhagic shock and by other injuries and a break through special predisposed sites can then be caused by increased venous pressure.

Åberg, B., (Institute of Physiology and Medical Biophysics, Bio-Medical Center, Uppsala, Sweden): MODEL EXPERIMENTS ON TRANSCAPILLARY TRANSPORT. 159

An isolated, perfused intestinal loop with an autodigested mucosa has been used as a model for paracapillary circulation and for studies of passive exchange of electrically neutral molecules over the capillary walls. The intestinal lumen has been regarded as an extracapillary space with free acess to catheters varying and measuring pressure and sampling.

It has been shown, that the velocity and direction of the fluid exchange between intestinal lumen is important to the speed and direction of the transport of raffinose and glucose. Raffinose has to a larger extent appeared to follow a bulk-flow compared with glucose. As the concentrations of the test-molecules are higher in the vessels than in the intestinal lumen, there is, at the venous end of the capillaries, a bulk-flow from the intestinal lumen and a solute counter-diffusion flow into the lumen. This contributes to a separation between the two molecules depending on the magnitude of the linear speed of the capillary resorption flow.

160 Åberg,B and S.-O. Lindahl (Institute of Physiology and Medical Biophysics, Biomedical Center, Uppsala, Sweden): THE SINGLE-INJECTION TECHNIQUE DURING VENOUS CONGESTION.

Earlier studies of transcapillary transport of substances have shown a restriction for penetration either by molecular/porous size (c.f. Grotte, G., Acta chir. Scand. 1956. suppl. 211) in steady-state or by diffusion coefficient in non-steady state (Crone, C., Acta physiol. Scand. 1963. 58. 229).

In penetrating the problem further we have adopted the method of Crone, i.e. given a single injection to the hindlimb of a cat without and with venous congestion. In case of free venous flow we obtained a volume-distribution for glucose somewhat larger than that for raffinose. During congestion the volume-distribution for both molecules was increased but more equal.

At least one explanation for the phenomena studied can be that resorptive flow at the venous end of the capillary might separate the molecules transported from the intracapillary to the extracapillary space on basis of their size. During free venous flow raffinose is transported with a bulk-flow back to the capillary to a greater extent than the smaller glucose molecule. During venous congestion this resorptive flow is disturbed, and the two molecules are transported in a more similar way.

161 Lassen, N.A. and Trap-Jensen, J. (Laboratory of Clinical Physiology and Department of Medicine C, Bispebjerg Hospital, Copenhagen, Denmark): EVIDENCE OF RESTRICTION IN DIFFUSION OF INULIN OVER THE CAPILLARY MEMBRANE IN EXERCISING HUMAN SKELETAL MUSCLE.

The transcapillary passage of hydrophilic molecules show a pattern consistent with simple diffusion through water-filled pores or slits. For larger molecules a restriction in the diffusion must occur since for such molecules the pore dimensions reduces the effective diffusion area. In resting skeletal muscle Crone (Proc.Soc.Exp.Biol.Med. 112:453,1963) found no evidence of restricted diffusion of inulin compared to sucrose, suggesting a much larger pore diameter than hitherto thought. We restudied this problem in rythmically exercising human skeletal muscle in order to minimize interference from flow limitation.

The single injection technique employing a mixture of inulin (or sucrose) and T-1824 was used in 6 normal subjects. The extraction, E, defined as the average fractional loss of diffusible indicator during the initial raising part of the first passage venous time-concentration curves, averaged 0.25 for sucrose and 0.01 for inulin at a blood flow level of 35 ml/100g/min. The capillary diffusion capacity ratios almost equals the ratio of these E values which markedly differ from the ratio of the free diffusion coefficient in water of about 3 (sucrose/inulin) This implies restricted diffusion for inulin compatible with the dimensions of the intercellular slits between the endothelial cells seen by electronmicroscopy.

Garlick, D. and C. Crone. (Institute of Medical Physiology A, University of Copenhagen, Denmark): IS THE INTERSTITIAL SPACE IN MUSCLE WELL-MIXED? 162

It has been suggested (Lassen, N.A. & J. Trap-Jensen, Scand. J. Clin. Lab. Invest. 1968. 21. 108) that capillary permeability can be assessed from the clearance rate of extracellular tracers from the interstitial space. This approach presupposes that the interstitial compartment is well-mixed so that no gradients of the tracers occur outside the capillary wall.

This assumption was tested in experiments where the fractional removal of extracellular markers (mannitol, sucrose, inulin) was studied in resting cat gastrocnemius muscle, perfused with Ringer-colloid, after equilibration with these substances. It was possible to determine extraction from the start of the washout period. The initial value was similar to that found with the indicator dilution technique in the same muscle. However, the extraction fell steadily from this initial value. The most likely explanation is the occurrence of concentration gradients in the interstitial fluid. The fact that the rate of diffusion in the interstitial space becomes a limiting factor in the removal of substances from the tissue, as well as limitations in the capillary wall, means that only extraction values obtained immediately after the start of washout can give information about capillary permeability.

Skadhauge, E. (Institute of Medical Physiology A, University of Copenhagen, Denmark): SOLUTE-LINKED WATER FLOW ACROSS THE CLOACAL EPITHELIUM IN THE NORMAL AND IN THE DEHYDRATED HEN. 163

Several authors have proposed that the water flow from gut lumen to plasma when the lumen has the same osmolality as plasma may be due to an osmotic flow into hypertonic confined regions in or between the cells. The hypertonicity is thought to be created by the active NaCl transport.

If this model is correct the water flow for a constant net NaCl flow should vary with the osmotic permeability of the epithelium.

The large intestine and coprodeum of normal and dehydrated hens was perfused with NaCl solutions of osmolalities varying from 150 mOs below to 300 mOs above the plasma osmolality. The transmural NaCl and water flow were measured. The lumen to plasma Na^+ transport was 220 μeq/kg·h in the normal and 240 μeq/kg·h in the dehydrated fowl. The lumen to plasma net water flow from luminal fluids of plasma osmolality was 180 μl/kg·h in the normal state, 380 μl/kg·h in the dehydrated.

Since the osmotic permeability estimated from the water flow in these experiments when the luminal fluids were far from plasma osmolality and measured in other experiments with impermeant perfusion fluids doubled in the mucosa to serosa direction from the normal state to dehydration, the fact that the solute-linked water flow, too, doubles, provides support for the confined-space-hypertonicity model.

164 Bindslev, N. (Institute of Medical Physiology A, University of Copenhagen, Denmark): HYDRAULIC CONDUCTIVITY, REFLECTION COEFFICIENT, AND SOLUTE MOBILITY OF THE CLOACAL WALL IN THE NORMAL AND THE DEHYDRATED HEN.

To elucidate the physiological changes in the passive transport parameters the hydraulic conductivity Posm, the reflection coefficient σ_s, and the salt mobility ω_s of the cloacal epithelium, the lumen of the coprodeum and large intestine was perfused. The perfusion fluids contained an impermeant solute (raffinose or $MgSO_4$) with osmolalities varying from 200 mOs below to 200 mOs above plasma osmolality. For the determination of Posm and σ_s the infusion rates were 5-9 ml/h. Posm (µl/kg·h·mOs) appears as the slope of the curve relating transmural volume flow to the difference in osmolality between plasma and perfusion fluid. σ_s is found as the ratio of the luminal osmolality and the plasma osmolality at zero volume flow. For the determination of passive salt flow the perfusion rate was varied from 5 to 225 ml/h. The passive salt (NaCl) flow was assessed at zero volume flow. The transmural potential difference was close to zero.

The Posm in the normal birds in the serosa (s) to mucosa (m) direction was 2.0-3.0 and in the m-s direction 4.4-6.1 µl/kg·h·mOs. During dehydration Posm in the s-m direction was unchanged, but in the m-s direction Posm nearly doubled (10.0-10.2 µl/kg·h·mOs). In both states σ_s was unity, and ω_s zero.

165 Persson, E. (Institute of Physiology and Medical Biophysics, University of Uppsala, Sweden): WATER PERMEABILITY IN RAT DISTAL TUBULES IN WATER DIURESIS AND ANTIDIURESIS.

In order to investigate how ADH affected water permeability in rat distal tubules, microperfusion experiments were performed to determine the osmotic water permeability (Lp) and the diffusional water permeability(P_D). The electrolyte concentrations were chosen to give no net electrolyte fluxes.The experiments were performed both in antidiuresis and in water diuresis after waterloading of hypophysectomized animals. In antidiuresis the Lp was $6.1\pm1.2 \times 10^{-8}$ $cm^3/cm^2 \cdot sec \cdot cm\ H_2O$ (mean value, ±1 SD)(n=8) and P_D was $327\pm132 \times 10^{-6}$ cm/sec (n=25). In waterdiuresis Lp was $1.6\pm1.6 \times 10^{-8}$ $cm^3/cm^2 \cdot sec \cdot cm\ H_2O$ (n=7) and P_D was $157\pm32 \times 10^{-5}$ cm/sec. When converted into the same dimension the Lp value was much higher than P_D, a result which cannot be explained by the existence of unstirred layer, but is additional support to the idea of pores through which the water flow at osmotic gradients mainly is a bulk flow and not a diffusional flow.The question of the modification of water structure in microcapillaries may be important for water flow, but yet too little is clarified.The larger ratio(Lp/P_D) in antidiuresis than in water diuresis may indicate that ADH changes the pore area-pore diameter relation so that the bulk flow increase is larger than the diffusional flow increase.

Wieth, J.O., (Institute of Biophysics, University of Copenhagen, Denmark): ANION-INDUCED CHANGES OF ERYTHROCYTE PERMSELECTIVITY. 166

Sodium and potassium permeabilities of human red cells are increased if chloride is replaced by other monovalent anions of the lyotropic series (J.Funder and J.O.Wieth, Acta physiol. scand. 1967.71.1968). Additional information about the permselectivity has been obtained by studying the permeation of ions (Na, K, Cl, SO_4) and of an uncharged molecule (erythritrol).

Anion-induced enhancement of cation permeability was always accompanied by a reduced permeability to negatively charged ions. A maximum effect on permselectivity was found in a 120 mM salicylate medium at 0 °C. The rate of chloride exchange decreased by a factor of 100,000, and chloride and potassium permeabilities were of comparable magnitudes (P-Cl: 2×10^{-9} cm/sec, P-K: 3.5×10^{-9} cm/sec). Passive fluxes of sodium and potassium showed a paradoxical temperature dependence in the presence of the adsorbable anions thiocyanate and salicylate with a minimum at 18 °C, and steep increases both below and above this temperature. Red cell permeability to erythritrol was not affected by the substitution of chloride with other anions. Because the anion-induced effect on permeability is determined by the electric charge of the permeating species, it is concluded that anions interact with fixed positive charges, which control the ionic permeability of the red cell.

Ahlgren, L. and Grampp, W. (Institute of Physiology, University of Lund, Lund, Sweden): MEMBRANE EFFECTS OF OUABAIN IN THE SLOWLY ADAPTING STRETCH RECEPTOR NEURONE OF LOBSTER. 167

Ouabain has previously been shown to cause an immediate and reversible depolarization of the smooth muscle membrane (Casteels, R., J. Physiol. 1966. 184:131). A similar effect was found in the slowly adapting stretch receptor neurone of lobster. Thus, in 5×10^{-4} M concentration ouabain produced a depolarization of 10-25 mV, the higher values obtaining with higher degrees of stretch activation of the cell. This effect which persisted in the presence of tetrodotoxin was abolished by substitution of choline for Na^+. The depolarization was parallelled by a reduction of the membrane resistance which manifested itself particularly after that spike activity had been blocked by tetrodotoxin. During development of the depolarization both amplitude and rates of rise and fall of the action potential diminished at a rate which increased with the firing frequency during the action of the glycoside. The described effects started immediately on arrival of the drug in the experimental chamber and attained a maximum 10-15 min later. Complete reversibility was obtained by washing the preparation for about 40 min. Initially during the washing procedure there was a 20 min long hyperpolarization of maximally 10-15 mV.

The results suggest a dual effect of ouabain on the membrane: 1) The drug causes an unspecific increase in permeability for Na^+ resulting in the depolarization. 2) Ouabain blocks the channels for specific ionic fluxes leading, perhaps in combination with the depolarization, to the decline of the action potential.

172 Poupa, O. and Carlsten, A. (Department of Clinical Physiology, University of Goteborg, Sweden): ANEURYSMS OF FROG HEART OBSERVED IN VIVO.

Almost all frogs (Rana Pipiens) accidentally kept for a few days in the winter period at +28 - +30°C died and at autopsy we observed aneurysms of the ventricle in most of them. This was never observed at +4 - +8°C.

Isoproterenol (150 - 200 mg/kg b.w.) injected twice with 1 day's interval in the lymphatic sac of Rana pipiens causes changes in the heart of frogs at +25°C but not at +4°C. In contrast to the reptiles this frog has only one type of musculature of the heart, the sponge-like, corresponding to the inner part of the reptilian ventricle, supplied by blood from the lacunae. No true coronaries are present. The ventricular lesions of the beating frog heart, provoked by isoproterenol and perfectly studied in 6-40 times magnification, consist of aneurysms and/or blister-like formations of typical localization and certain reversibility. Preliminary microscopy shows no infiltration of polymorphs even in large lesions, neither in the spontaneously dying nor in the isoproterenol group. The cardiac aneurysms produced in this way can be studied for hours and provide us with a living model of heart lesions suitable for hemodynamic and pharmacodynamic research.

The isoproterenol induced aneurysms seem to be very similar to those occurring in the spontaneously dying noninjected frogs kept at +28 - +30°C.

173 Kadefors, R., Magnusson, R., Nilsson, N.J. & Petersen, I. (Chalmers University of Technology, Sahlgren and Vasa Hospitals, Göteborg, Sweden): EFFECTS OF ISCHEMIA ON THE MYOELECTRIC SIGNAL SPECTRUM.

Maximum voluntary muscle contractions are accompanied by an increase of the low-frequency signal components (Kogi, K. & Hakamada, T., Rep. Inst. Sc. Lab. 1962. 60. 27.) and a reduction of the high-frequency signal components, possibly reflecting different sensitivities of different types of motor units (Kaiser, E. & Petersén, I., Electromyography. 1963. 3. 5; Kadefors, R., Kaiser, E. & Petersén, I., Electromyography. 1968. 7. 39.). The present authors have found that this high-frequency decrease is not materially affected by interruption of blood flow through the muscle. Also the reactive hyperemia after the contraction as well as the oxygen saturation, PCO_2, lactic and pyruvic acid concentration of the venous blood from the muscle were of about the same magnitude with arrested blood flow.

On the other hand, in a series of increasing submaximal loads to the biceps brachii muscle of healthy subjects an influence of blood flow arrest was observed. Typical signal spectrum changes were observed under control conditions at wrist loads of about 5 kp and during ischemia of the muscle at about 2 kp. The changes disappeared within a few seconds after restoration of blood circulation.

The results indicate an insufficient blood perfusion of the muscle as a cause of the power spectrum changes.

Molbech, Svend and Sophus H. Johansen (Polioinstitutet, Hellerup and Gentofte Hospital, Danmark): ENDURANCE TIME IN STATIC WORK DURING PARTIAL CURARIZATION. 174

In 3 normal subjects a 50% reduction of normal maximum isometric strength in elbow flexion was produced with (+)-tubocurarine. Under these circumstances the endurance for static work was found relatively much more reduced than strength. Similar experiments with decamethonium in one subject demonstrated no change in the normal relation between endurance and strength. Our findings can be explained by the different sensitivity of the "slow" and "fast" muscle fibers towards the 2 neuromuscular blockers: (+)-tubocurarine attacks mainly the slow fibers, which are fit for sustained contractions, and decamethonium the fast fibers, which are of more importance in maximum isometric strength mobilizations. The sensory feedback from the muscle spindles will be diminished by (+)-tubocurarine (but increased by decamethonium) This difference might contribute to the results, and explain why normal feeling of fatigue was depressed with (+)-tubocurarine, whereas after decamethonium the subject felt an initial increase in strength, subsequently followed by normal fatigue.

Andersson, K.-E. and K.A.P. Edman (Department of Pharmacology, University of Lund, Lund, Sweden): THE MECHANICAL OUTPUT OF SINGLE MUSCLE FIBRES AT DIFFERENT DEGREES OF HYDRATION. 175

According to the sliding-filament model the tension generated by the muscle fibre is dependent upon the number of active links formed and hence to the area of overlap between the A and I filaments of the myofibril. The characteristic relationship between sarcomere length and tetanic tension in single muscle fibres is accordant with this idea. It has previously been reported from this laboratory (Experientia 1968. 24. 134) that the changes in lateral spacing between the filaments that are normally associated with muscle shortening do not affect tension production. In the present study we have analyzed the mechanical output after equilibration of the fibre in different osmotic media. Ordinary Ringer's solution, Ringer + 50 mM sucrose (hypertonic solution) and Ringer containing 80 % of the standard NaCl concentration (hypotonic solution) were compared. The tetanic tension (P_{max}) and the maximal speed of shortening (V_{max}) both increased (by approx. 15 %) when the fibre was equilibrated in the hypotonic medium. The shape of the force-velocity curve was unaffected. A parallel decrease (by approx. 20 %) of P_{max} and V_{max} occurred by immersion of the fibre in the hypertonic solution As the measurements refer to given sarcomere lengths, the results would seem to make clear that the mechanical output per unit overlap area between the A and I filaments can be varied in a tetanically stimulated fibre. This suggests, in terms of the sliding-filament model, that the output of the individual active link, its contribution to fibre shortening as well as to tension production, can be varied by changing the state of hydration of the fibre.

180 Jokinen, Y. (Institute of Physiology, University of Helsinki, Finland): *THE MECHANISM OF ACTIVATION OF NEURONS IN THE RABBIT HYPOTHALAMUS IN* RESPONSE TO LOCAL THERMAL STIMULATION.

Like many other physiologic control systems, the thermoregulatory system receives its principal input from receptors located in hypothalamus. The existence of these receptors was verified at the cellular level by Nakayama et al. (Am.J.Physiol. 204:1122-1126).

To study the properties of the temperaturesensing elements in the preoptic area of adult rabbit, we recorded the single cell responses to the heating of the anterior hypothalamus, using glass capillary microelectrodes under light urethane anesthesia. The stimulus range was 2°C.

Among 210 preoptic neurons examined for temperature sensitivity we found 42 repeatedly responding cells. 24 of these were socalled "warm cells" and 18 "cold cells". 27 of them showed a linear relationship between the mean firing rate and the hypothalamic temperature. The remainder showed a relation which resembled an exponential function. Marked differences in the time structure of interspike interval sequences were found between these categories.

We concluded that the different types of cell cannot be activated by one and the same mechanism. Theoretically, a neuron can be activated by a change either in membrane properties or in synaptic input, and a combination of these components is also possible. Because it was not possible to differentiate the cell types by means of their location, our next step has been to construct a model where the activation mechanisms can be simulated with the aid of digital computer techniques. The output of the model is then subjected to the same procedures as the experimental material.

181 Wiesel, T. and Hubel, D. (Department of Neurobiology, Harvard Medical School, U.S.A.): PERIOD OF SENSITIVITY TO MONOCULAR DEPRIVATION.

A series of neurophysiological experiments were done in monocularly deprived kittens to determine the period of sensitivity to light and form deprivation. Our finding was that kittens were maximally sensitive to monocular deprivation between the 3rd and the 6th week after birth. During this period as little as 3 days of monocular closure was sufficient to produce a clear shift in the normal binocular dominance distribution for cortical cells, and detectable morphological changes in the lateral geniculate body. A closure for 6 days (23-29 day) was enough to cause a complete shift, so that the large majority of recorded cells responded only to the normal eye, just as was seen in kittens deprived for the first 3 months. The sensitivity to monocular deprivation began to decline after the 6th week, disappearing at about 12 weeks of age.

The interruption of normal connections in the visual cortex through monocular deprivation seems to be to a large extent permanent. An animal allowed to recover while still in the sensitive period, for example after a 5-week closure from birth, regained some slight control from the deprived eye over cortical cells over a period of several months or years but the large majority of cells nevertheless remained influenced by that eye. Animals deprived through the entire sensitive period show still less sign of physiological recovery.

Hultborn, H. and M. Udo (Department of Physiology, University of Göteborg, Sweden): SPATIAL CONVERGENCE OF SUPRASPINAL FACILITATION AND RECURRENT INHIBITION IN THE RECIPROCAL IA INHIBITORY PATHWAY. 182

The disynaptic reciprocal Ia inhibition from muscle spindles to antagonistic motoneurones is facilitated at an interneuronal level from many supraspinal structures (cf. Lundberg, Nobel Symposium I, 1966. 275-305). Recent experiments (Hultborn, Jankowska and Lindström, Life Sci. 1968. 7. 337-339) have shown that antidromic stimulation of ventral roots via motor axon collaterals and Renshaw cells causes a depression of the transmission in the Ia inhibitory pathway at an interneuronal level.

The technique of spatial facilitation has now been used to investigate whether the Ia inhibitory interneurones receiving excitatory action from supraspinal centres can be inhibited via recurrent collaterals. Intracellular recording was made from motoneurones. For each motoneurone the stimulation strength was chosen so that separate stimulation of either the supraspinal system or Ia afferents did not evoke an IPSP, while combined stimulation at suitable intervals gave a large IPSP. When preceded by stimulation of ventral roots this test IPSP was abolished or decreased This was found in more than 100 motoneurones belonging to different hindlimb muscles in which an IPSP appeared after facilitation from cortico-, rubro- or vestibulospinal tracts.

We conclude that a convergence from supraspinal and recurrent inhibitory pathways occurs on the same Ia inhibitory interneurones. This indicates that these two pathways are mutually interacting in the control of transmission in the Ia inhibitory pathway

Vallbo, Å.B. (Physiological department, Biological Institute, University of Umeå, Sweden): SINGLE UNIT ANALYSIS OF SLOWLY ADAPTING MUSCLE RECEPTORS IN MAN. 183

Impulses of single afferent fibres in muscle nerves were recorded from human subjects with tungsten electrodes inserted percutaneously. The tip diameters of the electrodes were 5-15 μ and the impedances were 50-150 kohm at 1,000 Hz. General or local anaesthesia was not used. Activities from 72 slowly adapting muscle receptors were studied. The main purpose was to experimentally penetrate the possibilities to define physiological characteristics which would allow an identification of the receptor type, and, second, to determine, in broad, the discharge patterns of the receptors during voluntary contractions without external shortening. For the majority of the endings characteristics of muscle spindles could be demonstrated such as high dynamic sensitivity to passive joint movements, discharge on relaxation after a voluntary contraction or irregular discharge in relaxed muscles indicative of fusimotor activity. During weak voluntary contractions without external shortening the majority of the units responded with a sustained discharge which started and stopped almost simultaneously with the onset and the cessation of the EMG activity. It was concluded that the motor cortex exerts a very powerful control of the intrafusal muscle fibres of the majority of the muscle spindles.

184 Lindquist, Chr. and A. Mårtensson (Department of Physiology, Karolinska Institutet, Stockholm, Sweden): THE POSSIBLE ROLE OF EXTEROCEPTORS IN THE CONTROL OF FACIAL MUSCLES.

It is still a matter of dispute whether muscle spindles are involved in the proprioceptive control of mammalian facial muscles. Morphological and physiological studies have not yielded conclusive data as to the possible presence of such receptors (Hosokawa, H., Texas Rept. Biol. Med. 1961. 19. 405). - Experiments undertaken to study the proprioceptive control of the cat's facial muscles have established that multisynaptic reflex responses of appreciable size are set up in facial muscles on stimulation of the corresponding intact muscle nerve. After section of this nerve no reflex response results when stimulating and recording from the central part of the nerve but is again obtained when the stimulating electrode is shifted to its peripheral part. This latter reflex response cannot be evoked in curarized animals and thus derives from the muscle contraction evoked. Similar reflexes were set up on stimulation of peripheral trigeminal nerve branches. - Afferent discharges induced in trigeminal nerve branches on contraction of facial muscles were analyzed in order to study the physiology and site of the receptors. These were found to respond to a sustained muscle contraction with a rapidly adapting impulse flow. The units studied so far were excited by, respectively, bending of coat hairs or of vibrissae or light touch of the skin. No evidence pointing to the presence of intramuscular contraction-sensitive receptors was found. These findings indicate that contraction-sensitive exteroceptors with afferent fibers in the trigeminal nerve may serve a proprioceptive control of the facial muscles.

185 Järvilehto, T. and Fruhstorfer, H. (Institute of Physiology, University of Helsinki, Finland): CHARACTERISTICS OF SLOW CORTICAL POTENTIALS ASSOCIATED WITH MOTOR AND MENTAL ACTS IN MAN.

The differences between CNV (Walter, W.G. et al., Nature 1964. 203. 380) and readiness potential (Kornhuber, H.H. and Deecke, L., Pflügers Arch. ges. Physiol. 1965. 284. 1) were studied in four situations. In the first situation (A) self-paced movements of the right index finger were used to elicit the readiness potential. The same motor act was timed by an oscilloscope sweep (B). Uncertainty about the quality of the motor act was added by a discrimination task determining the motor response which immediately followed the discrimination (C). In situation D the response followed 15 sec after the discrimination. The EEG was monopolarly recorded from electrodes located at Cz, Fz, and Fpz. Additionally, eye movements were recorded from an electrode situated just below the right eye. The EEG before and during the tasks was selectively summated in a computer omitting EEG epochs contaminated by eye movement, drop-out, or electrode artefacts. Preparation for a motor or mental task was associated with a negative wave maximal at the vertex, that disappeared during the initiation of the task. If uncertainty a[bout] the task was added, a frontal-negative wave appeared, which lasted the readiness potential and disappeared when the task was finished. The CNV seems to be composed of at least two components, one of which is the readiness potential and the o[ther] a frontal-negative wave associated with uncertainty.

Ungerstedt, U. (Dept. of Histology, Karolinska Institutet, Stockholm, Sweden): BEHAVIOURAL REGISTRATION OF DOPAMINE SYNAPTIC ACTIVITY IN THE BRAIN AFTER 6-HYDROXY-DOPAMINE LESIONS.

Several recent studies indicate that 6-hydroxy-dopamine (6-OH-DA) depletes peripheral nerves of noradrenaline by inducing a selective degeneration of the noradrenaline nerve terminals (Tranzer and Thoenen, N-S's Arch. exp. Path. Pharmak. 1967. 257. 74; Malmfors and Sachs, Europ. J. Pharmacol. 1968. 3. 89). In order to induce a degeneration of central monoamine neurons 6-OH-DA was injected stereotaxically in rats into the group of dopamine (DA) cell bodies located in the substantia nigra. The effect was studied with the histochemical fluorescent technique for monoamines. The injection resulted in a rapid degeneration of the whole DA-neuron system to the caudate nucleus with little damage to the brain tissue surrounding the DA cell bodies. Unilateral degeneration of this DA system produced marked turning of the animals to the side ipsilateral to the lesion. The turning could be increased, decreased or reversed by monoaminergic drugs. This was interpreted as an evoked imbalance in DA transmittor activity between left and right side of the brain and a method was worked out for the continous recording of turns per minute. With this method it has been possible to study DA synaptic activity under various pharmacological conditions. Amphetamine has been shown to produce a preferential release of DA from the lesioned neurons during the time of degeneration and the eventual disappearance of DA from the caudate nucleus seems to result in a DA receptor supersensitivity to e.g. apomorphine. The effect of DA release can be modulated by cholinergic drugs.

187

Arbuthnott, G.W. and Ungerstedt, U. (Department of Histology, Karolinska Institutet, Stockholm, Sweden). LOCOMOTOR BEHAVIOUR AFTER ELECTRICAL STIMULATION OF DOPAMINE CONTAINING NEURONES.

Recent experiments in this laboratory (Ungerstedt - this congress) have indicated that an unilateral activation of the dopamine receptors in the caudate nucleus will cause rats to rotate towards the unstimulated side. This result suggested that electrical stimulation of the dopamine neurones to the caudate nucleus should also cause rotation. Rats were prepared with electrodes chronically implanted among the axons of the dopamine neurones at the level of the lateral hypothalamus Electrical stimulation (200-600 μ A, 0.1 - 1.0 msec pulses at 50-100 Hz) through these electrodes did cause the animals to rotate towards the contralateral side. Not unexpectedly, since the electrodes were close to other extrapyramidal structures, other locomotor activity in response to stimulation was also observed: the rats reared up on their hind legs or flexed their contralateral limbs. It is possible to test which of these effects is due to stimulation of the dopamine neurones by pharmacological means, using drugs which have been shown to affect central monoamine transmission. Reduction of the catecholamine stores with tetrabenazine (10 mg/kg 1-2 hrs previously) or blockade of catecholamine receptors with Haloperidol (1 mg/kg 1-10 hrs previously) both reduce the rotation while the other movements associated with stimulation were hardly affected. Thus it seems possible that in this preparation the level of activation of the dopamine receptors can be estimated by measuring the rotation of the animals.

Dr Arbuthnott is in receipt of a Travelling Fellowship from the Wellcome Trust.

188 Clark, F.J.[x] and C.J. Hoffman (Purdue University, Lafayette. Ind. USA): A HIGH RESOLUTION MICROELECTRODE FOR MEASURING SPECIFIC IMPEDANCE IN THE CEREBRAL CORTEX.

Specific impedance at various depths in the cerebral cortex was measured at 1.6 KH_z using a novel thin film coaxial microelectrode technique which permitted measurement of very small volumes of tissue (0.01 mm^3). Experiments using rats indicated that spreading depression produces increases in specific impedance some 3 to 4 times the resting value, increases which are more than 10 times greater than those previously reported. (Van Harreveld, 1966, Brain Tissue Electrolytes. Washington: Butterworth). These higher values obtained in the present work were attributed to the very high resolution of the microelectrode method used.

Classical models relating specific impedance to extracellular volume were investigated to test their validity in application to suspension of complex insulating structures with high packing density. This investigation coupled with the results of the cortical specific impedance studies suggested that the classical models are indeed valid and that a large extracellular space exists in the cerebral cortex.

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189 Rosén, I. and Sjölund, B. (Institute of Physiology, University of Lund, Sweden): NATURAL STIMULATION OF GROUP I ACTIVATED CELLS IN THE CUNEATE NUCLEI OF THE CAT.

This investigation supplements earlier studies of group I activated cuneate cells, which were based on electrical stimulation of nerves. — One forelimb was denervated except for the deep radial nerve which was mounted for stimulation and left in continuity with ext.carp.rad., dig.comm., dig. long. and carp.uln. The muscle tendons could be connected to a strain gauge or loaded with weights. Identification of receptors was based on: (1)Threshold and (2)adaptation on stretch. (3)Discharge pattern on contraction. (4)Effect of succinyl choline. — Out of 44 slowly adapting group I afferents found in the deep radial nerve 20% originated from tendon organs and 72% from muscle spindles. The tendon organ afferents and 86% of the spindle afferents could be antidromically activated from the dorsal funiculus at C3. In the main cuneate nucleus (MCN) 34 cells were activated from group I spindle afferents and 3 from tendon organ afferents. 14 cells could be antidromically activated from the medial lemniscus and were all activated from spindles. In the external cuneate nucleus (ECN) 44 cells were activated from spindle afferents and 4 from tendon organ afferents. About 70% of the cells in both nuclei were activated from only one of the four muscles. 19 of 39 ECN cells but only 2 of 33 MCN cells could be inhibited by stretch in one or more of the muscles not producing excitation. — The results show that (1) tendon organ as well as group I spindle afferents ascend through the dorsal funiculus, (2) the majority of cuneate cells and presumably all cuneothalamic cells are activated from spindles, (3) inhibitory actions from synergists are less common in MCN than in ECN.

Landgren, S. and Silfvenius, H. (Depts. of Physiology, University of Umeå and University of Goteborg, Sweden): THE MEDULLARY RELAY IN THE PATH FROM HINDLIMB MUSCLE SPINDLES TO THE CEREBRAL CORTEX OF THE CAT. **190**

Projections from hindlimb group I muscle afferents to the postsigmoid gyrus of the cat's cerebral cortex were described by Landgren and Silfvenius (J. Physiol. 1968. 200. 353). It was demonstrated that the ascending path travelled in the dorsolateral fascicle, and that it remained intact after removal of the cerebellum. The location of the spinal, medullary and thalamic synapses was unknown. In this series of experiments the rostral part of nucleus gracilis and the caudal part of nucleus vestibularis medialis was explored with penetrating microelectrode tracks in search of responses to electrical stimulation of group I muscle afferents from the quadriceps, posterior biceps semitendinosus, and gastrocnemius-soleus muscles. Cats anaesthetized with chloralose were used.

Focal potentials and action potentials of single neurones were recorded in response to threshold stimulation of the group I afferents. The latency was 4 to 5 msec. Cells responding to stimulation of group I afferents were discharged antidromically from the contralateral thalamus (entrance of the medial lemniscus into the ventrobasal nuclei, latency: 1 msec, frequency following: 800 per sec). The cortical group I response disappeared after a saggittal section in the midline of the IV ventricle. The response in the medulla was abolished by transsection of the dorsolateral fascicle at C 1 and was greatly reduced by transsection of the dorsal columns at L 5 thus just caudal to Clarke's column. The findings suggest that the medullary relay in the group I path from the hindlimb is located in nucleus Z of Brodal and Pompeiano (J. Anat. 1957. 91. 438).

Baldissera, F. and G. ten Bruggencate (Department of Physiology, University of Göteborg, Sweden): RUBROSPINAL EFFECTS ON SPINAL BORDER CELLS. **191**

Spinal border cells (SBC) which belong to the ventral spinocerebellar system (Burke, Lundberg and Weight, Acta physiol. scand. 1968. 74. 16A) receive monosynaptic connexions, both inhibitory and excitatory, from supraspinal pathways descending in the ventral quadrants of the spinal cord (Baldissera and Weight, Acta physiol. scand. 1969, in press). It will be shown here that a dorsal descending pathway, the rubrospinal tract, is also monosynaptically connected with the SBC. In chloralose anesthetized cats the contralateral red nucleus was stimulated with tungsten electrodes while recording intracellularly from SBC in the lumbar cord. In 10 of 56 SBC, monosynaptic EPSPs (segmental latencies of 0.4-0.6 msec) were elicited by red nucleus stimulation; in addition, in most of the cells EPSPs and/or IPSPs with longer latencies, suggesting a di- and/or trisynaptic linkage, have been found. These late effects were often facilitated by volleys in the flexor reflex afferents, mainly from the ipsilateral side. On the other hand the rubrospinal volleys were effective in facilitating the disynaptic IPSP evoked in SBC by group I afferent stimulation. The present and previous results demonstrate that these spinocerebellar neurones are monosynaptically influenced by several descending systems, i.e. vestibulospinal, reticulospinal and rubrospinal tracts, which are known to be the main target of cerebellar output.

196 Przybylski, A. (*Department of Pharmacology, Karolinska Institutet, Stockholm, Sweden; Experimental and Clinical Medical Center, Polish Academy of Sciences, Warsaw, Poland*): SPECIFIC AND UNSPECIFIC STRENGTH OF THE STIMULUS.

It is assumed that every biological stimulus S has two components - specific S_s and unspecific S_n, and that $S = S_s + S_n$, (1)
which with regard to certain values of energy and information of a given receptive channel of the living system would be

$$S_s = I-I_o/E-E_o \approx \Delta I/\Delta E, \quad S_n = E-E_o/I-I_o = \Delta E/\Delta I; \tag{2}$$

where: I and E - the quantity of information and energy of the stimulus percepted by the living system, I_o and E_o - the actual quantity of "steady" information and energy of a given receptive channel of the system. Substituting ΔE by its corresponding change of entropy we have: $S_s = \Delta I/\Delta ST, \quad S_n = \Delta ST/\Delta I.$ (3)
Assuming that the specific strength of the adequate stimulus S_{as} is higher than its unspecific strength S_{an} we can write: $\Delta I_{as}/\Delta S_{as}T > \Delta S_{an}T/\Delta I_{an}$, (4)
from which inequality results - with supposition that $\Delta S_{as}T \leq \Delta S_{an}T$ and $\Delta I_{as} \geq \Delta I_{an}$ - that $\Delta I_{as} > \Delta S_{an}T$. (5)
Putting the value of $\Delta S_{an}T$ from the equation for the whole change of energy of a given receptive channel produced by the adequate stimulus

$$\Delta E_{as} = \Delta S_{as}T + \Delta S_{an}T \tag{6}$$

into the inequality (5) we have: $\Delta I_{as} > \Delta E_{as} - \Delta S_{as}T$ (7)
(phenomenon of gain of information of adequate specific stimulus).
From this inequality results that the informational component of the adequate specific stimulus exceeds its energy component.

AUTHOR INDEX

The figures refer to the abstract numbers

Aars, H. 137
Ahlgren, L. 167
Ahrén, K. 102
Ahtee, L. 86
Aitio, A. 105
Akre, S. 137
Andén, N.-E. 34
Andersson, K.-E. 175
Arbuthnott, G. W. 187
Areskog, N.-H. 121
Arfors, K.-E. 133, 134, 158
Arvela, P. 129
Asmussen, E. 2
Axelsson, J. 39, 177

Baldissera, F. 191
Ballard, K. 155
Balldin, U. 119
Banister, E. W. 127
Bergendorff, A. 78
Bergström, J. 8, 9
Bergqvist, U. 22
Bevegård, S. 4
Beviz, A. 112
Biber, B. 150
Bill, A. 52
Bindslev, N. 164
Bjorklund, A. 23
Bloom, G. D. 20
Bolme, P. 139
Borg, E. 192
Broen Christensen, C. 90
Bruggencate, G. ten 191
Bugge-Asperheim, B. 113
Bygdeman, S. 61
Bø, G. 147

Carlsson, E. I. 104
Carlsten, A. 172
Carstensen, H. 100
Cedergren, E. 35
Clark, F. J. 188
Cobb, C. 155
Crone, C. 57, 162

Dahlquist, R. 75
Delin, N. A. 152
Diamant, B. 32, 74, 75, 124
Donner, K. O. 49
Duncan, W. R. 127

Edman, K. A. P. 38, 175
Ehinger, B. 58
Ekstrom-Jodal, B. 154
Emmelin, N. 12
Emås, S. 97
Enger, P. S. 55
Elwin, C.-E. 96
Ericson, L. E. 60
Eriksson, E. 149
Euler, C. von 47
Euler, U. S. von 25

Falck, B. 58
Farnebo, L.-O. 70
Flemström, G. 13
Folkow, B. 141, 145
Forster, H. 115, 116
Frankenhaeuser, B. 36
Fredholm, Bertil 106, 107
Fredholm, Bo 76, 101
Frederiksen, O. 17
Frisk-Holmberg, M. 79, 107
Fruhstorfer, H. 185
Fuxe, K. 53

Garlick, D. 162
Giacobini, E. 168
Giere, F. A. 111
Gliemann, J. 98
Grampp, W. 167
Greenleaf, J. 125

Hallbäck, M. 141
Hamberger, B. 28
Hamberger, L. 102
Harri, M.-P. 92
Hartiala, K. 51
Hauge, A. 147
Hedqvist, P. 71
Helander, H. F. 14
Helle, K. B. 21
Hermansen, L. 111, 123, 130, 131, 132
Hietanen, E. 108
Hint, H. H. 158
Hirvonen, L. 138
Hjalmarson, Å. 112, 114
Hoffman, C. J. 188
Hohwü-Christensen, E. 1
Holm, J. 90
Holmberg, B. 177
Hubel, D. 181
Huikko, M. 129
Hultborn, H. 182
Hultman, E. 8, 9
Hyvärinen, J. 195
Håkanson, R. 24, 62
Håkansson, B. A. S. 146
Hägermark, Ö. 20, 76
Häggendal, E. 153, 154

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ACTA PHYSIOLOGICA SCANDINAVICA
SUPPLEMENTUM 332

ON MOTONEURON SYNAPTOLOGY IN ADULT CATS

An electron microscopic study
of the structure and location of neuronal
and glial elements on cat lumbosacral motoneurons
in the normal state and after
dorsal root section

BY

SEBASTIAN CONRADI

STOCKHOLM 1969

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Comparison of the fixation methods

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Perfusions performed at a constant pressure, regardless of whether a pressure of 150 or 70 mm Hg was used, resulted in a rapid flow of fixative through the animal and the fixation was very poor. The spinal cord was soft and white, while the spinal muscles were often brown and hard. When, however, the perfusion pressure was varied periodically between about 50 mm Hg and zero, the fixation of the cord was good. In these cases, the deep spinal muscles appeared hard, although the superficial ones seemed nearly unfixed.

Quantitative study

Two animals were used for the quantitative investigation. Equal halves of the material were taken from each of the animals, which were fixed through perfusion at a discontinuous pressure, with 5% purified glutaraldehyde in 300 mOsm Millonig buffer. 2.7% Rheomacrodex was added to the rinsing fluid and fixative. Consecutive semithin transverse sections were cut in cranio-caudal direction, through a specimen from the most cranial millimetre of L 7, in animals in which the size of the lumbosacral roots, on macroscopic examination, seemed to indicate a prefixed lumbosacral plexus (Sherrington 1892).

When a motoneuron was cut through the nucleus, 2—4 grids with ultrathin sections, the depth of some microns apart, were made. About half of the specimens were cut through in this way. The ultrathin sections of each animal included, in total, about 20 motoneuron cell bodies, which were cut roughly through the middle of the nucleus, judging from inspection of the actual profile in the electron microscope. On the fluorescent screen there were, namely, some markings, through which it was possible to control that the profiles of all the motoneuron nuclei had about the same diameter as the profiles of those nuclei which were cut through the nucleolus. The nucleolus was cut through in about half the cases. Profiles of motoneurons, both those in which long profiles of proximal parts of the dendrites were included and those in which no dendrite was seen to project, were chosen from these sections. The profiles of 10 motoneurons were photographed in each animal. Three [illegible] of proximal

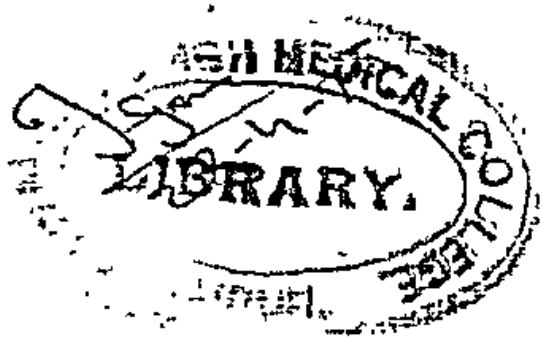
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CONTENTS

CONRADI, S., Ultrastructure and distribution of neuronal and glial elements on the motoneuron surface in the lumbosacral spinal cord of the adult cat 5

CONRADI, S., Ultrastructure and distribution of neuronal and glial elements on the surface of the proximal part of a motoneuron dendrite, as analyzed by serial sections 49

CONRADI, S., Observations on the ultrastructure of the axon hillock and initial axon segment of lumbosacral motoneurons in the cat . . . 65

CONRADI, S., Ultrastructure of dorsal root boutons on lumbosacral motoneurons of the adult cat, as revealed by dorsal root section . . . 85

Ultrastructure and distribution of neuronal and glial elements on the motoneuron surface in the lumbosacral spinal cord of the adult cat

by

SEBASTIAN CONRADI

INTRODUCTION

In order to define the ultrastructural correlate of the monosynaptic connexions of dorsal root origin, Conradi and Skoglund (1968) studied both the normal and the degeneratively-changed boutons on lumbar spinal motoneurons in the cat. The reason for the inconclusive results in that paper is explained by the fact, that such a study requires a thorough knowledge of the bouton types on the motoneurons. This must be obtained by studying serial sections, which were not at hand at that time. A thorough understanding of the ultrastructure of spinal motoneuron synaptology in the adult cat is also of great interest for the neurophysiologist (see Smith *et al.* 1967) and it is furthermore an indispensable prerequisite for a study of the postnatal development of the motoneurons. As pointed out by Mellström and Skoglund (1969), the synaptology of the growing motoneuron poses some special problems. There are thus several reasons for undertaking ultrastructural studies of the spinal motoneurons. The guiding aim of the present investigation has been to obtain morphologic correlates to the postnatal maturation of the spinal reflex system, which shows considerable functional changes during this period of development (for reviews, see Skoglund 1966).

Following this intention, a series of six papers will be presented, dealing with different aspects of the ultrastructure of spinal motoneurons, first in the adult cat, and then in the newborn and growing kitten. In the present paper which is the first in the series, the fixation and sampling methods are de-

scribed. The normal ultrastructure of the neuronal and glial extensions on the motoneurons and in the surrounding neuropile in the adult animal is described. The different types of boutons are presented, and the size of the boutons and the distribution of them on the motoneurons in a restricted part of the ventral horn is studied. In the second paper, (Conradi 1969 b), the proximal part of a motoneuron dendrite, one section of which was included in the quantitative estimation in the first paper, is analyzed through serial sections. The three-dimensional distribution of the boutons on the surface of the dendrite is presented. The third paper (Conradi 1969 c) describes the ultrastructure of the axon hillock and initial motoraxon segment and the distribution of the boutons on these parts of the motoneuron. In the fourth paper (Conradi 1969 d), the changes after dorsal root section are studied. The population of boutons on the motoneurons six weeks after homolateral dorsal root section is studied and the findings compared with the normal state. It is concluded that two types of boutons disappear and the normal structure of these boutons is analyzed through serial sections. The last two papers deal with the postnatal development of the above-mentioned parameters and the results are discussed against the background of earlier physiologic findings. In the fifth paper (Conradi and Skoglund 1969 a), the development of the ultrastructure of the neuronal and glial extensions on the motoneurons is described beginning at the newborn stage. The size and distribution of the boutons of different types on the motoneurons in the newborn and at three weeks are analyzed in the same region of the ventral horn as in the adult. The proximal part of a motoneuron dendrite in the newborn stages is studied through serial sections. In the sixth paper (Conradi and Skoglund 1969 b), the postnatal development of the initial motoraxon segment and the dorsal root boutons is presented.

The lumbosacral motoneurons of the adult cat have been extensively studied with physiologic methods. Many studies with the light microscope have given a rather complete picture of the morphology of these cells. Earlier investigations (v. Lenhossek 1895, Ramon y Cajal 1909, for references, see Bok 1928) have been extended through detailed studies on the size-distribution (Schadé and van Harreveld 1961, Mellström and Skoglund 1969) and arrangement of the dendrites of the motoneurons (Lhermitte and Kraus 1925, Weil 1927, Chu 1954, Aitken and Bridger 1961, Scheibel and Scheibel 1966). The peripheral connexions of different motoneurons (Romanes 1951, Balthasar 1952) and the cytoarchitectonic organization of the ventral horn (Rexed 1954) have been worked out. The boutons on the motoneurons have also been investigated with light microscopy (Held 1897, Auerbach 1898,

Hoff 1932, Gibson 1932, Phalen and Davenport 1937, Lorente de No 1938, Barr 1939, Barnard 1940, Minckler 1940, Schadewald 1941, Haggar and Barr 1950, Aitken and Bridger 1961, Gelfan and Rapisarda 1964, Illis 1964) and with electron microscopy (see for example Wyckoff and Young 1956, Rosenbluth 1962, Gray 1963, Bodian 1964, 1966 a, b, Charlton and Gray 1966, Uchizono 1966, Ralston 1967). From the latter studies on several species, the boutons on the motoneurons have been shown to be of different morphologic types and it has been suggested that the morphologic typing has a physiologic counterpart (Bodian 1966 a, b, Uchizono 1966).

In the present study, the ultrastructure of the boutons and other extensions on the motoneuron cell membrane and in the surrounding neuropile is studied in the lateral motoneuron nucleus in segment L 7 (Peroneus: Romanes 1951, Balthasar 1952), after perfusion with buffered glutar aldehyde. The above-mentioned earlier classification of the boutons is used and somewhat extended. The different bouton types are followed through serial sections, but the types are, as far as possible, defined from the appearance in single sections, so that all bouton profiles in a section could be typed.

The size and distribution on the motoneurons of the boutons of different types is then investigated quantitatively on single sections. The technique and the results are used for the evaluation of the changes after dorsal root section and during the postnatal development. A complete mapping of the number and types of boutons on the motoneurons would require lengthy series of consecutive sections, which work would be too time-consuming at present. Instead, an attempt has been made to estimate the size and distribution of the boutons from single sections. The following factors seem to be of importance for such a study:

a) The work must be performed at a strictly localized region, so that values from different animals can be compared.
b) Neurons with the same morphologic characteristics should be used and the countings made at defined areas of the neurons.
c) The plane of section ought to be the same in all animals used.

The present study was performed on transverse sections through the mid-nucleus level of motoneurons in the lateral motoneuron nucleus in the most cranial millimeter of L 7. This region of the spinal cord was used since direct comparisons with earlier data on the postnatal development of some quantitative parameters (Mellström and Skoglund 1969) can be made, and because the population of cells in the different animals are topographically, thus functionally related.

MATERIAL AND METHODS

33 adult cats of both sexes were used. The fixation method has been published previosuly (Berthold 1968) in an ultrastructural investigation on the spinal roots on roughly the same material as that used here. Briefly, the method was the following:

Fixation method

a) Fixative: 5%, or in a few cases 2.5% glutar aldehyde was used throughout. In most cases, the stock solution, containing 25% glutar aldehyde in water (KEBO, Stockholm) was purified with active charcoal (Fahimi and Drochmans 1965, Anderson 1967) before use. The osmolality of 5% purified glutaraldehyde in distilled water, as determined cryoscopically, was about 500 mOsm.

The purified glutaraldehyde was dissolved either in the buffer system of Millonig (see Karlsson and Schultz 1965), which has an osmolality of 440 mOsm at pH 7.4, or during the earlier experiments, in a phosphate buffer of similar composition. The Millonig buffer was mostly diluted with distilled water so that the osmolality of the buffer in the fixative was 200—300 mOsm, thus isotonic to cat serum (Handbook of Biological Data 1956). Consequently, the total osmolality of the fixative was about 800 mOsm at pH 7.4.

In 4 animals, the buffer was 100 mOsm and 500 mOsm, respectively. In about 2/3 of all animals, 2.7%, or in a few cases, 8.1% Rheomacrodex® (Pharmacia, Sweden) was added to the fixative and rinsing fluid.

b) *Perfusion technique:* The cats were anaesthetized intraperitoneally with pentobarbital, 40 mg/kg body weight, and kept on artificial respiration through a tracheal cannula. The chest was opened and the left lung removed. One of the following two methods was then used:

1) (10 animals). During the earlier part of the study, the aortic arch was clamped, the descending aorta opened, and the perfusion cannula, a teflon tube, was inserted downward. The right auricula was opened and the coeliac, mesenteric and femoral arteries were clamped. Rinsing fluid (Ringer's solution) and the fixative were admin*istered from 50 ml syringes in a manner similar to that described by Osvaldo and* Latta (1966). 50 ml rinsing fluid and 100 ml fixative, both at 37°C, and 400 ml icecold fixative were perfused for 15 min. In this method the ionic strength of the buffer in the fixative was 360 mOsm.

2) In 23 animals, the perfusates were administered from three complete infusion sets, containing Ringer's fluid at 37°, fixative at 37° and icecold fixative, respectively, and connected to the perfusion catheter through a three-way stopcock. Each set was connected *to a blood pressure balloon and a manometer, by which the pressure in the sets could* be changed between zero and 300 mm Hg and continuously controlled. The fluid level in the sets was 10—20 cm above the heart level of the animals. The perfusion catheter, a sharpened teflon tube, was inserted directly downward into the descending aorta *through the arterial wall: the rinsing fluid was immediately delivered. The inferior* v. cava was opened and the coeliac and mesenteric arteries clamped. The perfusion pressure was kept at 100 mm Hg during the rinse (about 15 sec.). During the fixation,

the pressure was either constantly maintained at 150 mm Hg (2 animals) or 70 mm Hg (2 animals) or brought to fluctuate periodically between about 50 mm Hg and zero, 2—3 times per minute (15 animals). 50 ml prerinse at 37°C, 100 ml fixative at 37° and 500—1000 ml icecold fixative were perfused for 20—30 minutes. In these animals, the ionic strength of the buffer was 250 or 300 mOsm. The majority of the operated cats (Conradi 1969 d) were fixed in the same way. Some of the observations refer to the unoperated side of these cats. The above-mentioned procedure was also used with buffers of 500 mOsm and 100 mOsm. Rheomacrodex was used only with this fixation technique.

c) *Postfixation*: Immediately after the perfusion, the spinal canal was opened and the lumbosacral part of the spinal cord excised. Thin transverse slices of the segment L 7 were cut and divided into two symmetric halves, which were postfixed for four hours in a fixative quite similar to the perfusate, but lacking Rheomacrodex. After a rinse (30 min.) in icecold veronal acetate buffer (Andersson-Cedergren 1959) or 300 mOsm Millonig buffer, the blocks were postfixed in icecold 2% OsO_4 in veronal acetate, or 300 mOsm Millonig buffer. After another rinse for 30 min., the tissue blocks were dehydrated in increasing concentrations of acetone for a total of 12 hours, then embedded in Vestopal W.

d) *Sectioning:* On an LKB 4801 Ultrotome, semithin transverse sections of half the spinal cord were prepared; the sections were stained with toluidine blue and examined in the light microscope. The lateral part of the ventral horn was trimmed out and cut into ultrathin sections. In some cases, vertical sections of the lateral motor column were also studied. The ultrathin sections were collected on one-hole grids according to the method of Galey and Nilsson (1966). In most cases, 5—15 consecutive sections were collected on the grids. Longer series, comprising 50—300 consecutive sections, in which a few random single sections were lost, were also studied. Neither in the present nor in the forthcoming papers, were any attempts made to calculate the tissue compression, or errors due to an uneven plane of section, at sectioning. The ultrathin sections were stained with uranyl acetate and lead citrate and examined in an RCA 3A, Philips EM 200 or Philips EM 300 electron microscope.

Fixation quality

The quality of the fixation could be roughly determined by macroscopic examination of the spinal cord. After successful perfusions, the cord was an even yellow colour, firm, and somewhat elastic; the blood vessels on the surface were filled with a clear fluid. The gray substance was usually better fixed than the white, the latter often exhibiting small white spots. In the light microscope, lacunae around the capillaries, extracellular rims around the neuron perikarya and/or markedly split myelin were observed in some specimens. Such phenomena were taken as indications of an unsatisfactory fixation.

Ultrastructurally, the general appearance of the gray matter in well fixed material was similar to that reported earlier from the CNS (Karlsson and Schultz 1965), after aldehyde perfusion. Common criteria for a good ultrastructural preservation of central nervous tissue (Palay *et al.* 1962, Pease 1964, Karlsson and Schultz 1965) were applied in evaluating the different fixation methods. With all methods it was very difficult to

obtain a proper fixation of the heavily myelinated motoraxons (see Conradi 1969 c). They nearly always showed splitting of the myelin sheath.

Comparison of the fixation methods

The general ultrastructural appearance of the gray matter with regard to membrane relationships was rather constant, when buffers between 100 mOsm and 500 mOsm were used (cf. Karlsson and Schultz 1965, Johnston and Roots 1967, Westrum 1967). With stronger buffers, however, the outline of the neuron cell bodies and dendrites was often somewhat wrinkled, as compared to the smooth outline, obtained with hypotonic or isotonic buffers. The addition of Rheomacrodex to the fixative did not change the fixation quality markedly, but the fixation of the white matter seemed to be improved.

The quality of the fixations was related to the perfusion method. With the first method mentioned above, in which the aorta was clamped before perfusion, a few experiments resulted in excellent fixation, but usually the result was poor. Insertion of the perfusion catheter directly into the aorta, without previous clamping, produced the following results:

Perfusions performed at a constant pressure, regardless of whether a pressure of 150 or 70 mm Hg was used, resulted in a rapid flow of fixative through the animal and the fixation was very poor. The spinal cord was soft and white, while the spinal muscles were often brown and hard. When, however, the perfusion pressure was varied periodically between about 50 mm Hg and zero, the fixation of the cord was good. In these cases, the deep spinal muscles appeared hard, although the superficial ones seemed nearly unfixed.

Quantitative study

Two animals were used for the quantitative investigation. Equal halves of the material were taken from each of the animals, which were fixed through perfusion at a discontinuous pressure, with 5% purified glutaraldehyde in 300 mOsm Millonig buffer. 2.7% Rheomacrodex was added to the rinsing fluid and fixative. Consecutive semithin transverse sections were cut in cranio-caudal direction, through a specimen from the most cranial millimetre of L 7, in animals in which the size of the lumbosacral roots, on macroscopic examination, seemed to indicate a prefixed lumbosacral plexus (Sherrington 1892).

When a motoneuron was cut through the nucleus, 2—4 grids with ultrathin sections, the depth of some microns apart, were made. About half of the specimens were cut through in this way The ultrathin sections of each animal included, in total, about 20 *motoneuron cell bodies, which were cut roughly through the middle of the nucleus,* judging from inspection of the actual profile in the electron microscope. On the fluorescent screen there were, namely, some markings, through which it was possible to control that the profiles of all the motoneuron nuclei had about the same diameter as the profiles of those nuclei which were cut through the nucleolus. The nucleolus was cut through in about half the cases. Profiles of motoneurons, both those in which long profiles of proximal parts of the dendrites were included and those in which no dendrite was seen to project, were chosen from these sections. The profiles of 10 motoneurons were photographed in each animal. Three long profiles of proximal

parts of dendrites, projecting from motoneurons, in which the nucleus had not been ideally cut, plus 15 long profiles of thin, longitudinally-sectioned dendrites from the neuropile just outside the motoneurons, were also photographed in each of the animals. Thus, the majority of the countings on the dendrites refers to horizontally-directed processes. The origin of the thin dendrites could not be determined; nevertheless, an investigation of the membrane relationships of the thin dendrites was thought to be of interest since at least some of them ought to belong to motoneurons (see the discussion). The primary magnification of the photographs was about 3000X and the paper magnification 5X. This magnification was chosen since the surface contact of every bouton with the actual profiles could easily be recognized. The copies were taped together to montages. Usually 5—10 copies were required to cover the circumference of one motoneuron cell body. On the montages, the border between the motoneuron cell body and the dendrites, roughly defined as the inflexion point of the membrane in the plane, was demarcated.

The long profiles of proximal dendrites were divided into two parts. The most proximal part, (DA), was defined as the region covered at one side by the first 20 boutons, counting from the cell body. The part distal to this was denoted DB. The cell body was depicted SO and the thin dendrites d. Hereafter, the relationships of these regions of the motoneuron were treated separately. When proximal parts of dendrites had been cut obliquely, forming only a slight hump on the profile of the cell body, the membrane relationships were included in the DA group.

The dimensions of the profiles of the cell bodies and dendrites were measured on the montages. The least diameter was noted for the cell bodies, since occasional ovoid contours were thought possibly to have been due to the nearness of the bases of a thick dendrite, which was out of the plane of the section. The diameter of the nucleus and presence of nucleolus were noted. The diameter was measured on the dendritic profiles except in those cases, in which only very short stumps projected from the motor cell bodies. The length of the membrane of every profile was measured with a rolling map distance measurer.

The type of every bouton or other tissue component on the cell bodies and dendrites was determined through direct inspection of the actual section at high magnification in the electron microscope and the result of this typing was depicted on the montages. Finally, the length of apposition to the profiles of the motoneurons and dendrites, of the different boutons and other tissue components and the height of every bouton profile were measured on the montages. The values were set up in table form. Apart from the total number of bouton profiles on every motoneuron or dendritic profile, the relative proportion and mean length and height of the different bouton types was calculated. The membrane covering of the boutons on the profiles was expressed either as

$$\frac{\text{total length of apposition of the boutons}}{\text{membrane length}} \quad \text{or} \quad \frac{\text{membrane length}}{\text{no. of boutons}}.$$

The former was calculated both for all boutons, or for the different types and requires knowledge of the dimensions of the boutons. The latter, which can be translated as mean distance along the membrane between the centres of adjacent boutons, does not require knowledge of the size of the boutons. The tables in the present and the following papers (Conradi 1969 d, Conradi and Skoglund 1969 a) are

neuron cell membrane, but they are most often found at a greater distance, in the surrounding neuropile (Fig. 2). Proximal parts of the motoneuron dendrites are covered by astrocytic extensions to a lesser extent than the cell bodies. A greater proportion surface area of the proximal parts of the dendrites is covered by boutons. The dendrites in the neuropile are surrounded by many flattened astrocytic extensions.

Oligodendrocytes (see Magnaini and Walberg 1964, Kruger and Maxwell 1966, Wendell-Smith et al. 1966) are usually found in the neuropile (Fig. 2) or as satellite cells at the motoneuron cell bodies (Fig. 4), but seldom at proximal parts of the dendrites and thin dendritic profiles. In nearly all cases, a thin astrocytic lamella is interposed between the cell membrane of the oligodendrocyte and the motoneuron (Fig. 4). In fact, direct apposition between oligodendrocytic and neuronal cell membranes, except at the myelin, is seldom observed anywhere in the neuropile. Some glial cells, similar to microgliacytes, were also observed in the neuropile (Fig. 2) (for references and further information see Conradi 1969 c and d).

Myelinated fibres and capillaries were sometimes observed just outside the motoneuron cell membrane, but in the majority of the cases, thin astrocytic lamellae were interposed.

Dendrites

At some places, most often at the dendrites, the motoneuron membrane has immediate contact with adjacent dendritic profiles. Two types of contacts were observed.

The most common type consists of a mere apposition of the motoneuron cell membrane and an adjacent dendrite without any visible membrane specialization and little, if any, extracellular material interposed (Fig. 5). The second type consists of a desmosome-like arrangement, similar to the contact between adjacent neurons in the lateral geniculate nucleus as described by Peters and Palay (1966), which shows a symmetric apposition of contrasting material at the cytoplasmic side of the membranes and an intervening 100—200 Å wide cleft, containing a dense material (Fig. 6). In addition to this, two adjacent dendritic profiles, separated only by a single flattened astrocytic process, were often observed.

Boutons

In an extensive review of the ultrastructure of synapses, the term "synaptic complex" is defined as a region of specialized contact between two neuronal

extensions (Gray and Guillery 1966). The presynaptic extension is most often an axon enlargement, named bouton or synaptic knob, which is filled with synaptic vesicles. This specialization exhibits the following characteristics:

a) A presynaptic membrane, at the inner side of which the synaptic vesicles and usually a thin layer of contrast-rich material are apposed.
b) A synaptic cleft, which has a uniform thickness and seems usually to contain a contrast-rich material.
c) A postsynaptic membrane, which is covered by a layer of contrast-rich material.

The above-mentioned criteria for the recognition of synaptic complexes have been adopted in the present study and proved very useful for an analysis of the synaptic complexes on the motoneurons.

General features of the synaptic complex. The synaptic cleft usually occupies only a minor part of the apposition region between the boutons and the motoneurons cell membrane, and except at C boutons (see below), it very seldom reaches the periphery of the apposition region (Figs. 7 and 8). The contrast-rich material at the presynaptic membrane is usually less pronounced than at the postsynaptic membrane. Sometimes "dense projections" (Gray 1963) could be observed at the presynaptic membrane. In boutons with a large synaptic cleft, the synaptic vesicles are most often apposed only at part of the presynaptic membrane. The contrast-rich material filling the synaptic cleft is very dense and homogeneous in well-fixed specimens (Fig. 8).

As revealed by serial sectioning, practically all boutons seemed to establish a synaptic complex or a structure similar to this, many of them establishing more than one synaptic complex with the motoneuron cell membrane. It is rather seldom observed that boutons in synaptic contact with a motoneuron profile make synaptic contact with still another profile. When observed, such "double synapses" occur most frequently at thin dendritic profiles in the neuropile (Fig. 7).

Membrane relationships of the boutons

The convex side of the boutons is generally surrounded by thin astrocytic lamellae (cf. Wolff 1965) which can be observed in Fig. 13. Some of the boutons, especially bigger ones, are also contacted at the convex side by thicker astrocytic extensions. In many cases the glial encapsulation was incomplete and adjacent boutons were directly apposed (Fig. 8). In some rare instances, a desmosome-like junction was present at apposing boutons.

Near the synaptic complex "coated" vesicles and invaginations were sometimes observed (cf. Andres 1964). Lateral to the synaptic cleft, the cell membrane of the bouton and postsynaptic profile were mostly tightly apposed, and arrangement quite similar to that in earlier descriptions from various regions of the CNS. Small desmosome-like junctions (Hamlyn 1962, Karlsson 1966 b, Gray and Guillery 1966, Mugnaini *et al.* 1967) were frequently observed in this region (this is seen in Fig. 13) and seemed to be present at all bouton types. They extended for about 400—1000 A; the membranes were 100—200 A apart. These desmosomes were often isolated from the synaptic cleft, although in some cases they seemed to form the lateral border of the synaptic cleft. At low magnification, large desmosome-like figures are difficult to distinguish from synaptic complexes.

In many places, especially around motoneuron cell bodies, some of the boutons were not perfectly aligned to the postsynaptic membrane. Instead, a 200—700 A wide extracellular space (Fig. 9), commonly containing a fine, granular material, was interposed. Sometimes the space was observed to extend into the region between two adjacent boutons, where "coated" invaginations and vesicles could be found at the cell membranes. Such spaces were also observed, when a 100 mOsm buffer system was used. A small, flattened, membrane-bound sac was frequently situated below the cell membrane of the postsynaptic profile at these spaces (Fig. 9). Continuity between the sac and granular endoplasmic reticulum was noticed in some cases, and a dense material could often be observed between the sac and the motoneuron cell membrane (cf. Siegesmund 1968). Some boutons were apposed at spinous projections from the motoneurons (cf. Bodian 1964, 1966 b).

Preterminal axons. In single sections, connexions between preterminal axons and boutons are rather seldom observed. Many observations, both from single and serial sections, however, revealed that the majority of the preterminal axons are unmyelinated 2—5 μ, before the bouton (Fig. 10). This also holds true for the biggest boutons. Boutons *de passage* were rather infrequently found. At myelinated preterminal axons, a thin rim of extracellular material was observed around the axon just distal to the myelin sheath. A similar arrangement has previously been described at boutons in the rat brain (Karlsson 1966 b).

Different types of boutons

The boutons and the synaptic complexes adjoining them could be divided into several morphologic types, some of which have been described earlier

in the spinal cord of different species (Rosenbluth 1962, Gray 1963, Bodian 1964, 1966 a, b, Uchizono 1966, Ralston 1967). The description and abbreviations used by Bodian will be followed as far as possible in this study. Boutons containing spherical synaptic vesicles and in which the layer of postsynaptic material was of even thickness, were denoted S (spherical) (Fig. 11). In those cases where postsynaptic dense bodies were observed, the term T (Taxi) was used (Fig. 12). Boutons containing flattened synaptic vesicles were denoted F (flattened) (Fig. 13). Some of these contained a dense ground substance and often membrane-bound dense bodies; they were called FD (dense) (Fig. 14). Big boutons, containing spherical synaptic vesicles, and in which a subsynaptic cistern was situated below the boutons, were denoted C (cistern) (Fig. 16). Some big boutons containing synaptic vesicles showed a large synaptic complex of irregular shape, almost completely covered by the postsynaptic bodies of Taxi (1961). Since these boutons seemed to disappear after dorsal root section (Conradi 1969 d), they were denoted M (monosynaptic) (Fig. 18). At the convex side of these and apparently at some big S boutons, small boutons, containing small vesicles of irregular shape, were apposed; these boutons were designated P (presynaptic) (Fig. 18). Some boutons, often those with spherical synaptic vesicles also contain dense-cored vesicles, 700—100 A in diameter (Fig. 20). These dense-cored vesicles are usually not apposed at the presynaptic membrane. Since the relative number of such vesicles varies between the boutons, it is difficult to define a separate bouton type based on the presence of these vesicles. Recent work indicates that large, dense-cored vesicles form an unspecific characteristic, not believed to be linked to, for instance, any particular transmitter substance (Pellegrino de Iraldi and de Robertis 1968, Hökfeldt 1968).

The different types of boutons were investigated through serial sections. Generally, the shape of the synaptic vesicles in each bouton remained constant throughout the series. In a few cases, some of the vesicles inside the same bouton appeared spherical, others appeared to be flattened. These were classified as belonging to the F-type.

In nearly all instances, it is rather easy to distinguish S from F boutons on single sections when the tissue is well fixed. A curious difference between F boutons and the other types was noted in this study. When the specimens were rinsed and osmificated in veronal acetate buffer, a fine, granular precipitate was often present in the tissue, even in unstained sections. The precipitate was usually concentrated on membranes, mitochondria and synaptic vesicles. On the synaptic vesicles, however, it was much more intense in F

than S boutons (Fig. 21). When Millonig buffer was used instead of the veronal acetate, most of the precipitate disappeared.

In some big boutons, mostly those containing spherical synaptic vesicles, neurofilaments could be observed. These frequently formed a bundle, which seemed to be continuous with the neurofilaments inside the preterminal axon. Membrane-bound dense bodies were observed in some boutons, often those of the FD-type.

The S-type. (Fig. 11). Many 0.5—4 μ long profiles of boutons containing spherical vesicles are observed at all regions of the motoneuron cell membrane. The diameter of the vesicles is 350—500 A and these boutons generally exhibit a clear ground substance. The synaptic cleft is about 150 A thick, and the layer of postsynaptic material varies somewhat in thickness, between 50 and 200 A. Some contrast-rich material is usually attached to the presynaptic membrane.

The T-type. (Fig. 12). The boutons exhibiting postsynaptic dense bodies have the same general characteristics as the S-type. The synaptic cleft is often somewhat larger and about 150 A thick. Especially at thin dendritic profiles, boutons of this type show comparatively large synaptic complexes. There is a 200—300 A thick layer of postsynaptic dense material, below which the postsynaptic dense bodies are situated; 3—7 of these bodies can ordinarily be seen in one section. This type of synaptic complex has earlier been found in the spinal cord (Gray 1963, Bodian 1964, 1966 b) and in other regions of the CNS. In the present material, it was often observed that several boutons showing this type of synaptic complex were situated near each other.

In some cases, the same bouton made synaptic complexes of both the S- and T-type (see Conradi 1969 b). In the quantitative estimation, such boutons were classified as the T-type.

The F-type. (Fig. 13). Boutons containing flattened synaptic vesicles, 200—300×400—600 A in diameter (cf. Uchizono 1966, Bodian 1966 b), are intermingled with boutons of other types on all regions of the motoneuron surface. These boutons nearly always contain a clear ground substance, usually each bouton establishes several synaptic complexes. The synaptic cleft, 90—130 A thick, is narrower than at the S-type (cf. Ralston 1967). A thin layer of contrast-rich material, 50—150 A thick, is attached to the postsynaptic membrane (cf. Ralston 1967, Colonnier 1968). As a rule, no postsynaptic bodies of Taxi were observed below boutons with flattened

vesicles. The synaptic clefts of the F boutons are either small, roughly 0.1—0.5 μ, or large, roughly 0.5—2 μ (Figs. 13 and 15).

Some of the F boutons with the large synaptic cleft could be very big; usually showing a dense ground substance containing dense membrane-bound profiles and often small, circular, contrast-rich profiles (Figs. 14 and 15). Such boutons could be seen to be partially invaginated in the postsynaptic profile. The number and appearance of these boutons, which were denoted FD, varied between the specimens; they were therefore not included in the statistical evaluations as a separate entity.

The C-type. (Figs. 16 and 17). On the motoneuron cell bodies and proximal parts of the dendrites, 3—7 μ long profiles of boutons are often observed. These boutons form the biggest bouton type on the motoneurons and, as Bodian (1964) noted, two or more of these boutons are frequently grouped together. The type has also been described by Rosenbluth (1962), Gray (1963), Bodian (1964, 1966 b), Charlton and Gray (1966) and van Harreveld and Khattab (1967). The boutons are filled with tightly-packed spherical vesicles, 350—500 A in diameter, among which some dense-cored vesicles are often intermingled. A subsynaptic cistern is located just inside the postsynaptic membrane. The synaptic cleft, which is very large and very narrow, 30—80 A, extends under almost the entire bouton. Furthermore, no material is attached at the pre- or postsynaptic membranes, and the synaptic vesicles are not apposed along the whole of the presynaptic membrane. The very flat subsynaptic cistern extends inside the entire postsynaptic membrane and sometimes the cistern is seen to communicate with the sacs of granular endoplasmic reticulum. One single cistern may extend below 2 or 3 adjacent boutons. Below the cistern, the postsynaptic cytoplasm often contains granular endoplasmic reticulum. This is especially prominent on the proximal parts of the dendrites, where several sac-like profiles of the endoplasmic reticulum can often be seen. These profiles are oriented parallel to the cell membrane, which frequently bulges below the boutons (Fig. 16). No C boutons were observed on the smaller neurons in the neuropile. The presence of C boutons was, in fact, one of the criteria for distinguishing the neurons, assumed to be motoneurons, in this study.

The M-type. (Figs. 18 and 19). At proximal parts of the motoneuron dendrites, profiles of big boutons, 4—7 μ in length, and containing spherical synaptic vesicles, 350—500 A in diameter, are sometimes found. The vesicles are usually somewhat scattered and distributed throughout the bouton, which otherwise exhibits a very clear ground substance. These boutons form large

synaptic complexes of irregular shape, in which the whole postsynaptic membrane is covered by a 200—300 A thick layer of contrast-rich material and the postsynaptic dense bodies. Several profiles of synaptic complexes are commonly seen in one section, where, due to this, 15—20 postsynaptic dense bodies often can be visible below one bouton. In some cases, M boutons were situated at several dendritic spines. On the convex side of these boutons, small P boutons, containing small vesicles of irregular shape, 250—400 A in diameter, are regularly apposed. The large size of the extension of the synaptic complex and the presence of the P boutons differentiates the M from the T boutons.

When longer series of consecutive ultrathin sections are studied (Conradi 1969 b, d), P boutons are sometimes also found to be apposed at big boutons containing spherical vesicles. These boutons have then to be classified as belonging to the S-type, since they do not exhibit the large synaptic complex covered by a great number of postsynaptic dense bodies, typical for the M-type. In the quantitative estimation, all boutons on which P boutons were apposed were classified under the same heading. In single sections, it is not always possible to judge whether the actual bouton profile is an M bouton, sectioned outside the synaptic cleft, or a big S bouton, of the variety just mentioned.

Sometimes the P boutons were apposed both at one of these big boutons and at the motoneuron cell membrane. If these P boutons were cut through and no synaptic complex of the bouton could be seen, it was not possible to judge by inspection alone whether the profile was of the P- or F-type. No separate heading for the P boutons was used in the quantitative estimation, which accounts for some P boutons possibly having been included under the F heading in the quantitative study of single sections.

The M boutons and the big S boutons, having P boutons apposed at the convex side, will be further dealt with in connexion with the findings after dorsal root section (Conradi 1969 d).

Quantitative estimations

The size and distribution on the motoneurons of bouton profiles of the different types was investigated on single transverse sections through the lateral motoneuron nucleus, in the most cranial millimeter of L 7 (see Material and Methods). Four regions were investigated: the motoneuron cell bodies (SO), the proximal parts of the motoneuron dendrites (DA, DB) and small, longitudinally-sectioned dendritic profiles in the neuropile (d). The

bouton profiles have been typed according to the criteria given above. Data from the different motoneuron profiles are presented in Table 1. The ensuing description will concentrate on the mean values of the different parameters presented in Table 2.

Profiles of cell bodies and dendrites (Table 2)

The mean least diameter of the cell bodies was about 49 μ, a figure which agrees well with that in the earlier light microscopic studies from this region (Mellström and Skoglund 1969). There are some variations between the different cells. Since the sections cut the cell bodies at a defined level (see Material and Methods), the diameters obtained here are probably a closer estimate of the true diameter than is the case for the dendrites, which were sectioned at random. The mean diameter of the dendritic profiles decreases with increasing distance from the cell body, and the mean value for the diameter of the dendrites in the neuropile is less than half that from the proximal parts of the motoneuron dendrites.

Location of the different bouton types (Table 2)

S, T and F boutons are found on all regions of the motoneuron surface. There is an increase in the relative proportion in the number of apposing F boutons in the sections from the distal dendrites towards the cell body, where these boutons form more than half the number of apposing boutons. The situation for the proportion of apposing S boutons is the reverse. T boutons are most frequently encountered at thin dendritic profiles. C boutons are restricted to motoneuron cell bodies and proximal parts of the dendrites: about twice as many occur at the former location. The M boutons are almost exclusively found at the proximal parts of the motoneuron dendrites. Thus, each of the regions of the motoneurons seems to be surrounded by a specific spectrum of the various types of boutons. The occurrence in the sections of bouton profiles of these types is, of course, dependent upon the size of the boutons. There is a greater chance for big boutons to be included in the sections. This must be taken into account when estimating the true numerical proportion of boutons of different types on the regions of the motoneurons (see the Discussion). The most appropriate way to express the population of boutons seems to be as the percentage of bouton covering on the cell membrane of the motoneuron cell bodies and dendrites. This expression can be calculated both for all boutons as well as for each type separately, and is independant of the size of the boutons (see below).

Table 1. Quantitative estimation

Cell no. 1–10, D 1–3, d 1–15 = Cat 1

Cell no. 11–20, D 4–6, d 16–30 = Cat 2

		1			2			3			4			5			6		
		SO	DA	DB	SO	DA	DB	SO	DA	DB	SO	DA	DB	SO	DA	DB	SO	DA	DB
Diameter, μ	1	38.2	10.7	6.9	47.2	10.4		47.9	12.8	11.4	43.0	9.5		49.6	9.5	7.6	46.8	7.5	
Membrane length, μ	2	147.2	156.9	101.7	145.1	98.6		132.6	153.4	35.4	141.6	178.8		133.6	198.6	47.2	174.3	148.6	
Boutons																			
S, number	3	9	17	14	15	15		15	26	7	12	13		14	36	10	11	16	
T, number	4		1	2					1		1	6			1	1	2	1	
F (+ FD), number	5	14	42	25	20	22		16	26	7	25	36		19	39	14	19	25	
C, number	6	5		1	4	5		3	5		3	4		3			3		
M and big S, number	7							1	1									1	
Spines, number	8																		
Apposing dendrites																			
number	9					1													
length, μ	10					1.7													
Apposing oligodendrocytes																			
number	11													1					
length, μ	12													4.2					
Boutons, total number	13	28	60	42	39	42		35	59	14	41	59		36	76	25	35	43	
Boutons, total length, μ	14	57.3	114.2	79.4	70.3	90.7		77.6	131.5	26.9	79.5	118.1		66.7	144.6	42.9	90.6	101.0	
Membrane length, μ / Boutons, total number	15	5.3	2.6	2.4	3.7	2.3		3.8	2.6	2.5	3.5	3.0		3.6	2.6	1.9	5.0	3.5	
Boutons, total length, μ / Membrane length, μ	16	0.39	0.73	0.78	0.48	0.91		0.56	0.86	0.76	0.56	0.66		0.50	0.73	0.91	0.52	0.68	

	7			8			9			10			11			12			13		
	SO	DA	DB	SO	DA	DB	SO	DA	DB	SO	DA	DB	SO	DA	DB	SO	DA	DB	SO	DA	DB
1	48.6	9.5		41.6	10.4	12.5	43.7	11.8	6.9	47.2	6.4		46.1	11.8	5.5	41.6	6.9		59.0	15.6	11.1
2	144.4	131.9		122.2	193.7	132.6	133.3	143.0	36.1	145 8	171.1		165.2	136.8	49.3	180.5	47.9		180.5	92.0	40.6
3	9	22		17	26	15	8	19	6	10	22		15	28	10	14	3		12	11	4
4		3		1	2	3			2		1		1	1	1					1	
5	14	18		18	33	13	11	26	8	26	26		24	31	11	22	4		24	22	9
6	4	2		2	1	2				2	1			2		4	1		3	3	
7		2			2	1		2			2										
8											1										
9									1												
10									1.3												
11																					
12																					
13	27	47		38	64	34	19	47	16	38	52		40	62	22	40	8		39	37	13
14	55.8	97.3		87.6	137.4	89.6	32.6	88.1	29.7	78.4	116.7		76.3	104.5	37.4	100.6	24.8		89.9	76.8	28.8
15	5.3	2.8		3.2	3.0	3.9	7.0	3.0	2.3	3.8	3.3		4.1	2.2	2.2	4.5	6.0		4.6	2.5	3.1
16	0.39	0.74		0.72	0.71	0.68	0.24	0.62	0.82	0.54	0.68		0.46	0.76	0.76	0.57	0.52		0.50	0.83	0.71

	14			15			16			17			18			19			20		
	SO	DA	DB	SO	DA	DB	SO	DA	DB	SO	DA	DB	SO	DA	DB	SO	DA	DB	SO	DA	DB
1	52.4	8.6		55.6	10.2	9.9	39.9	6.9		54.1	13.8	13.1	58.3	11.8		64.2	6.9		55.5	9.3	8.2
2	168.7	127.0		141.0	386.1	143.0	146.5	59.0		172.2	136.8	81.9	152.7	104.7		193.0	16.9		129.8	278.4	131.9
3	14	21		7	36	11	10	4		14	17	13	10	8		9	2		9	37	18
4	1				1								1	1					1	1	1
5	30	15		16	92	28	14	10		17	19	17	30	28		19			15	59	29
6	2	2		1	9	2	2			1	3		2	2		2			1	1	
7																				3	1
8																					
9																					
10																					
11																					
12																					
13	47	38		24	138	41	26	14		32	39	30	43	39		30	2		26	101	49
14	92.7	76.6		62.2	301.2	103.4	59.6	36.3		60.8	96.1	63.4	86.8	76.2		58.3	3.5		52.2	212.6	84.8
15	3.6	3.3		5.9	2.8	3.5	5.6	4.2		5.4	3.5	2.7	3.6	2.7		6.4	8.5		5.0	2.8	2.7
16	0.55	0.60		0.44	0.78	0.72	0.41	0.62		0.35	0.70	0.77	0.57	0.73		0.39	0.21		0.40	0.76	0.64

Table 1. (cont.)

		D1		D2		D3		D4		D5		D6	
		DA	DB	DA	DB	DA	DB	DA	DB	DA	DB	DA	DB
Diameter, μ	1	18.6	8.3	10.4	7.0	11.1		7.3		21.5	7.6	19.6	10.4
Membrane length, μ	2	122.2	239.6	54.9	239.6	99.7		136.5		223.3	160.4	135.4	51.4
Boutons S, number	3	20	47	13	27	12		23		19	17	23	7
T, number	4	1	3	1	2	1					1		2
F (+FD), number	5	26	51	9	39	19		30		26	32	34	10
C, number	6	2			6			2		1	2	4	
M and big S, number	7	1		1	2								
Spines, number	8											1	
Apposing dendrites number	9				2						1		
length, μ	10				5.6						3.5		
Apposing *oligodendrocytes, number*	11												
length, μ	12												
Boutons, total number	13	50	101	24	76	32		55		46	52	61	19
Boutons, total length, μ	14	87.2	184.2	43.2	155.7	59.5		116.5		95.5	115.9	115.7	37.5
$\frac{\text{Membrane length, } \mu}{\text{Boutons, total number}}$	15	2.4	2.4	2.3	3.2	3.1		2.5		4.9	3.1	2.2	2.7
$\frac{\text{Boutons, total length, } \mu}{\text{Membrane length, } \mu}$	16	0.71	0.77	0.83	0.69			0.85		0.43	0.74	0.85	0.73

Table 1. (cont.)

		d1	d2	d3	d4	d5	d6	d7	d8	d9	d10	d11	d12	d13
Diameter, μ	1	6.3	4.2	3.5	4.2	3.5	5.2	5.9	7.3	4.2	4.2	3.1	6.9	4.9
Membrane length, μ	2	205.6	103.1	86.1	60.4	64.6	107.6	104.2	137.5	120.1	103.5	55.6	209.0	109.7
Boutons: S, number	3	41	14	7	11	9	22	11	20	11	15	4	38	13
T, number	4		2	1	1		2	2	1	2	2	3	1	3
F (+FD), number	5	38	4	7	18	13	11	19	20	17	11	1	36	13
C, number	6								1	1				
M and big S, number	7								1	1				
Spines, number	8					1					1			
Apposing dendrites, number	9								1		1			
length, μ	10								2.8		0.3			
Apposing oligodendrocytes, number	11													
length, μ	12													
Boutons, total number	13	79	20	15	30	22	35	32	43	32	28	8	75	29
Boutons, total length, μ	14	126.3	34.9	25.0	45.1	37.9	65.3	57.8	93.2	65.5	41.0	12.7	119.2	45.6
$\frac{\text{Membrane length, } \mu}{\text{Boutons, total number}}$	15	2.6	5.2	5.7	2.0	2.9	3.1	3.3	3.2	3.8	3.7	7.0	2.8	3.8
$\frac{\text{Boutons, total length, } \mu}{\text{Membrane length, } \mu}$	16	0.61	0.34	0.29	0.75	0.59	0.61	0.55	0.68	0.55	0.40	0.23	0.57	0.42

Table 1. (cont.)

	d14	d15	d16	d17	d18	d19	d20	d21	d22	d23	d24	d25	d26	d27	d28	d29	d30
1	4.2	5.2	5.6	2.8	4.2	3.5	5.6	5.2	3.1	5.9	3.8	5.9	5.6	3.5	3.5	5.2	6.9
2	71.5	212.5	71.5	50.0	61.1	106.9	74.7	119.8	101.7	107.6	131.3	104.5	86.1	70.1	71.5	312.5	227.1
3	8	23	13	3	5	7	2	21	10	18	6	9	9	9	7	41	22
4	2	5				2	1	1		1	3	1		2		9	8
5	10	20	9	1	6	7	1	20	9	17	4	21	10	10	15	57	18
6								2									
7																	
8																	
9		1															
10		1.0															
11																	
12																	
13	20	48	22	4	11	16	4	44	19	36	13	31	19	21	22	107	48
14	36.9	101.4	34.9	4.9	25.1	25.1	5.4	77.2	27.8	61.0	25.2	66.8	39.9	43.1	41.9	200.6	100.8
15	3.6	4.4	3.3	14.6	5.6	6.7	18.7	2.7	5.4	3.0	10.1	3.4	4.5	3.3	3.3	2.9	4.7
16	0.52	0.48	0.49	0.10	0.41	0.24	0.07	0.64	0.27	0.57	0.19	0.64	0.46	0.61	0.59	0.64	0.44

Table 2. Quantitative estimation, mean values

	CAT 1				CAT 2				CATS 1+2			
	SOMA	DA	DB	d	SOMA	DA	DB	d	SOMA	DA	DB	d
Mean diameter, μ	45.4	10.7	8.7	4.9	52.7	12.7	9.4	4.7	49.0	11.7	9.0	4.8
Mean membrane length, μ	142.0	142.4	118.9	116.7	163.0	144.7	94.1	113.7	152.5	143.5	106.5	115.2
Boutons: S mean number	12.0	19.8	18.0	16.5	11.4	17.8	11.4	12.1	11.7	18.8	14.7	14.3
proportion of all bouton profiles, %	35.7	39.2	40.9	47.9	32.9	36.2	35.4	43.6	34.3	37.7	38.6	46.0
mean length, μ	1.7	1.7	1.6	1.6	1.5	1.8	1.9	1.7	1.6	1.7	1.7	1.6
mean height, μ	*0.84*	*0.88*	*0.81*	*0.70*	*0.98*	*1.0*	1.1	0.94	0.90	0.94	0.91	0.80
T mean number	0.4	1.5	1.9	1.8	0.4	0.4	0.7	1.9	0.4	0.9	1.3	1.8
proportion of all bouton profiles, %	1.2	2.9	4.2	5.2	1.2	0.8	2.2	6.7	1.2	1.9	3.4	5.9
mean length, μ	1.8	2.2	1.8	2.0	1.9	2.8	1.5	2.2	1.9	2.3	1.7	2.1
mean height, μ	1.1	1.2	0.70	0.90	0.76	1.2	1.2	1.0	0.93	1.2	0.84	0.97
F (+FD) mean number	18.2	26.7	22.4	15.9	21.1	28.5	19.4	13.7	19.7	27.6	20.9	14.8
proportion of all bouton profiles, %	54.2	53.0	51.0	46.1	60.8	57.7	60.2	49.2	57.5	55.3	54.9	47.5
mean length, μ	2.1	2.1	2.0	1.8	2.2	2.1	2.3	1.9	2.1	2.1	2.1	1.9
mean height, μ	0.96	1.0	0.95	0.97	1.0	1.1	0.99	1.1	1.0	1.1	0.97	1.0
C mean number	2.9	1.5	1.3	0.1	1.8	2.3	0.6	0.1	2.4	1.9	0.9	0.1
proportion of all bouton profiles, %	8.6	3.1	2.9	0.4	5.2	4.7	1.8	0.5	6.9	3.9	2.4	0.4
mean length, μ	3.8	3.3	5.2	5.0	5.7	5.4	5.7	3.9	4.5	4.5	5.4	4.5
mean height, μ	1.0	1.1	0.96	0.97	1.4	1.4	1.3	1.4	1.2	1.2	1.1	1.2
M and big S, mean number	0.1	0.9	0.4	0.1		0.3	0.1		0.05	0.6	0.3	0.07
proportion of all bouton profiles, %	0.3	1.8	1.0	0.4		0.6	0.4		0.1	1.2	0.7	0.2
mean length, μ	5.7	4.5	3.7	4.0		4.0	5.8		5.7	4.4	4.2	4.0
mean height, μ	1.5	1.5	1.3	1.4		1.7	1.9		1.5	1.5	1.7	1.4
mean number		0 08		0.1						0.04		0.07
ng dendrites mean number		0.08	0.4	0.2			0.1			0.04	0.3	0.1
mean length, μ		1.7	4.2	1.4			3.5			1.7	4.0	1.4
osing oligodendrocytes mean number	0.1								0 05			
mean length, μ	4.2								4.2			
otal number of boutons	33.6	50.4	44.0	34.4	34.7	49.3	32.3	27.8	34.2	49.8	38.1	31.1
Total length of boutons, μ	69.8	101.8	86.6	60.5	73.6	105.1	70.5	51.7	71.8	103.2	78.5	56.2
membrane length, μ / total no. of boutons	4.2	2.8	2.7	3.4	4.7	2.9	2.9	4.1	4.5	2.9	2.8	3.7
total length of boutons, μ / membrane length, μ	0.49	0.72	0.73	0.52	0.45	0.73	0.74	0.46	0.470	0.721	0.737	0.488

Mean size of the different bouton types (Table 2)

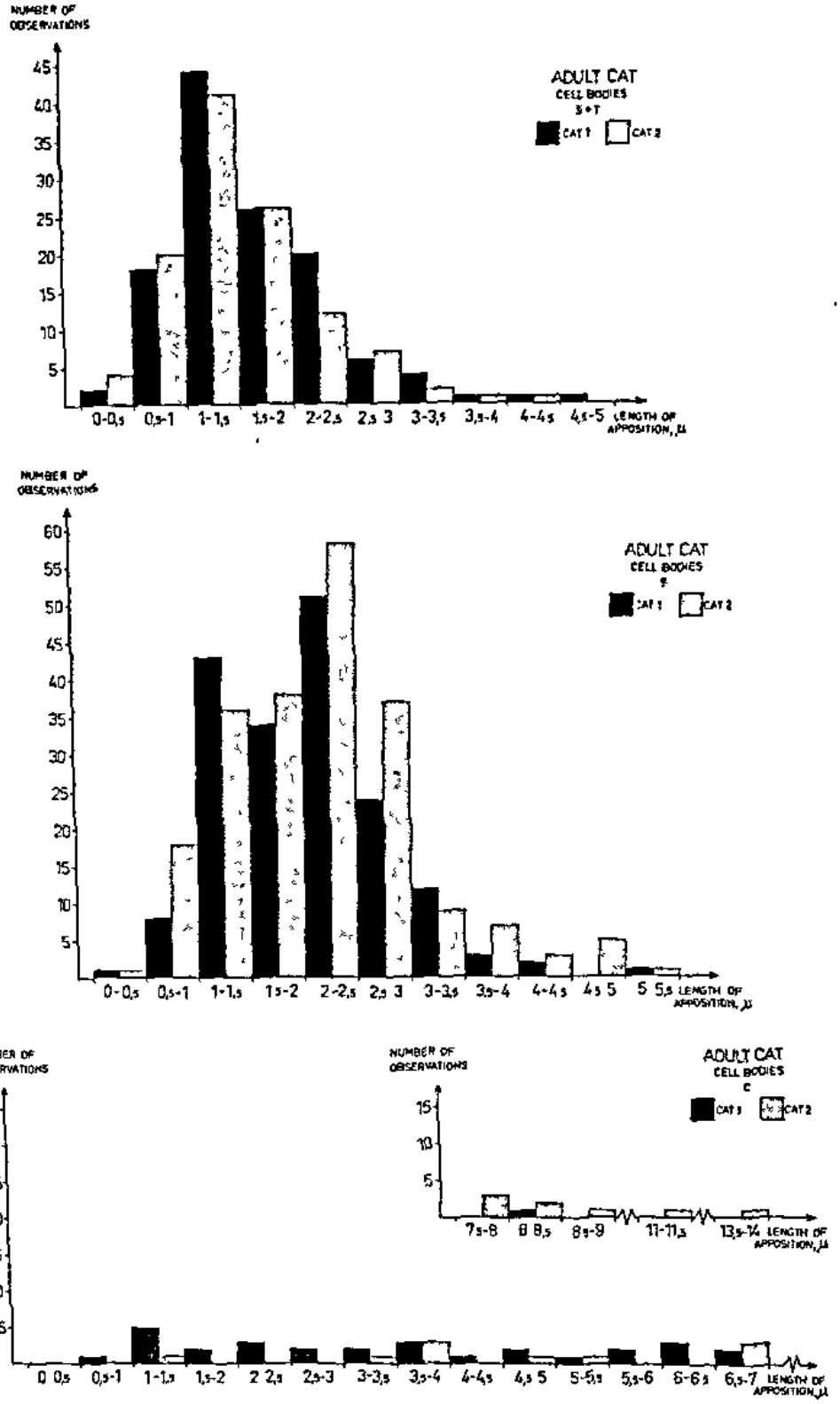
The mean length of apposition of the profiles of S, T and F boutons is around 2 μ. The F boutons are slightly bigger than the S boutons, especially on the cell bodies ($10\% > p > 5\%$) while the C- and M-types are 2—3 times bigger than the others. Boutons with higher values for length of membrane apposition usually are greater in height. For the S and T boutons, the mean length of apposition is rather similar in the different regions, but there is a tendency for the F boutons on the cell bodies to be bigger than those on the thin dendrites ($10\% > p > 5\%$).

The range in the length of apposition for the profiles of the boutons of different types on the cell bodies is depicted in Text-Fig. 1. Since one and the same bouton could be observed to establish synaptic complexes of both S- and T-types (see above), these two types are included under the same heading in the figure and in the ensuing text. The size-distribution of the bouton profiles for the S+T and F boutons, is roughly bell-shaped. In both cases, the diagrams are slightly asymmetric, with the size-range larger above than below the mean values (see Table 2), although the length of very few profiles is greater than twice the mean value. Furthermore, the size-distribution is similar between the two animals. For the F boutons, the total size-range is greater than for the S+T boutons, and it is clear that the profiles of F boutons are generally somewhat larger than the S+T boutons. The majority of the observations also cover a wider range. Possibly there are two peaks in the diagram for the F bouton profiles. The statistical analysis showed that the size-range of both S+T and F boutons was similar at both the cell bodies and the thin dendrites.

Bouton covering (Tables 1, 2 and 3)

The highest bouton covering, i.e. above 70%, is found on the proximal parts of the motoneuron dendrites. Only about half the surface of the cell bodies is covered by boutons, which seems also to be true of the thin dendrites. These regional differences are significant ($p < 5\%$). The bouton covering for the different types separately is presented in Table 3.

From the table it is seen that the covering of the S+T boutons on the cell bodies is only about 12%, which is significantly ($p < 5\%$) lower than on the proximal parts of the dendrites, where it is about 25%. There seems to be little if any difference in the covering of the S+T boutons between the proximal dendritic profiles and the thin dendrites in the neuropile. The covering of the F boutons on the cell bodies is about twice that of the



Text-Fig.: Diagram showing the length of apposition of all the bouton profiles on the motoneuron cell bodies in the quantitative study.

Table 3. Percentage bouton covering, for the different bouton types separately

Type	SOMA		DA		DB		d	
	Covering, %	% of covering	Covering, %	% of covering	Covering, %	% of covering	Covering, %	% of covering
S+T	12.6	26.8	24.5	34.0	26.0	35.3	23.8	48.9
F	27.2	57.8	39.7	55.0	41.9	56.9	24.2	49.6
C	7.0	14.9	6.1	8.5	4.7	6.4	0.5	1.0
M	0.2	0.4	1.9	2.6	1.1	1.5	0.2	0.4

S+T boutons, though the regional differences are not significant. The values for the proximal parts of the dendrites are higher than on the cell bodies and thin dendrites, but the total bouton covering is also the highest in this region. The quotient between the bouton covering of S+T- and F-types, however, seems to vary between the regions ($10\% > p > 5\%$). The covering of the C boutons decreases from the cell body towards the dendrites ($10\% > p > 5\%$), and the covering of the M and big S boutons is greatest on the proximal parts of the dendrites ($10\% > p > 5\%$).

Other profiles on the motoneuron surface (Table 2)

Only a very small part of the motoneuron cell membrane is seen to lie in apposition at adjacent dendrites, an arrangement which seems to be most common at proximal parts of the dendrites. Apposition between the motoneuron cell membrane and oligodendrocytes is restricted to the cell bodies (those cases were omitted in which a single astrocytic sheath was interposed). Rather few dendritic spines were encountered.

DISCUSSION

Fixation

Perfusion-fixation with buffered aldehydes is a generally accepted method for ultrastructural studies (Sabatini *et al.* 1963, for references see Maunsbach 1966). Earlier work has presented general information on the appearance of central nervous tissue after such a fixation procedure, and on changes in the appearance of the tissue according to the fixation method (Bodian and Taylor 1963, Westrum and Lund 1966, Karlsson and Schultz 1965, Johnston and Roots 1967). Considering these data plus current criteria for

a good ultrastructural preservation of central nervous tissue, it is obvious that perfusion-fixation of the spinal cord is more involved than that of the brain.

The fact that the cord often showed poor fixation when the circulation had been arrested before perfusion, confirms previous results from the CNS and other regions (Palay *et al.* 1962, Pease 1964, Karlsson and Schultz 1965, Maunsbach 1966, Osvaldo and Latta 1966). It is more difficult, however, to explain the reason for perfusions at a discontinuous pressure giving the best fixation of the spinal cord and roots (cf. Berthold 1968), when the circulation was not interrupted before perfusion. Perfusion with a discontinuous pressure from the infusion sets was introduced in this study since those at constant pressure resulted in a poor fixation, whereas some of the perfusions with syringes gave a very good preservation. The fluctuations in the pressure were thought to mimic the effect which must have occurred when the syringes were changed. In earlier studies on vascular perfusion of the renal medulla, the best perfusion resulted from free-hand injection from syringes (Moffat and Fourman 1963). The fact, however, that there are no earlier similar reports on the brain makes it tempting to suggest that at least one reason for the poor fixation of the spinal cord after perfusions at a continuous pressure might involve the complicated vascular supply, via small segmental vessels (see Jellinger 1966), to the spinal cord. Continuous pressure might either: 1) force the fixative too fast through the large arteries so that much of it bypasses rather than enters the very thin, long vessels which branch directly off from the arteries, or, 2) cause a rapid fixation with ensuing rigidity of the big spinal muscles which perhaps affects the flow of fixative to the smaller vessels.

Discontinuous pressure, on the other hand, through reducing the rate of flow of the fixative through the large vessels, might be sufficient to distribute it to the small vessels; moreover, discontinuous pressure more closely simulates the *in vivo* flow of blood.

Besides the fixation method, the dehydration and embedding procedures are also known to affect the appearance and dimensions of tissue components. Although there seems to be some swelling of retinal-rod membranes after aldehyde fixation alone (Robertson 1966), the interperiod distance of peripheral myelin shrinks about 30% following aldehyde fixation and Vestopal-embedding (Karlsson 1966 a). These data for tissue shrinkage must be born in mind when the diameters of various profiles, as obtained here, are compared with those in the earlier light microscopic works, or when the dimensions *in vivo* are sought.

Sampling

Earlier light microscopic studies (Schadé and van Harreveld 1961, Mellström and Skoglund 1969) show that the size of the motoneuron cell bodies varies considerably. There are, in fact, no clearcut criteria for separating the alfa-motoneurons from gamma-motoneurons, which are believed to be intermingled (Nyberg-Hansen 1965). The profiles of big neurons investigated in the present study, however, form a homogeneous population, in regards to size and cytologic characteristics, thereby justifying their being considered alfa-motoneurons. Furthermore, as mentioned above, the C boutons seemed to be present only at the bigger neurons.

The present investigation has been restricted to the lateral motoneuron nucleus in L 7. Due to the thin light microscopic sections used in the present work, the motoneuron pools described by Romanes (1951) are difficult to outline. This seems especially clear in light of the earlier observation (see for example Testa 1964), confirmed in the present study, that the horizontal extension of the motoneuron columns varies considerably between different levels of the cord. Some of the observations here might then refer to neighbouring motoneuron nuclei. In the quantitative estimation, however, the lateral motoneuron nucleus could be adequately distinguished by using a large number of consecutive transverse, semithin section.

Both transverse and longitudinal sections of the cord were used, but for the purpose of orientation, most observations were made on transverse sections. Since this is also true for the quantitative estimation, the values obtained refer thus only to horizontal dendrites. Although these are quite numerous, judging from Golgi studies (Sprague and Hongchien 1964), it has been shown that the system of vertical dendrites may be even more prominent (Lhermitte and Kraus 1925, Weil 1927, Scheibel and Scheibel 1966).

The description of the surface relationships of the motoneurons refers chiefly to cell bodies and proximal parts of the dendrites emanating from these. Although the contacts of the thin dendritic profiles in the neuropile were also studied, even in the quantitative estimation, the origin of these profiles was not known. Golgi studies (Sprague and Hongchien 1964, Scheibel and Scheibel 1966) show that the neuropile of the ventral horn contains dendritic ramifications, not only from adjacent and distant motoneurons, the dendrites of which can be 500—1000 μ long. There are also many dendrites belonging to adjacent small neurons (Balthasar 1952), possibly inter- and gamma-neurons. Since the distribution of boutons seemed to differ between small and big cell bodies, a similar difference might also be true for the thin dendritic branches. In the quantitative estimation, some

characteristics of the population of boutons on the thin dendrites were, however, common to most profiles, namely a low percentage bouton covering on the surface, few C and M boutons and a relatively high number of the S- and particularly T-types of boutons. At least some of the thin dendritic profiles might belong to alfa-motoneurons.

The synaptic complex

The morphologic criteria for the synaptic complex, as defined by Gray and Guillery (1966), were adopted and proved very useful in the present study. Synaptic complexes or structures similar to these seem to occur at practically all boutons investigated through serial sections (see also Conradi 1969 b).

The region of apposition of the C boutons at the motoneuron does not fulfill the criteria for a synaptic complex as described by Gray and Guillery, although in earlier descriptions of similar boutons, both in the spinal cord (Rosenbluth 1962, Gray 1963, Bodian 1964, Lewis and Shute 1966, Charlton and Gray 1966, Bodian 1966 b, van Harrevald and Khattab 1967) and in the cochlea (see for example Smith and Rasmussen 1965, Wersäll 1968), they have been classified as "synapses". This fact raises the question as to which criteria are crucial for the definition of a synaptic complex. At the C boutons, there is no contrast-rich material attached at the pre- and postsynaptic membranes and often the vesicles are not apposed along the whole presynaptic membrane. The synaptic cleft is extremely narrow, although a dense material is usually visible. However, the general membrane relationships in the CNS, as well as the occurrence of extracellular material, vary with the fixation method (see Discussion in Conradi 1969 c). Assuming that every bouton establishes at least one synaptic complex, nevertheless the results presented in this study indicate that the C boutons establish synaptic contact with the motoneurons. They were, namely, never observed to establish similar junctions with other neuronal structures.

Types of boutons

The boutons were classified into six types. All of these, apart from the M- and P-types, have been described by earlier authors, in studies on the cat or other species (Rosenbluth 1962, Gray 1963, Bodian 1964, 1966 a, b, Uchizono 1966, Charlton and Gray 1966, Ralston 1967). Some of the parameters underlying the typing will be briefly discussed.

The synaptic vesicles were described as being either spherical or flattened. This simple delimitation was used since it allows typing by mere inspection

of the boutons. When the boutons are thoroughly studied at high magnification, subgroups appear to be present in both the S- and F-types. Small variations in the size, shape and contrast of the vesicles in different boutons of the same type can than be observed. A further subtyping would, however, have complicated the statistical evaluation enormously, although in some other systems of the CNS, typing of boutons according to small variations in the size of the vesicles has been done (Mori 1966, Larramendi *et al.* 1967, Gobel 1968).

Boutons containing flattened synaptic vesicles, which seem to be present in specimens from the CNS fixed with aldehydes, were at first described by Uchizono (1965) in the cerebellum of the cat, where such boutons were exclusively distributed on the cell bodies. Other authors have later described such boutons in several regions in the CNS (see for example Walberg 1968) and the spinal cord (Uchizono 1966, Bodian 1966 a, b, Sedar and Moskowitz 1967, Ralston 1967).

Recently, it has been claimed that the proportion of boutons with flattened vesicles can vary with the fixation method (Holländer *et al.* 1969). In the present study, several differences other than shape of the vesicles were noted between boutons with spherical and flattened vesicles, namely:

a) The synaptic cleft of F bouton type is narrower and the layer of postsynaptic dense material is often thinner than at the S- and, particularly, T-type.
b) Very few F boutons exhibit granular synaptic vesicles or postsynaptic dense bodies.
c) Big boutons, with a subsynaptic cistern, covering the whole apposition region of the bouton, were never observed to contain flattened vesicles.
d) In some fixations, there was a granular precipitate, which was most intense in F boutons.
e) The location and percentage covering of F boutons on the motoneuron cell membrane differed from that of other bouton types.

The above observations indicate that boutons with spherical and flattened vesicles, respectively, form separate systems. Needless to say, no evidence has been presented to claim that the shape of the synaptic vesicles after aldehyde fixation represents the shape *in vivo*.

In a few cases, it was difficult to type some boutons, especially in single sections. This was the case, for example, when very few vesicles were present, when the synaptic complex was not observed or when some vesicles appeared flattened and some spherical. Although this is a source of error in the quantitative estimation, it might not contradict the suggestion that there are

boutons of different morphologic types on the motoneurons. If it is assumed that the vesicles of the F bouton are discoid, then some vesicles will always appear rounded in single sections.

The presence of a granular precipitate in some fixations, mainly in F boutons, could be indicative of chemical differences between F and other bouton types.

Quantitative estimation

The primary aim of the quantitative estimation was thus to get an idea about the population of boutons of different types on the various regions of the motoneurons. Earlier studies on other types of neurons have shown that the spectrum of apposing boutons varies with the location on the neuron (see, for example, Purkinje cells: Larramendi and Victor 1967, Hippocampus Pyramidal Cells: Hamlyn 1962 and the neurons in Clarkes column: Sedar and Moskowitz 1967); this might thus also hold true for the motoneurons. Furthermore, the present quantitative study should serve as a basis for evaluating the changes after dorsal root section and during the postnatal development. This study, together with a forthcoming report (Conradi 1969 b), is, however, also intended as a contribution to the methodology for quantitative studies on nervous connexions. Rather few quantitative ultrastructural studies on the size and distribution of the boutons on neurons have appeared (see, for example, Blackstad and Dahl 1962, Green and Pease 1966, Karlsson 1966 b, Larramendi *et al.* 1967, Holländer *et al.* 1969) even though the interest for such studies seems to increase (see for example Lemkey-Johnston and Larramendi 1968).

It has to be pointed out that the material underlying the present study is very small. Only a restricted part of the ventral horn was studied, and single sections, which might not be quite representative, were used. Despite this, some of the tendencies described were quite consistent. In some respects, the present results confirm those of earlier light microscopic studies.

In the present study, the mean length of apposition of the most commonly-occurring boutons was 1.5—2.0 μ. The bigger bouton types (C- and M-types) had a mean length of apposition of about 4—6 μ. In several of the earlier studies, which were performed on different species, the majority of the boutons were reported to have a length of apposition of 1—2 μ and the biggest boutons were about 5 μ long (Hoff 1932, Gibson 1937, Phalen and Davenport 1937, Barr 1939, Minckler 1940, Barnard 1940, Haggar and Barr 1950, Wyckoff and Young 1956, Gelfan and Rapisarda 1964, Illis 1964). Barr (1939) also reports that 38% of the motoneuron cell body is

covered by boutons, a value quite similar to the one obtained in the present study. Several of the earlier authors have also noted that the thinnest dendritic branches are covered by boutons. There are only some slight differences between the earlier and present results. In the region investigated in this study, it was shown that the bouton covering of the motoneurons was highest on proximal parts of the dendrites, decreasing outward and towards the cell body; this does not seem to have been pointed out earlier. From the present study, the location of boutons with a big zone of apposition (C- and M-types) seems to be restricted to cell bodies and the proximal parts of the dendrites, whereas for the others, the mean length of apposition of the boutons is rather similar on different parts of the motoneuron surface. The percentage covering of the boutons on the motoneurons has been investigated with the electron microscope (Green and Pease 1966). Although no details on the sampling procedure and the methodology are given, these authors conclude that 48% of the proximal parts of the motoneuron dendrites is covered by boutons. The decrease in value towards thinner dendritic profiles agrees with the present findings. However, the authors obtain a higher percentage bouton covering on the cell bodies (49%) than on proximal parts of the dendrites. This differs from the finding in the present study and might be due to regional or sampling differences.

As mentioned, the percentage of covering of the motoneuron cell membrane varied for some bouton types amongst the various regions of the motoneurons. Besides the bouton covering for the different types, it is also of interest to calculate the numerical proportion of the bouton types on the different regions of the motoneuron surface. Values can then be compared for example with three-dimensional reconstructions of parts of the motoneurons (see Conradi 1969 b, c). It is obvious that the relative number of the different bouton types, as obtained from single sections, cannot be representative for the true situation, since there is, as mentioned, a greater chance for big boutons to be included in the sections. Also, the size-range of the bouton profiles could be of importance. If there are relatively many big bouton profiles in one of the types, this type might be over-represented, since a big bouton cut through the periphery is easier to recognize than a smaller bouton cut in the same way. However, the diagram on the length of apposition of the bouton profiles on the cell bodies showed a peak near the mean values for both the S + T- and F-types. The statistical analysis showed that the variance of the values for these two bouton types was similar on the cell bodies and the thin dendrites

If it is assumed that the area of apposition on the motoneurons of all the bouton types shows the same size-range, the same shape, and the same

Table 4. Numerical proportion of boutons of the different types as seen in the sections ("present") and the corresponding values when the proportion has been corrected for mean length of apposition ("corrected")

		SOMA %	DA %	DB %	d %
S+T	Present	35.5	39.6	42.0	51.9
	Corrected	43.3	45.3	47.8	55.6
F	Present	57.5	55.3	54.9	47.5
	Corrected	53.9	52.3	50.7	44.2
C	Present	6.9	3.9	2.4	0.4
	Corrected	3.0	1.8	1.0	0.2
M	Present	0.1	1.2	0.7	0.2
	Corrected	0.04	0.6	0.4	0.1

orientation towards the plane of section, then an estimate of the true numerical proportion of boutons of different types can be obtained by dividing the proportion of one bouton type, as seen in the single sections, with the mean length of apposition of that type. The sum of the new percentages will be less than one hundred, but for clarity, these percentages can easily be adjusted upwards so that the sum reaches that value. This has been done in Table 4.

It can be seen in the table that the proportion of F boutons shows a slight decrease from the cell bodies towards the dendrites, whereas the reverse is true for the S+T boutons. On the cell bodies, the F boutons apparently outnumber the S+T boutons; the situation is reverse on the distal dendrites. The C boutons occur most commonly on the cell bodies, decreasing in number rather sharply towards the dendrites.

When this table is compared with Table 3, it is most noticeable that the covering of the bigger bouton types is much higher than the numerical proportion. The values in Table 3 for percentage of the total bouton covering by the different bouton types, however, show the same tendencies for location of the boutons as in Table 4.

Physiologic considerations

In the initial description of boutons containing flattened synaptic vesicles, these boutons were suggested to be inhibitory (Uchizono 1965), since they exclusively surround the cell bodies of Purkinje cells, where inhibitory influences should be localized according to the physiologic evidence cited. If a similar function is true for the F boutons on the motoneurons (cf. Bodian 1966 a, b), at least one inhibitory input is distributed both on the cell

bodies and dendrites, probably also on the distant branches. If it is further assumed, as in the original report, that boutons containing spherical vesicles are excitatory, then about 60% of the area of the motoneuron cell bodies covered by boutons could be subjected to inhibitory influences, compared to scarcely 50% at the distal dendrites.

The physiologic evidence for the localization of inhibitory synapses on the motoneurons is conflicting. Eccles (1964) argues that the localization of the inhibitory synapses is restricted to the cell bodies or neighbouring regions. Granit *et al.* (1966) and Terzuolo and Llinas (1966), have presented evidence to the contrary, showing that inhibitory synapses may also be localized at a distance far from the cell bodies. A recent report, based on a large number of measurements with the intracellular recording technique, combined with extensive theoretical calculations, concludes that the inhibitory synapses ought chiefly to be located on or near the cell bodies, whereas the excitatory synapses are distributed over the entire motoneuron surface (Smith *et al.* 1967).

Following post-asphyxial hind limb rigidity, which is thought to be caused by a mass destruction of perhaps mostly inhibitory interneurons in the spinal cord, there is a loss of boutons on the motoneurons and the loss is most pronounced at the cell bodies (Gelfan and Rapisarda 1964). The remaining boutons have also been studied ultrastructurally but no quantitative analysis has been performed (van Harreveld and Khattab 1967). The predominance of F boutons on the cell bodies found in the present study might confirm these results. The above assumption (Uchizono 1965), that the boutons containing flattened vesicles are inhibitory, does not, however, imply that all boutons containing spherical vesicles are excitatory. Following the assumption in the present material that the bouton covering and proportion of inhibitory boutons decrease from the cell body towards the dendrites, then the localization of the C boutons is compatible with an inhibitory function of these boutons. The C boutons occur most commonly on the cell bodies, and, to some extent, on the proximal parts of the dendrites, whereas they are scarcely seen at all on the thin dendrites. If both the F and C boutons are inhibitory, then more than 70% of the area of the motoneuron cell bodies covered by boutons is subjected to inhibitory influences.

Boutons similar to those of the C-type are formed by the presumably inhibitory endings of the olivocochlear bundle (see Smith and Rasmussen 1965, for references, see Wersäll 1968). The big size and characteristic appearance of these boutons must be of help in attempting to determine the site of origin. It might then be possible to study the significance of the

regularly-occurring apposition of subsurface granular endoplasmic reticulum, and of the fact that the synaptic cleft of these boutons seems to contain A Ch-E activity (Lewis and Shute 1966).

ACKNOWLEDGEMENTS

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SUMMARY

The membrane relationships of the motoneurons in the lateral part of segment L 7 were studied in 33 adult cats, fixed through perfusion with buffered glutaraldehyde. The motoneurons are contacted by glial cells, adjacent dendrites and boutons. Flattened astrocytic extensions are found on all parts of the motoneuron surface and cover nearly half of the motoneuron cell bodies. Oligodendrocytes are found both as perineuronal satellites and in the surrounding neuropile. The boutons on the motoneurons and in the neuropile can be divided into six morphologic types. Of the boutons containing spherical synaptic vesicles, 350—500 A in diameter, the S-type shows synaptic complexes with a 50—200 A thick layer of postsynaptic material. The T bouton type exhibits postsynaptic dense bodies at the synaptic complex. The type of big boutons, mainly localized on the motoneuron cell bodies and proximal parts of the dendrites, where a subsynaptic cistern was found below the synaptic complex was denoted C. The big boutons of the M-type contain spherical synaptic vesicles and a large, irregularly-shaped synaptic complex, covered by many postsynaptic dense bodies. On the convex side of these and some big boutons of the S-type, there are apposed small boutons, P-type, which contain small synaptic vesicles of irregular shape. Boutons with flattened vesicles are also found, F-type.

As a background for studies on operated adult cats and kittens, the size and distribution of the boutons of different types on the motoneuron cell bodies and dendrites, and in the neuropile, were studied quantitatively on single sections. The sections were taken from a restricted region of the ventral horn in two cats. The F boutons were the more frequent on or near the motoneuron cell bodies, whereas the S- and T-types occurred most frequently in the neuropile. The mean length of apposition to the motoneurons varied between the bouton types. The percentage of motoneuron and dendrite membrane covered by boutons was higher on the proximal parts of the motoneuron dendrites than on the cell bodies and in the neuropile. The percentage of membrane covering was also expressed for each type of bouton separately. A further attempt was made to calculate the numerical proportion of boutons of different types at different regions of the motoneurons. The results are discussed briefly against the background of the earlier suggested relationship between vesicle shape and function of the boutons.

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Fig. 12. T-type. Below the bouton, three postsynaptic dense bodies are seen. In the detail, the layer of postsynaptic dense material is seen to be somewhat irregular and thicker than at the S-type.

Fig. 13. F-type. The entire bouton profile is surrounded by a thin astrocytic lamella (arrow). The synaptic cleft is narrower than at the preceding types and the layer of postsynaptic dense material is thin. To the left, a desmosome-like junction (DE) is formed between the bouton and the postsynaptic dendrite.

Fig. 14. FD-variety of the F bouton type, located on the proximal part of a motoneuron dendrite. The ground substance of the bouton is rather contrast-rich and two big dense bodies are observed. The bouton is bigger than ordinary F boutons and the synaptic cleft is large (between the arrows). ×24,300

Fig. 15. Detail of an F bouton with a large synaptic cleft (between the arrows). The layer of postsynaptic material is thin and shows a low density. ×95,000

Fig. 16. A C bouton on the proximal part of a motoneuron dendrite. Note the abundance of granular endoplasmic reticulum below the bouton. ×18,900

Fig. 17. Detail of the region of apposition of a C bouton. Note the narrow and continuous synaptic cleft and the absence of pre- or postsynaptic material. The membranes forming the subsynaptic cistern (arrow) lie close together. ×113,000

Fig. 18. A bouton of the M-type on the proximal part of a motoneuron dendrite. Note the big size of this bouton and the clear ground substance. The synaptic vesicles are scattered throughout almost the entire bouton. Three profiles of synaptic complex, which are covered by postsynaptic bodies (arrow), are observed. On the convex side of the bouton, three small boutons of the P-type are apposed. ×22,200

Fig. 19. Detail of the synaptic complex of an M bouton. Note the extension of the synaptic complex. There is a thick and somewhat irregular layer of postsynaptic material, almost completely covered by postsynaptic dense bodies in this section. ×95,400

Fig. 20. Bouton containing several dense-cored vesicles (arrow). The synaptic vesicles are of somewhat irregular size and shape. ×73,600

Fig. 21. Two adjacent boutons on the proximal part of a motoneuron dendrite (M). A precipitate is present, especially on the membranes and the mitochondria, but also on the flattened vesicles in the bouton to the right, whereas almost no precipitate is found on the spherical vesicles in the left bouton. ×66,300


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Fig. 12. S-type. Below the bouton, three postsynaptic dense bodies are seen. In the detail, the layer of postsynaptic dense material is seen to be somewhat irregular and thicker than at the S type.

Fig. 13. F-type. The entire bouton profile is surrounded by a thin astrocytic lamella (arrow). The synaptic cleft is narrower than at the preceding types and the layer of postsynaptic dense material is thin. To the left, a desmosome-like junction (DJ) is formed between the bouton and the postsynaptic dendrite.

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Fig. 15. Detail of an F bouton with a large synaptic cleft (between the arrows). The layer of postsynaptic material is thin and shows a low density. ×95,000

Fig. 16. A C bouton on the proximal part of a motoneuron dendrite. Note the abundance of granular endoplasmic reticulum below the bouton. ×19,900

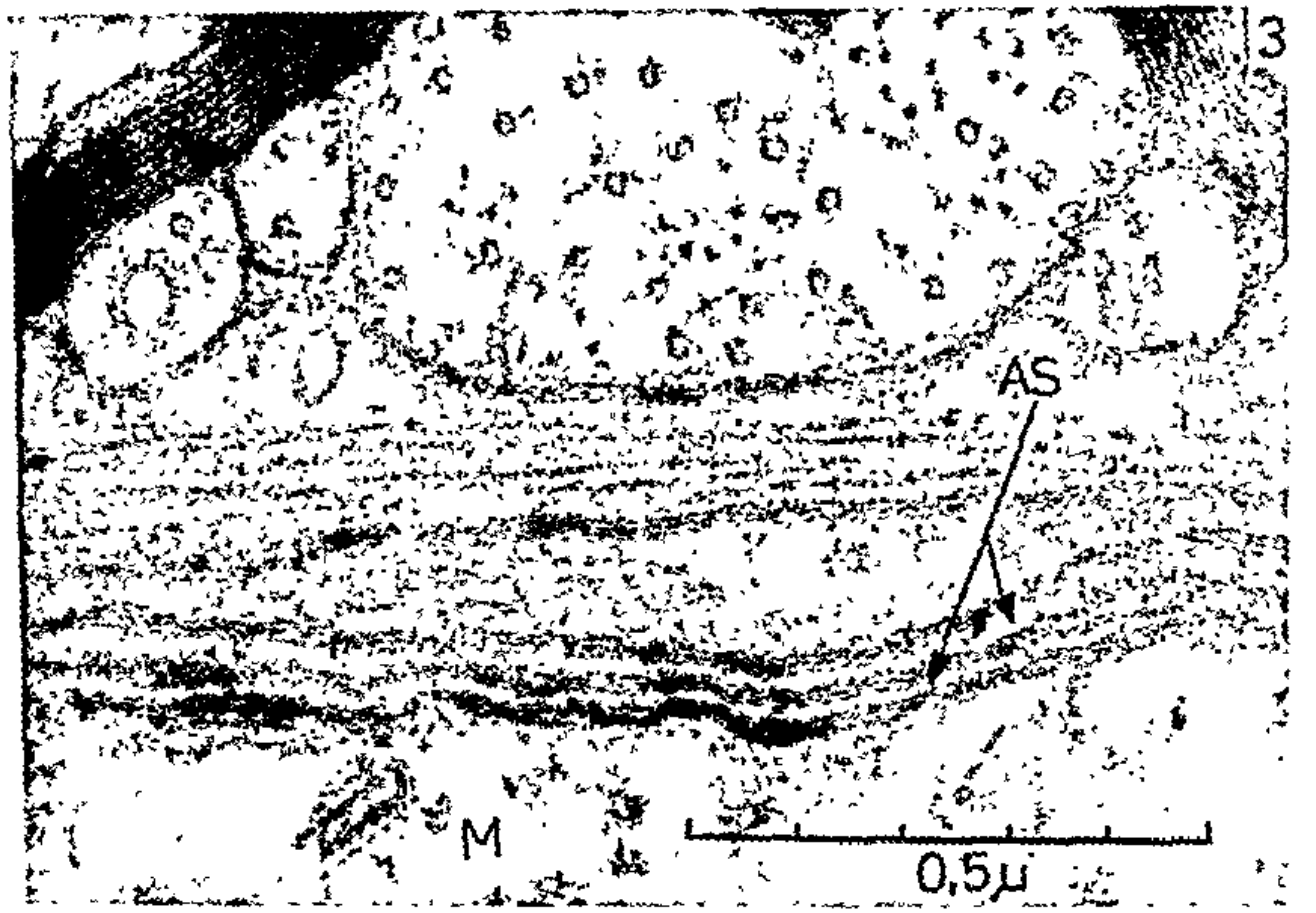
Fig. 17. Detail of the region of apposition of a C bouton. Note the narrow and continuous synaptic cleft and the absence of pre- or postsynaptic material. The membranes forming the subsynaptic cistern (arrow) lie close together. ×113,000

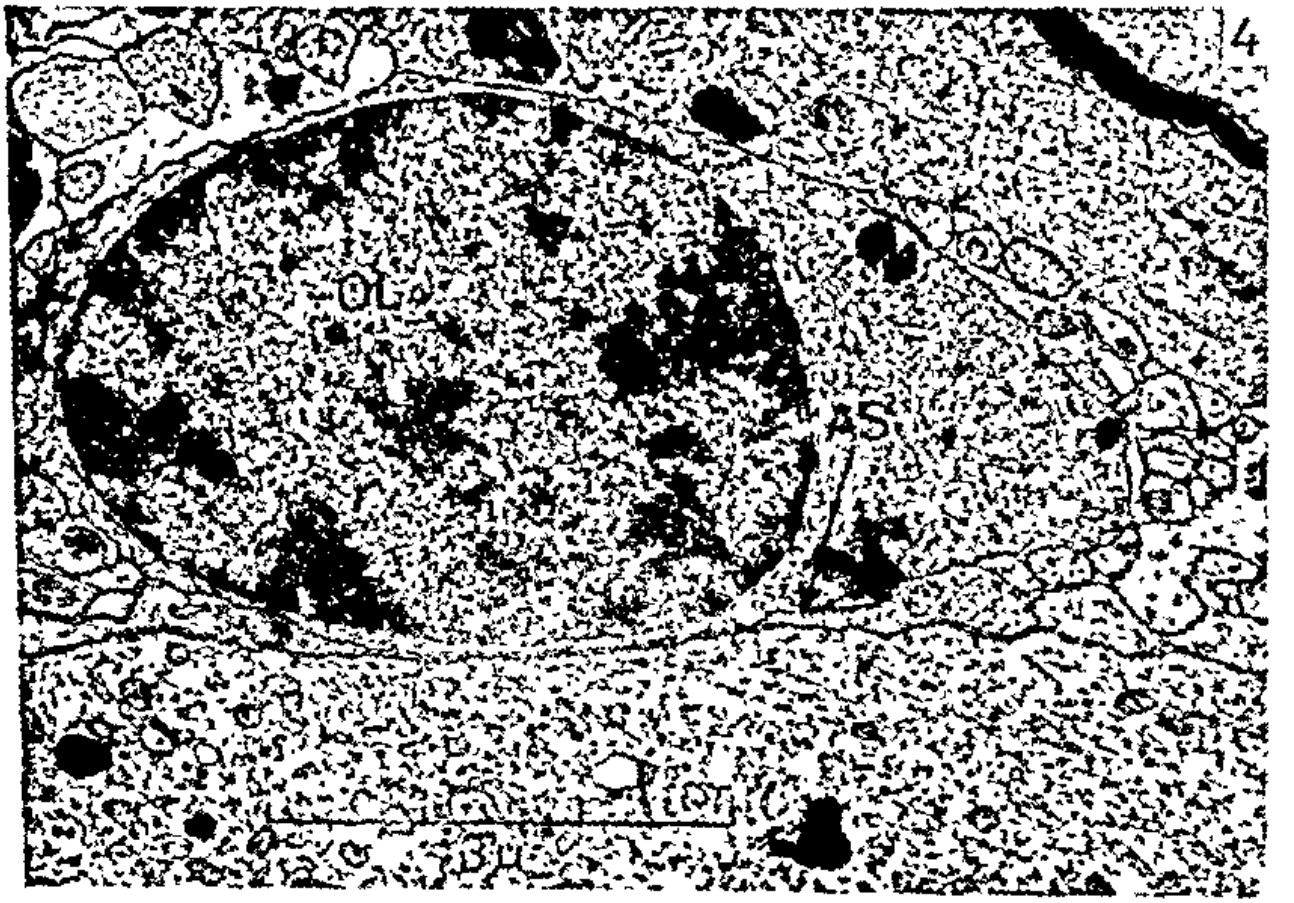
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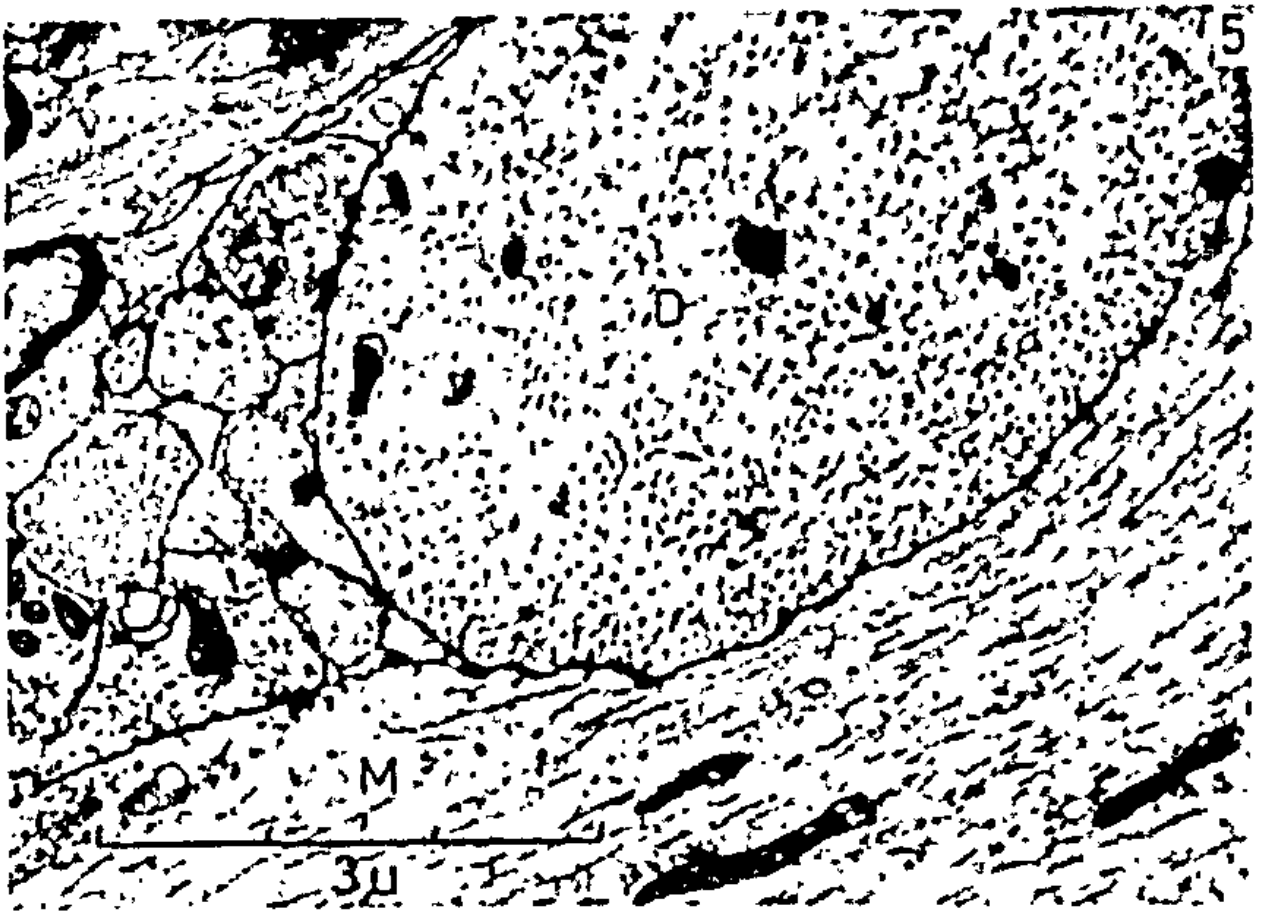
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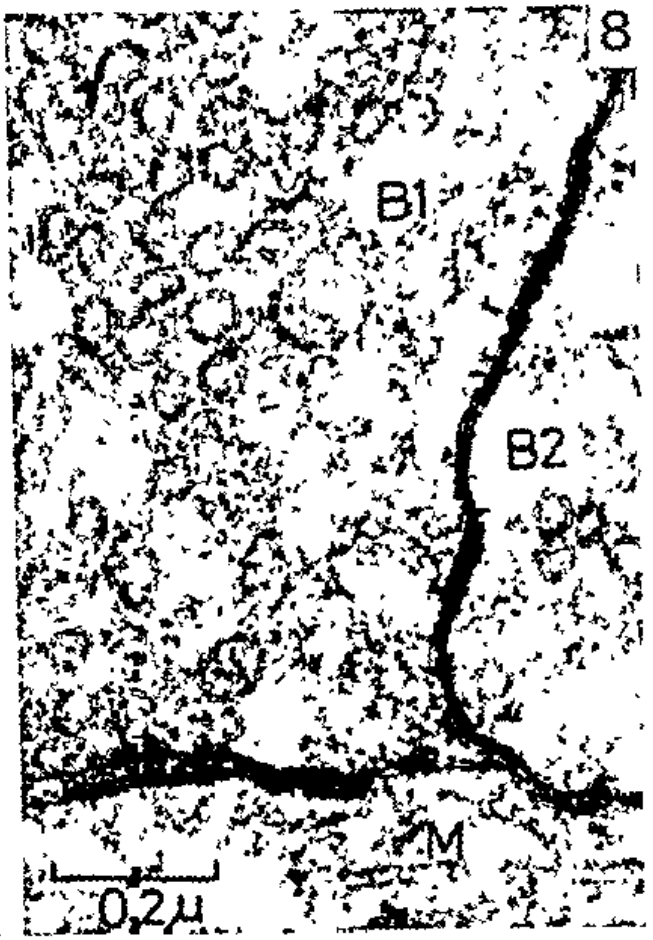

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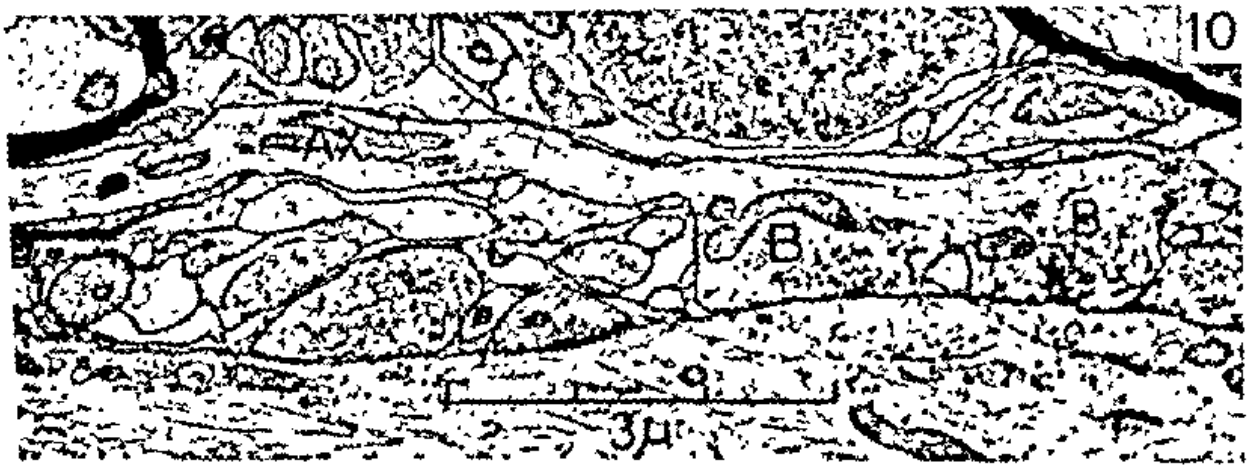

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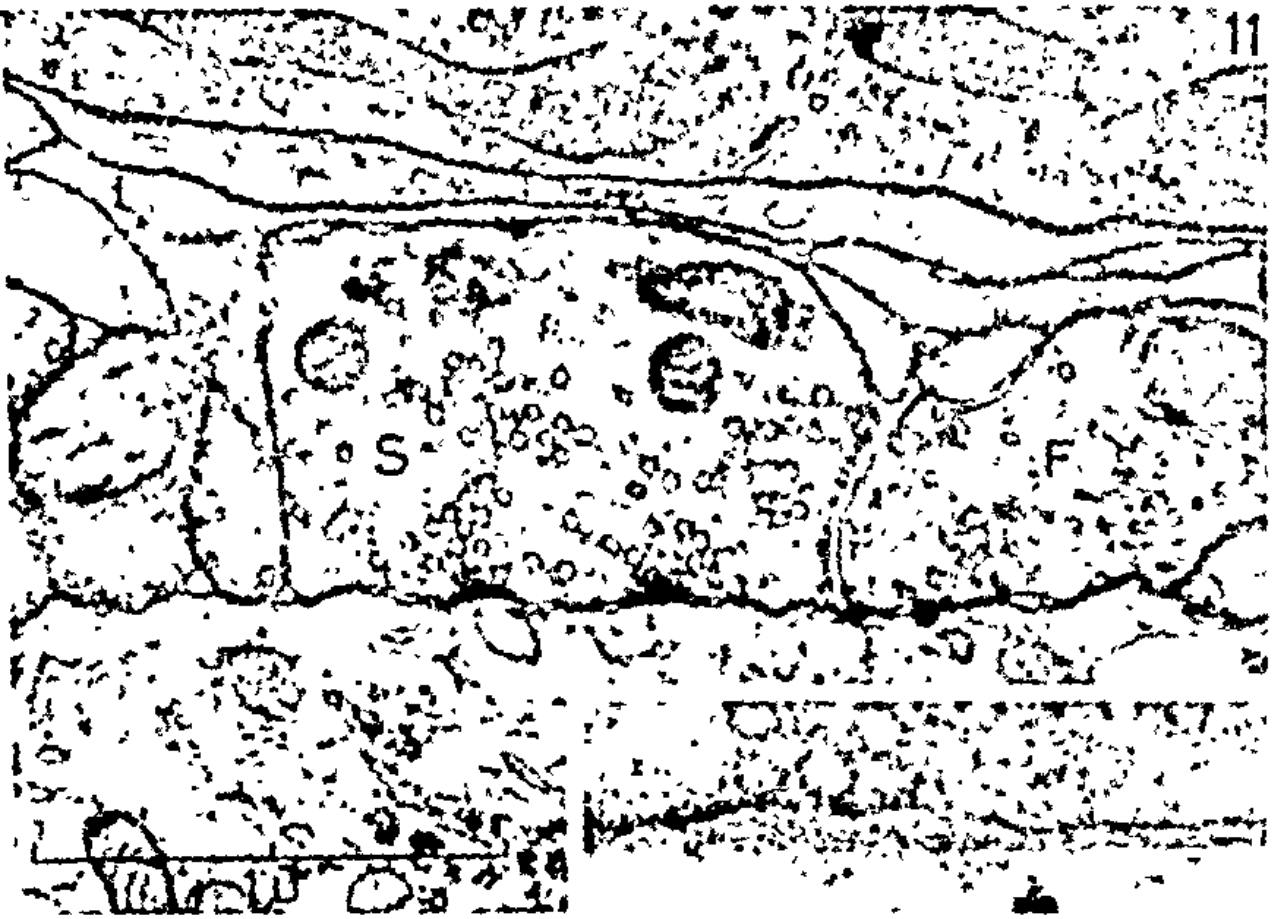

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7
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D1
1μ

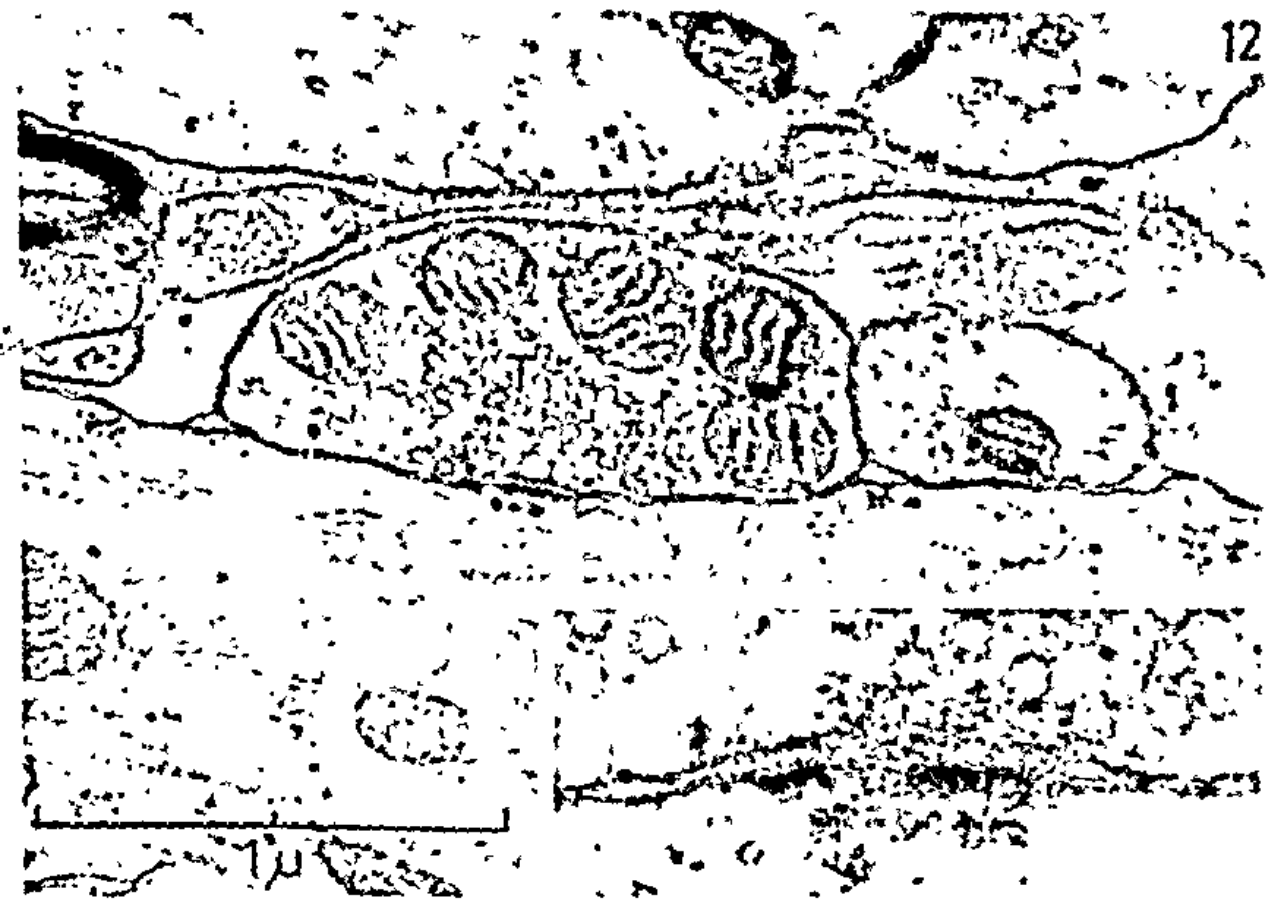

8
B1
B2
M
0,2μ


9
B
M
0,5μ

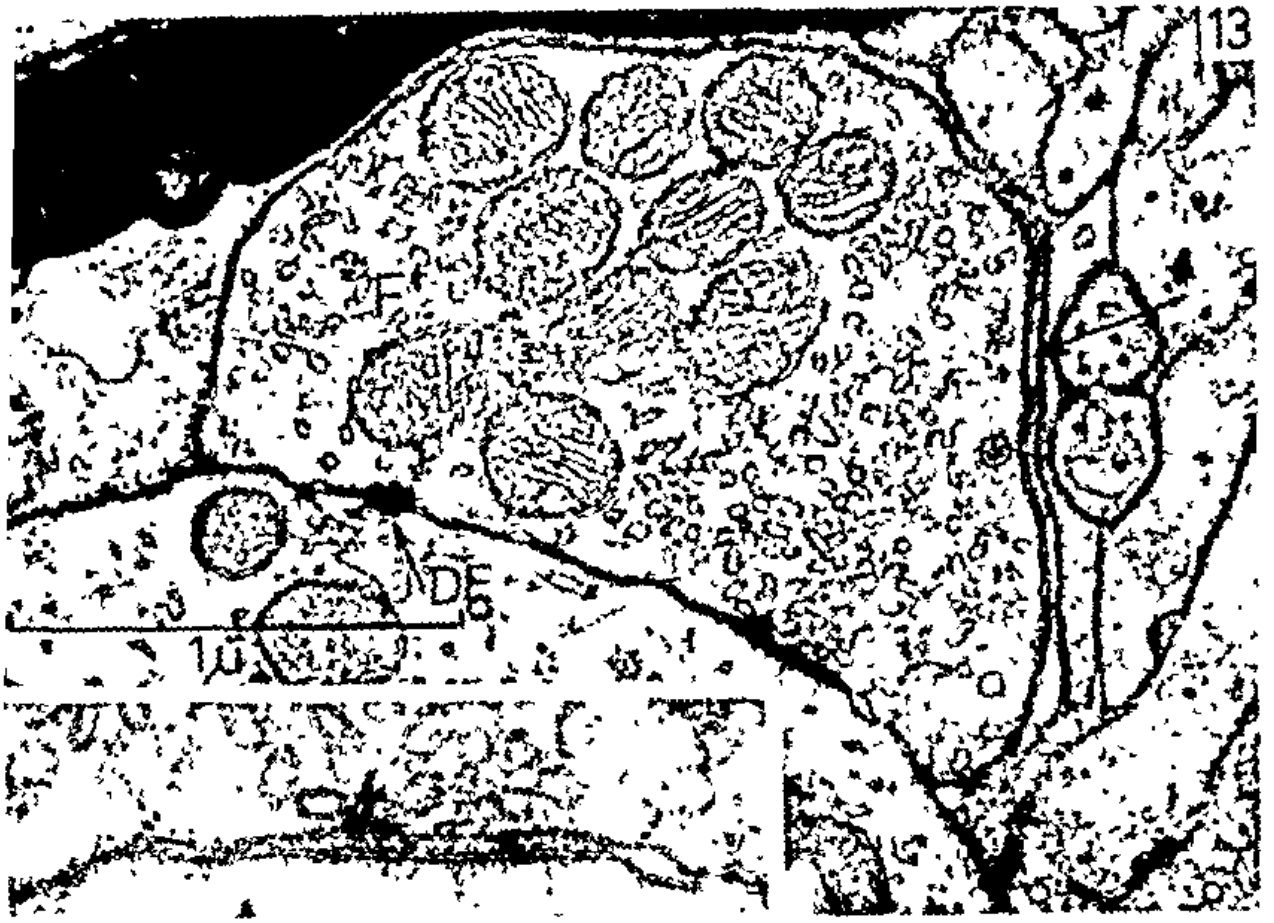

10
AX
B
B
3μ

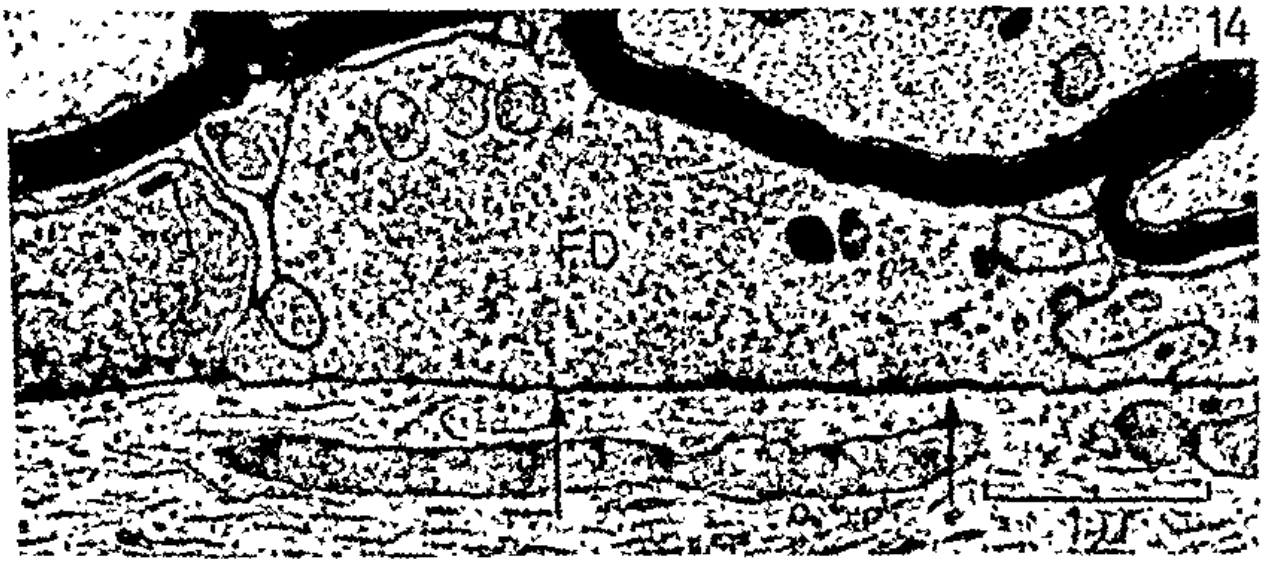


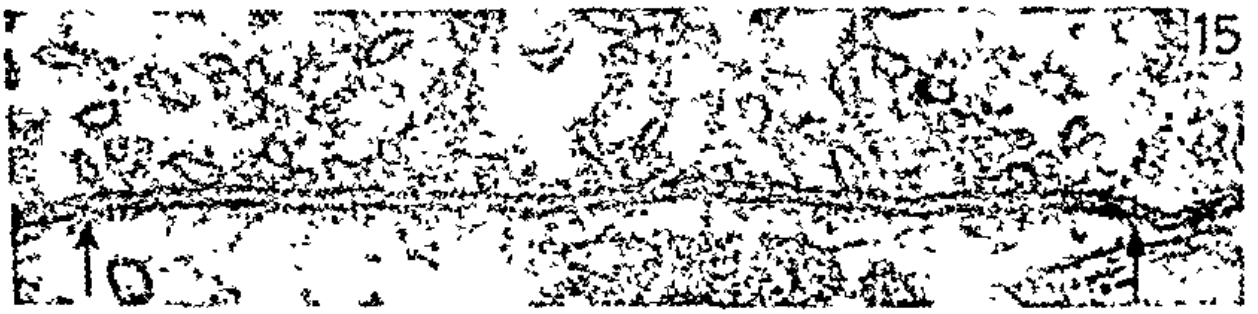
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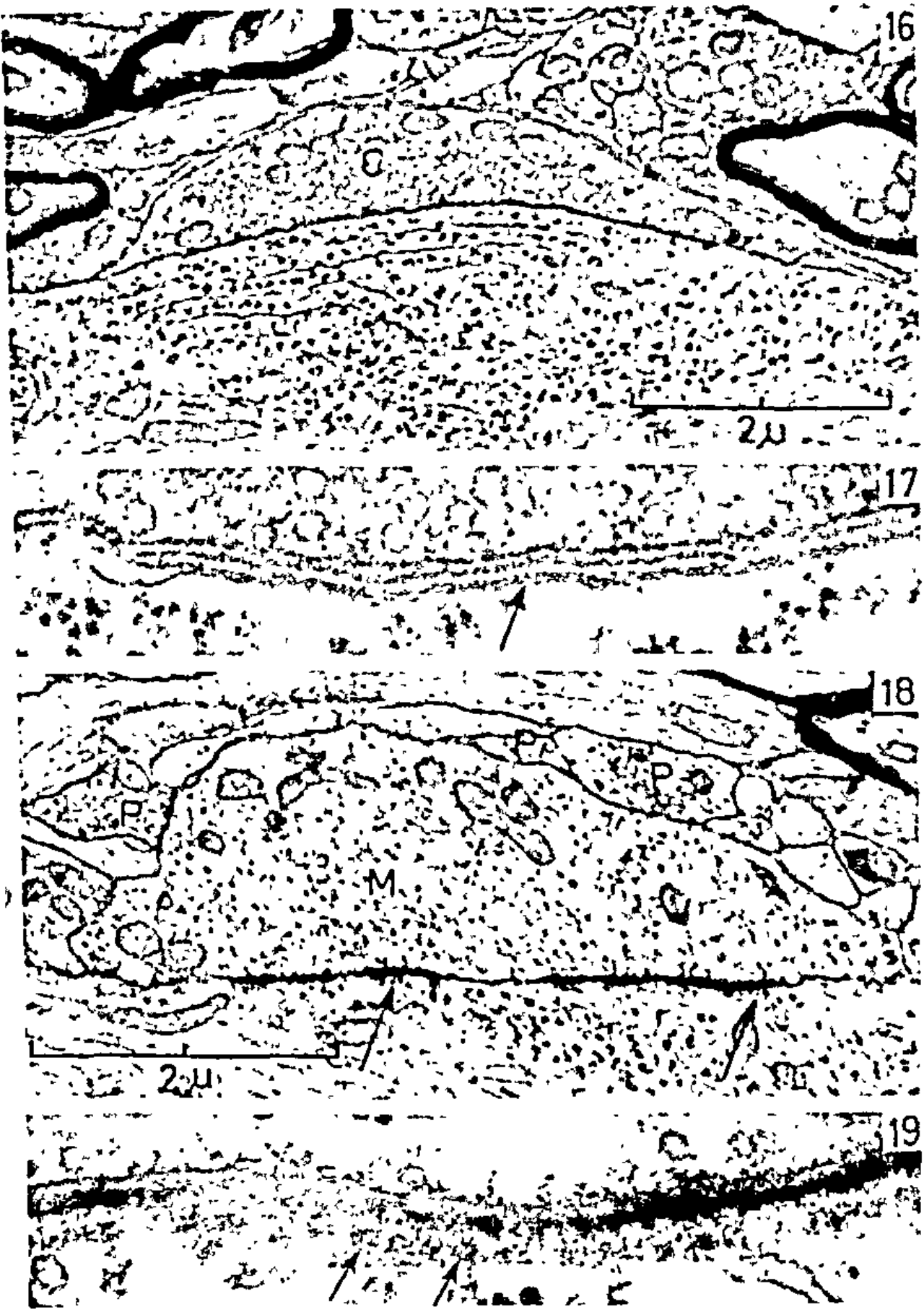


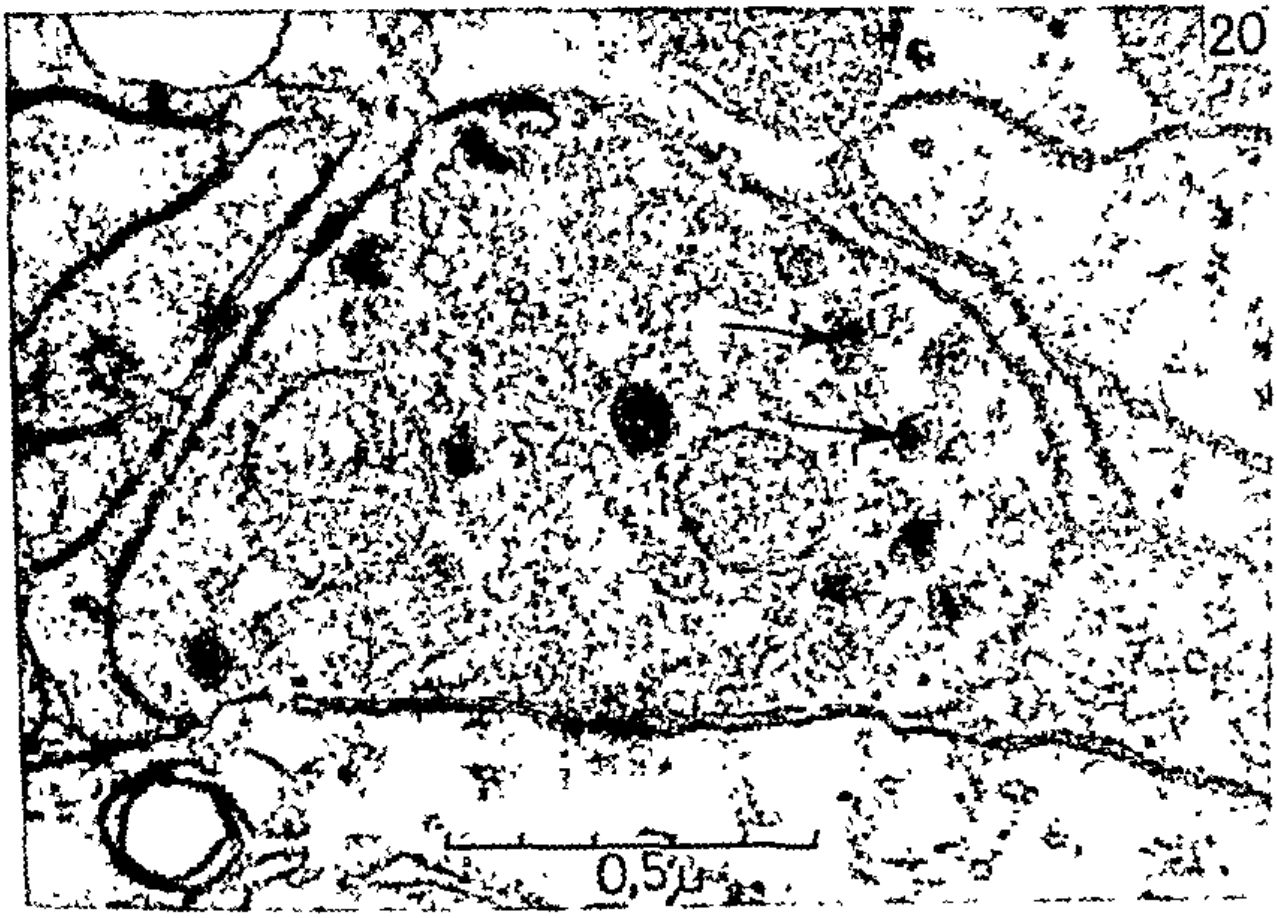
12

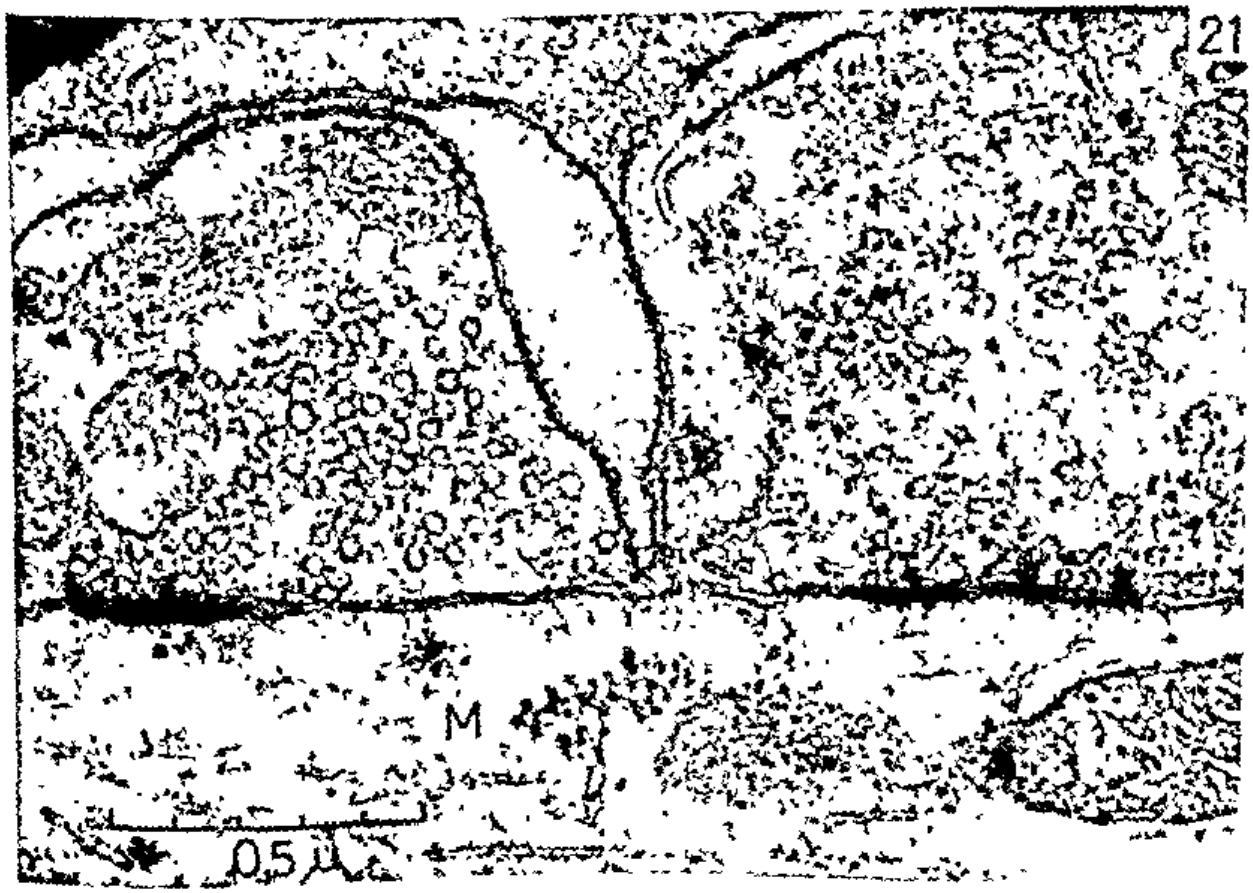

13
DE
1μ


14
FD
1μ


15
1μ


16
2μ
17
18
P
P
P
M
2μ
19


20
0,5 μ


21
M
0,5 μ

Ultrastructure and distribution of neuronal and glial elements on the surface of the proximal part of a motoneuron dendrite, as analyzed by serial sections

by

SEBASTIAN CONRADI

INTRODUCTION

In a preceding paper (Conradi 1969 a), the ultrastructure of the neuronal and glial elements contacting the motoneuron cell membrane was described. The boutons were classified into six different types, according to observations on single or short series of sections. The size and distribution of the bouton profiles on the motoneurons was investigated on single sections from a sharply defined region of the ventral horn.

It seemed desirable, however, to investigate further a part of the material in the quantitative study through longer series of consecutive sections. The aim was the following:

a) To study the shape of the apposition region, and the size and shape of the synaptic complex of the different types of boutons, in order to investigate further the criteria underlying the typing of the boutons. The latter seemed important also since there are few ultrastructural reports on the three-dimensional appearance of the synaptic complex.

b) To show the topographic relationships of the different types of boutons on the motoneuron surface. It even seemed possible to determine whether boutons of the same types could be connected to the same preterminal axon.

c) To get some information on the relevance of the observations from single sections. The values for the size of the area of apposition of the different bouton types could be controlled and the size-range estimated. Further-

more, the percentage of bouton covering, both total and for the different types separately, as well as the numerical proportion of boutons of the different types could be studied and compared with the values obtained in the earlier quantitative estimation.

Part of the motoneuron cell membrane was chosen to be depicted in one plane, because of the difficulty in adequately illustrating complex volumes, for example by wax-reconstruction. The surface of the proximal part of a motoneuron dendrite was analyzed, since all the types of boutons, according to the quantitative estimation, are found on this part of the motoneuron.

MATERIAL AND METHODS

The measurements on cell 20 in the quantitative study (see the preceding paper) were made on one of a longer series of consecutive sections. The series consisted of 750 consecutive ultrathin transverse sections through the lateral motoneuron nucleus in the most cranial mm of L 7. The series passed in cranio-caudal direction through a horizontal proximal part of a motoneuron dendrite, which was cut through in 220 sections and the observations in the quantitative study were made on one of these sections (see below). In one instance, 3 consecutive sections were lost; otherwise, only a few single ones were lost. Assuming a cylindrical shape of the dendrite, the mean section thickness was about 800 Å, as calculated from measurements on the diameter of the dendrite at several places.

The dendrite left the motoneuron cell body in a ventro-medial and horizontal direction (Fig. 3). The diameter of the first part of the dendrite was rather constantly at 11 μ. After a distance of about 30 μ, the dendrite curved medially and caudally, sending out a downward branch. Then the dendrite, now about 9 μ in diameter, again took a horizontal course. After a distance of about 40 μ, it curved slowly cranially and disappeared out of the sections. The horizontal parts of the dendrites were analyzed.

Every second section through the dendrite was photographed at a primary magnification of 3,700×. For each section, 5—6 photographs were required; they were enlarged 3 times and the copies taped together to montages. The type of each bouton on the dendrite was then determined on every 7—10th section through inspection in the electron microscope at high magnification, and the result was transferred to the montages.

The membrane relationships of the dendrite were visualized thus: the analyzed parts of the dendrite were cylindrical. The plane of sectioning was parallel to the longitudinal axis of the cylinder. From the diameter and length of the dendrite, the mantle surface at the magnification used was calculated and depicted on a transparent paper. Each section through the dendrite was represented on the paper as a pair of parallel, longitudinal, lines: the uppermost section, as two lines in the middle of the paper; the lowest section, as two lines on each side of the paper. (See Fig. 4). The transparent paper was then placed on the montages in consecutive order. Each time the outline of

the dendrite in the actual section was aligned with the corresponding line on the paper, and the length of apposition of every bouton and other types of extensions were depicted along this line.

Other parameters also transferred to the paper were: the presence, direction and myelination of the preterminal axons; the presence of small boutons (P-type) on the convex side of the boutons; the presence of a synaptic complex with an additional dendrite ("double synapse", in Figs. 7 and 8), and localization of dendritic spines and of granular endoplasmic reticulum just inside the surface of the dendrite.

The extension of synaptic complexes and desmosome-like junctions were depicted at some of the boutons, but were restricted to those parts of the dendrite, where the cell membrane was cut rather perpendicularly, i.e., some distance away from the upper and lower margin of the dendrite. In this part of the investigation, the boutons were studied at high magnification in the electron microscope at each section. The extension and position of the synaptic complexes and desmosome-like junctions were judged by inspection and transferred by free-hand to the transparent paper.

Some difficulties are encountered in the use of this method. The copying procedure often produces small differences in the magnification between the montages; thus when the large montages are analyzed, the distance between the proximal and distal border of the montages varies. In such cases, the transparent paper has to be moved slightly lengthwise along some of the montages, resulting in some inaccuracy, not so much as regards the size of the apposition areas of the boutons, as to the distance between the boutons.

There are also some difficulties involved in illustrating adequately the membrane relationships at the upper and lower margin of the dendrite. If the diameter of the dendrite varies slightly, some of the sections at the cranial and caudal edges will pass through the dendrite at those places where the diameter is greater. At the slightly narrower parts, the sections will pass just outside the dendrite (Fig. 2) In such instances, the site of the vertical plane through the centre of the dendrite had to be depicted as a straight line on the montages, and the apposition regions of the different boutons were drawn on the transparent paper according to the distance from this line, instead of on the line corresponding to the actual section. In region A of the dendrite, there was also a slight continuous decrease in the diameter of the dendrite away from the cell body. Due to this, the pair of lines representing each section was brought to converge slightly towards the distal part of the reconstruction (see Fig. 5).

The number and size of the parameters to be analyzed in the drawing determine the magnification of the montages. In the present study, the interest was primarily focused on the apposition areas of the boutons. With the final magnification, about 11,000×, it was not possible to analyze the astrocytic sheaths surrounding the boutons. The location and extension of the synaptic complexes had to be drawn merely from inspection, since if the magnification should have been big enough to permit recognition of these complexes on the montages, it would have been necessary to increase the number of photographs tenfold or more.

The outline of the apposition areas of the boutons at the regions A and B, respectively, on the transparent papers, was transferred to an ordinary paper in the same scale. Some measurements were made on the latter.

All the boutons on each drawing were counted and the numerical proportion of the different types calculated. The maximum length of apposition *in the direction of the*

longitudinal axis of the dendrite was measured for all the boutons, and from this, the mean value for each type of bouton was calculated. This definition of maximum length was used, since the measurements on the bouton profiles on dendrites in the single sections (Conradi 1969a) roughly represented the length of apposition along the longitudinal axis of the dendrites, which were cut lengthwise. Both the maximum longitudinal length and the length of the profiles were measured on those boutons which were included in section 114, used in the quantitative estimation in the preceding paper. To calculate the packing density of the boutons, the largest square on the surface of the dendrite was depicted on each of the two drawings and the number of boutons inside it was counted. In order to make possible comparisons with earlier light microscopical findings, the values were transferred to no. of boutons/100 μ^2.

Finally, the percentage covering of the boutons, both total and for each type separately, was calculated, and for this purpose, photographic copies of the two drawings, measuring about 25×15 cm, were used. At first, the whole drawings were each weighed with an accuracy of 0.001 g. Then the apposition areas of all the boutons were cut out and the profiles, representing the same type of bouton, were weighed together. During the cutting procedure, special care was taken in order not to contaminate the copies. The percentage covering of the boutons of different types was calculated as the ratio.

$$\frac{\text{weight of the bouton profiles}}{\text{weight of the whole drawing}}$$

RESULTS

General

All the six types of boutons, described in the preceding paper, were present on the dendritic segments (see Figs. 5—10). Thus, there were boutons with spherical synaptic vesicles, denoted S-type, except for those instances in which postsynaptic dense bodies (T-type) or a subsynaptic cistern (C-type) could be observed. It is clearly seen in the figures that some of the boutons with spherical synaptic vesicles establish synaptic complexes both with and without postsynaptic dense bodies. In the following description, therefore, the S and T bouton types are not dealt with separately. Some big boutons (M-type), containing spherical synaptic vesicles and showing a synaptic cleft of an obviously irregular shape and completely covered by postsynaptic dense bodies are also present. Small boutons (P-type), containing rather small synaptic vesicles of an irregular shape were found to be apposed at the convex side of the M boutons and some big S boutons. By mere inspection at high magnification in the electron microscope, profiles of P boutons cannot be distinguished with certainty from profiles of boutons with flattened vesicles (F-type). Due to this and to the fact that the type of the bouton

profiles was controlled in the microscope at about every 7th section, the P and F bouton types were demarcated in the same way in the figures 5—10. Some big boutons with flattened vesicles were found to have a rather dense ground substance (FD-variety).

In Figs. 5 and 6 it is seen that a large part of the dendritic mantle surface is covered by boutons, which are usually tightly packed. At a few places, the surface is covered by myelinated axons or adjacent dendrites, but in these cases, thin, intervening astrocytic sheaths are always present. The apposition areas of the boutons are mostly of similar shape, often slightly ovoid, with the longitudinal axis parallel to that of the dendrite. It is clearly seen, however, that there is a considerable size-range even among boutons of the same type. Most of the boutons are surrounded by thin astrocytic sheaths, but due to the thinness of the latter and the fact that they do not completely cover the boutons, they were, as mentioned, not included in the drawing.

Distribution of the different types of boutons

The F boutons seem to be clustered at some places on the reconstructed mantle surface. Some big boutons with spherical synaptic vesicles (S-, T- and M-types), in which each bouton is surrounded by several dendritic spines, form a row in proximo-distal direction at the left side in region A (Fig. 10). Apart from this, the distribution of the boutons of different types appears to be random.

Preterminal axons

The presence and direction of the identified preterminal axons are depicted in Figs. 7 and 8. The preterminal axons could be recognized at only some of the boutons. This could be due to the fact that the vast majority of these axons are unmyelinated some distance before the boutons. If such thin axons are cut transversely in the series, they are difficult to recognize. That few boutons *de passage* were observed might be due to the fact that rather few of the preterminal axons could be traced. None of the bigger boutons seemed to be formed *de passage*. Many of the identified preterminal axons were directed parallel to the longitudinal axis of the dendrite. In some cases, the preterminal axons were apposed to the dendritic surface immediately before the bouton. This can be seen by the shape of the apposition area, which shows slender lateral protrusions at some of the boutons. In two cases, an unmyelinated preterminal axon crossed the areas

of apposition of another bouton, an arrangement which thus formed a "tunnel" between the bouton and dendrite.

Tracing the course of the preterminal axons could have clarified whether several boutons belong to the same preterminal axon; however, such determinations require a higher magnification than that used in this study and would have increased the number of photographs markedly.

The synaptic complex

Practically all the boutons on the analyzed dendrite seemed to make synaptic contact with the dendrite, and out of all the boutons on the membrane region investigated, only 4 boutons established synaptic contact with an additional dendrite (Fig. 8). In these cases, the second synaptic complex was established to thin adjacent dendritic profiles.

The size and distribution of the synaptic complexes were analyzed on part of the dendritic membrane; the result is depicted in Fig. 9. Most boutons are seen to establish several circular or ovoid synaptic complexes, the size of which vary somewhat. The complexes of S, T, F and M boutons occupy only a part of the apposition area of the boutons and usually do not reach the periphery of it. Only boutons of the C-type give rise to one synaptic cleft of *the same size-order as the zone of apposition of the bouton. Since this bouton* type is the biggest, one single C bouton may have a synaptic cleft the size of 10—20 smaller boutons together.

The synaptic complexes of S and F boutons are of rather uniform size, apart from some big complexes belonging to F and, particularly, FD boutons. *The synaptic cleft of M boutons seems to be irregular in shape. It was in fact* rather difficult to judge whether there was one big, irregular synaptic cleft or several smaller ones, situated very close together at the M boutons. The entire postsynaptic membrane is covered by postsynaptic dense bodies and the size of the synaptic complex is bigger than at other boutons of the S- and T-type.

Small P boutons were apposed at the convex side of the M boutons. Some of these P boutons also made contact with *the surface of the dendrite.* P boutons were also apposed at the convex side of some big S boutons (Fig. 10). At one of these, part of the postsynaptic membrane was covered by postsynaptic dense bodies (Fig. 9). Apart from this, it can be clearly seen that the anatomy of the synaptic complex differs between the M boutons and these big S boutons.

*Besides the synaptic complexes, every bouton forms several desmosome-*like junctions with the dendrite. Due to the small size of these structures, it was not always possible to determine whether the profiles in the consecutive

sections belonged to the same or different junctions. Some "dense projections" (Gray 1963) from the presynaptic membrane may also have been misinterpreted as desmosomes.

Dendritic spines and postsynaptic granular endoplasmic reticulum

The dendritic spines are not distributed at random on the surface of the dendrite. Except in a few cases, most spines are arranged as palisades around some M boutons and some big S boutons, to which P boutons are apposed at the convex side (Fig. 10). Granular endoplasmic reticulum, localized just inside the cell membrane of the dendrite, was regularly present at C boutons. Many of the M boutons showed a similar relationship, but for the rest, the distribution of the endoplasmic reticulum appeared to be random.

Size of the boutons

The size of boutons of the same type shows rather large variations and there are several very small boutons of both the S- and F-types. The C and M boutons are, as a rule, bigger than boutons of the other types. The biggest S boutons are bigger than the biggest F boutons. It must be noted, however, that at some places several F boutons are observed to be interconnected by means of short bridges, (Figs. 5 and 6) making it difficult to define the delimitation of the single boutons.

In table 1, the dimensions of the boutons of different types, as obtained from the two reconstructions, are compared to those obtained on single sections in the preceding communication (Conradi 1969 a). Region A in this study has roughly the same extension as DA in the preceding paper.

In (a) and (b), the values for the mean maximum longitudinal length of apposition of all the boutons in the two reconstructions are compared with the mean length of apposition of all the bouton profiles, included in the quantitative estimation on single sections (Conradi 1969 a). In regard to the most commonly occurring bouton types, the values for the F-type are rather similar in (a) and (b), but the values for the S+T type are 10—25% higher in (a). In (c) is shown the sum of the figures for the maximum longitudinal length of apposition of the boutons, included in section 114, which was the section used in the quantitative estimation. The values exceed those for the sum of the length of apposition of the bouton profiles, which could be seen in section 114 (d). The difference is 25—35%.

Table 1. Comparison between dimensions of boutons of different types as obtained by serial or single sections

(a) Mean maximum longitudinal length of appositon of all the boutons		A, μ	B, μ
	S+T	2.1	1.9
	F	2.3	2.0
	C	6.2	3.4
	M	7.7	4.9
(b) Mean length of apposition of all the bouton profiles in the quantitative estimation (Conrad 1969 a)		DA, μ	DB, μ
	S+T	1.8	1.7
	F	2.1	2.1
	C	4.5	5.4
	M	4.4	4.2
(c) Sum of the values for maximum longitudinal length of apposition of the boutons, included in section 114		A, μ	B, μ
		68.5	85.5
(d) Sum of the length of apposition of the bouton profiles in section 114		A, μ	B, μ
		50.5	62.0

Percentage bouton covering

The values underlying this estimation are presented in Table 2. For comparison, the figures from the preceding paper are included.

The percentage bouton covering of A above, seems somewhat higher than at other proximal parts of the dendrites. Apart from this, the values agree rather well with the preceding estimation. In region A, the surface area covered by the S+T boutons is comparatively large. The findings are further dealt with in the discussion.

Table 2

	A weight g	Weight of total surface %	DA bouton covering %	B weight g	Weight of total surface %	DB bouton covering %
Total surface	5.388			5.983		
Boutons, total	4.293	79.5	72.1	4.285	71.7	73.7
S+T	1.586	29.4	24.5	1.490	24.9	26.0
F	2.253	41.9	39.7	2.323	38.9	41.9
C	0.332	6.2	6.1	0.230	3.8	4.7
M	0.122	2.2	1.9	0.242	4.0	1.1

Table 3. Numerical proportion of different bouton types

	A, number	A, %	DA, %	B, number	B, %	DB, %
S+T	83	41.5	45.3	102	42.5	47.8
F	113	56.5	52.3	131	55.0	50.7
C	3	1.5	1.8	3	1.3	1.0
M	1	0.5	0.6	3	1.2	0.4
SUM	200			239		

Numerical proportion of the boutons of different types

The relative numerical proportion of the boutons of different types is presented in Table 3. For comparison, the proportion, corrected for the mean length of apposition, as obtained in the quantitative estimation on single sections (Conradi 1969 a) is included.
The proportion of the boutons of different types is seen to be rather similar in the present and the preceding investigation. The findings are further dealt with in the discussion.

Packing density of the boutons

In region A, 161 boutons are situated within a square, measuring 30.5 × 30.5 μ^2. This corresponds to 17.5 boutons/100 μ^2. In region B, 202 boutons are found inside 26.5 × 35.0 μ^2, which means 22.0 boutons/100 μ^2.

DISCUSSION

It is obvious that serial sections have to be used, not only for the morphological definition of different bouton types, but also for the study of the size and distribution of the boutons. Present techniques, however, permit analyses of only restricted areas and a small number of sections. Therefore, the present study comprises only a limited region of one single motoneuron. This must be born in mind when comparisons with the results from the quantitative estimation in the preceding paper (Conradi 1969 a) or with earlier light microscopic findings (see below) are made. The interest in this study was focused on the methodology for investigations of the

membrane relationships of neurons through serial sections, and the topography of the location of boutons of different types.

For investigations, similar to the present one, it seems advantageous to depict the membrane of the nerve cell in one plane. Although some difficulties are involved—the same applies to three-dimensional reconstructions—the topography of the membrane relationships, at least in regard to the boutons, can be clearly seen and measurements on the drawings can easily be done. Longitudinally-sectioned cylindric profiles are the easiest to visualize in this way and require relatively few sections. It is, however, also possible to study obliquely- or transversely-sectioned cylindrical processes. In the former, the lines representing the sections on the transparent paper will be elliptical, while in the latter, the lines will be straight and parallel and directed at right angles to the longitudinal axis of the dendrite. It is also possible, with some limitations, to visualize the mantle surface of curved dendrites, in which the diameter is not constant (see Conradi and Skoglund 1969 a). For stereometrical reasons, however, it is very difficult to visualize the whole surface of a neuron cell body using the method in this study.

Distribution of the boutons

Apart from a possible clustering of the F boutons, the distribution of the boutons of different types on the dendritic mantle surface seemed to be rather random. Due to the typing used here a more exact pattern of localization of different bouton types might have been overlooked. Undoubtedly, it would have been advantageous if the preterminal axons could have been traced. In the present and preceding paper it was observed that one bouton can make synaptic contact with two dendrites of different dimensions and possibly also of different origin. Lorente de Nó (1941), when studying the synaptology of the motoneurons in the light microscope, also found that boutons from the same preterminal axon can make contact with several different neuronal processes. These observations might indicate that the spatial specificity for the establishment of synaptic contacts, at least with regard to the boutons, could be rather inexact.

Quantitative estimations

The aim of the quantitative studies on the reconstructions was, as mentioned, to get some idea on the relevance of the data from the quantitative estimation on single sections.

Bouton size. It could be seen that the C and M boutons formed a larger area of apposition than other bouton types. The area of apposition showed a relatively large size-range, even between boutons of the same type, but the shape of this area was rather similar between the boutons.

As expected, the mean maximum longitudinal diameter of the boutons in the reconstructions exceeded the mean length of all bouton profiles on the proximal parts of the motoneuron dendrites in the quantitative estimation on single sections (Table 1). The difference between the S+T and F boutons in the reconstructions, however, was smaller than in the quantitative study. This could mean that the boutons on the analyzed dendrite were not representative for the whole population (see below). Another possibility is that there are differences in the morphology of these two bouton types, introducing errors in investigations on single sections. The maximum longitudinal length of apposition of all the boutons in section 114, used in the quantitative study in the preceding paper, exceeded the length of apposition of the bouton profiles in that section by 25—35%. Nevertheless, the material is too small to allow any general conclusions on the ratio between the length of apposition of bouton profiles in single sections and the true values. For such studies, either a greater number of reconstructions must be used, or the measurements must be performed on several sections in each reconstruction.

In the earlier light microscopic studies on various species (Hoff 1932, Gibson 1937, Phalen and Davenport 1937, Barr 1939, Minckler 1940, Barnard 1941, Schadewald 1941, Haggar and Barr 1950, Illis 1964, Gelfan and Rapisarda 1964), most of the boutons on the motoneurons ranged in size between 1 and 2 μ, although bigger boutons were also observed. When these values are compared with those in Table 1, it is seen that the figures obtained in the present work are slightly higher. The discrepancies can be due to the different sampling and preparative procedures used. It should be pointed out, however, that the packing density of the boutons as obtained in this study (about 20 boutons/100μ^2) is of the size-order as the highest values (15—20 boutons/μ^2) from the light microscopic studies. (Barr 1939, Minckler 1940, Wyckoff and Young 1956, Gelfan and Rapisarda 1964). If the value obtained in the present study is representative, and the shrinkage factors are comparable, the impregnation of the boutons in the earlier works seems to have been effective.

Bouton covering. The total percentage bouton covering on the surface of the dendrite in region B, was rather similar to the one obtained for DB in the preceding study (Table 2). The value for region A was about 10% higher

membrane relationships of neurons through serial sections, and the topography of the location of boutons of different types.

For investigations, similar to the present one, it seems advantageous to depict the membrane of the nerve cell in one plane. Although some difficulties are involved—the same applies to three-dimensional reconstructions—the topography of the membrane relationships, at least in regard to the boutons, can be clearly seen and measurements on the drawings can easily be done. Longitudinally-sectioned cylindric profiles are the easiest to visualize in this way and require relatively few sections. It is, however, also possible to study obliquely- or transversely-sectioned cylindrical processes. In the former, the lines representing the sections on the transparent paper will be elliptical, while in the latter, the lines will be straight and parallel and directed at right angles to the longitudinal axis of the dendrite. It is also possible, with some limitations, to visualize the mantle surface of curved dendrites, in which the diameter is not constant (see Conradi and Skoglund 1969 a). For stereometrical reasons, however, it is very difficult to visualize the whole surface of a neuron cell body using the method in this study.

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SUMMARY

In order to study the three-dimensional morphology and distribution of neuronal and glial elements on the motoneuron surface, the membrane relationships of the proximal part of a motoneuron dendrite were analyzed through ultrathin serial sections. One of the sections through the dendrite was included in the quantitative estimation on single sections in the preceding paper (Conradi 1969 a). The membrane relationships were illustrated in drawings, showing the mantle surface of the dendrite unfolded. The morphology of the area of apposition of the boutons was investigated, as well as the distribution of boutons of different types on the surface of the dendrite. The arrangement of the preterminal axons of the boutons and the size and shape of the synaptic complexes and desmosome-like junctions were also investigated.

The boutons were tightly packed over nearly the entire analyzed surface. All the types of boutons described in the preceding communication were present, and two types of big boutons containing spherical synaptic vesicles were found to have smaller boutons apposed at the convex side. The distribution of boutons of different types seemed almost random. Most of the boutons established more than one synaptic complex and several desmosome-like junctions with the dendrite. Nearly all the preterminal axons were unmyelinated for the last distance before the boutons and a few boutons *de passage* were found.

Some quantitative parameters were also studied and the results were compared with the findings from single sections in the preceding communication. The mean maximum length of apposition of the boutons in the direction of the longitudinal axis of the dendrite exceeded the mean length of apposition of the bouton profiles in the single sections. The percentage bouton covering, both totally and for the different types of boutons separately, was similar to that found for proximal parts of the dendrites in the quantitative study on single sections.

It is concluded that a good estimate on the size and distribution of boutons can be obtained from observations on single sections.

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LEGENDS TO THE FIGURES

Fig. 1. Survey of the analyzed dendrite at section 127 (see Figs. 5 ff.). The extension of regions A and B are depicted. ×910

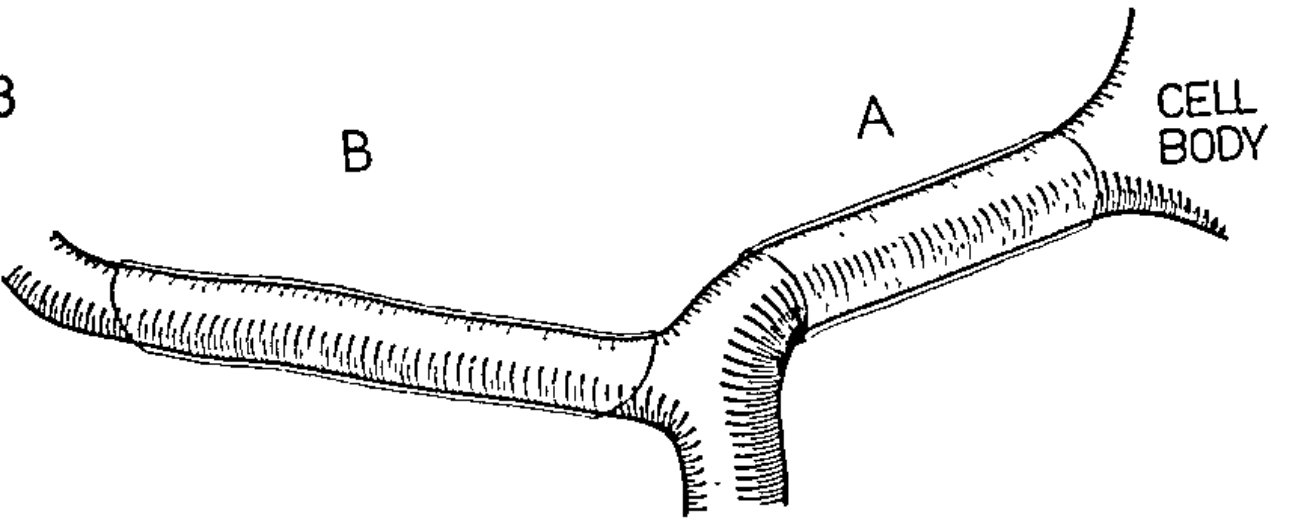
Fig. 2. Part of region B, just at the cranial edge of the dendrite. Part of the dendrite (D) is cut through to the left in the figure, whereas to the right, the apposing boutons are sectioned. The figure illustrates the difficulty in depicting the membrane relationships near the upper and lower edges, as described in the text. Note the dense packing of the boutons, some of which are labeled (B). ×7,900

Fig. 3. The figure shows a schematic drawing of the dendrite and the analyzed regions of the surface.

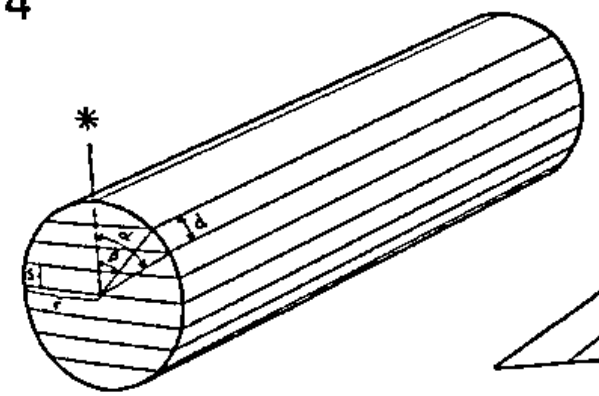
Fig. 4. The drawing illustrates the method by which the mantle surface of the serially sectioned cylindrical profile is visualized in one plane. The parallel straight lines represent the sections. S=mean section thickness, r=radius of the cylinder, d=distance between the sections on the surface.

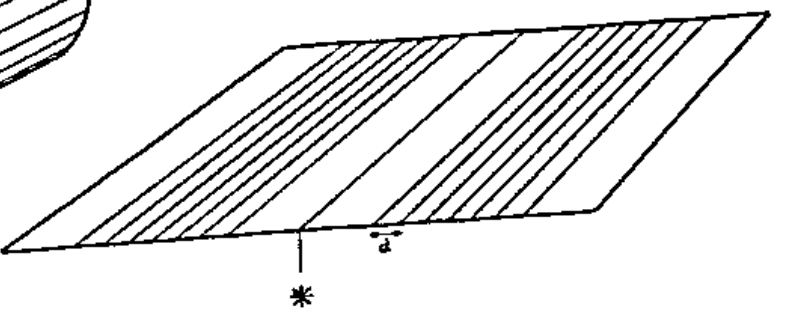
Figs. 5—10. Various details of the surface relationship of the analyzed dendrite are shown. Figs. 5, 7, 9 and 10 show region A, Figs. 6 and 8 region B. The explanations to the figures are included in the drawings.


1
A
B
50 Д
2
D
B
B
B
B
5Д



4





$$\cos \alpha = \frac{2s}{r}$$

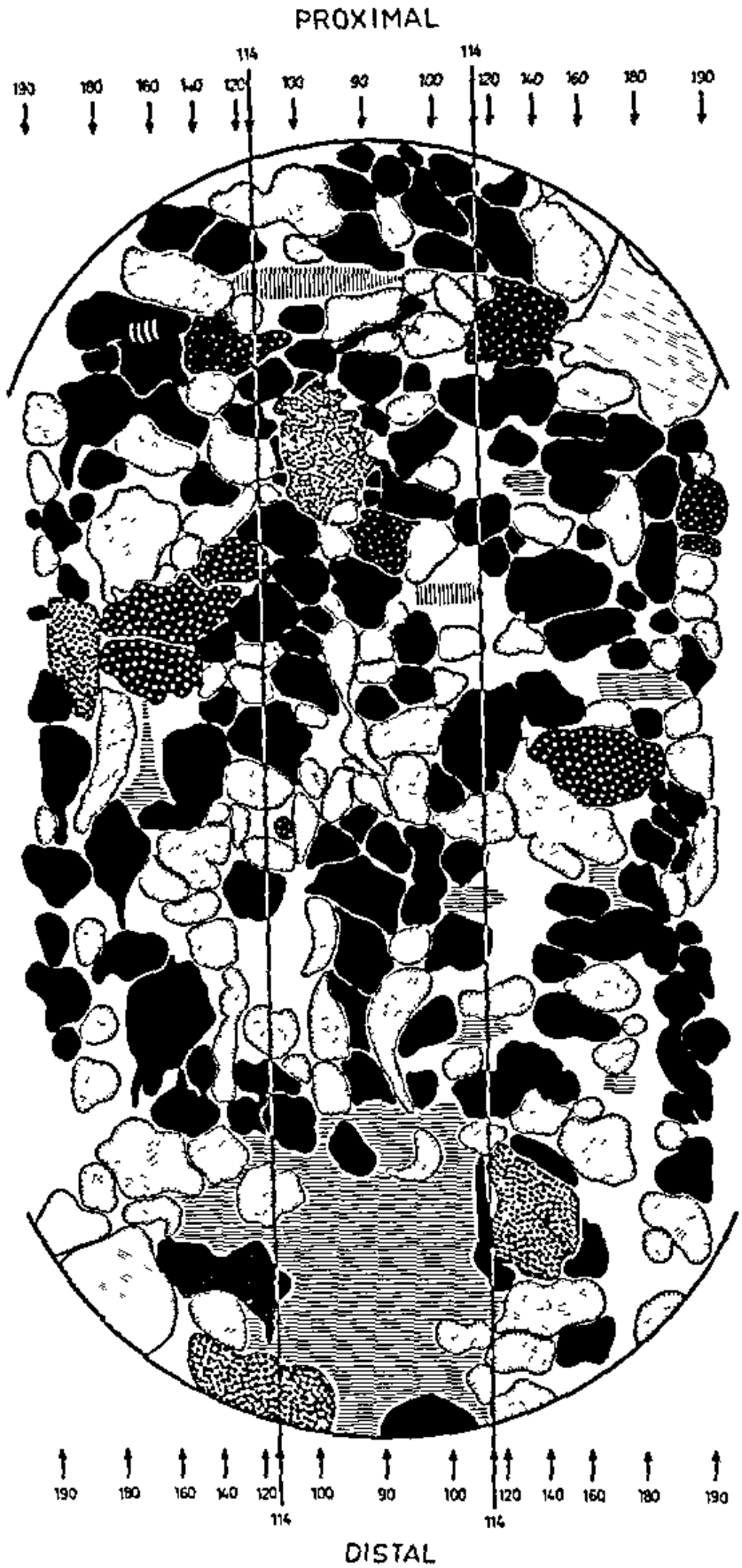
$$\cos \beta = \frac{3s}{r}$$

$$d = 2 \cdot \pi \cdot r \left(\frac{\alpha}{360^\circ} - \frac{\beta}{360^\circ} \right)$$

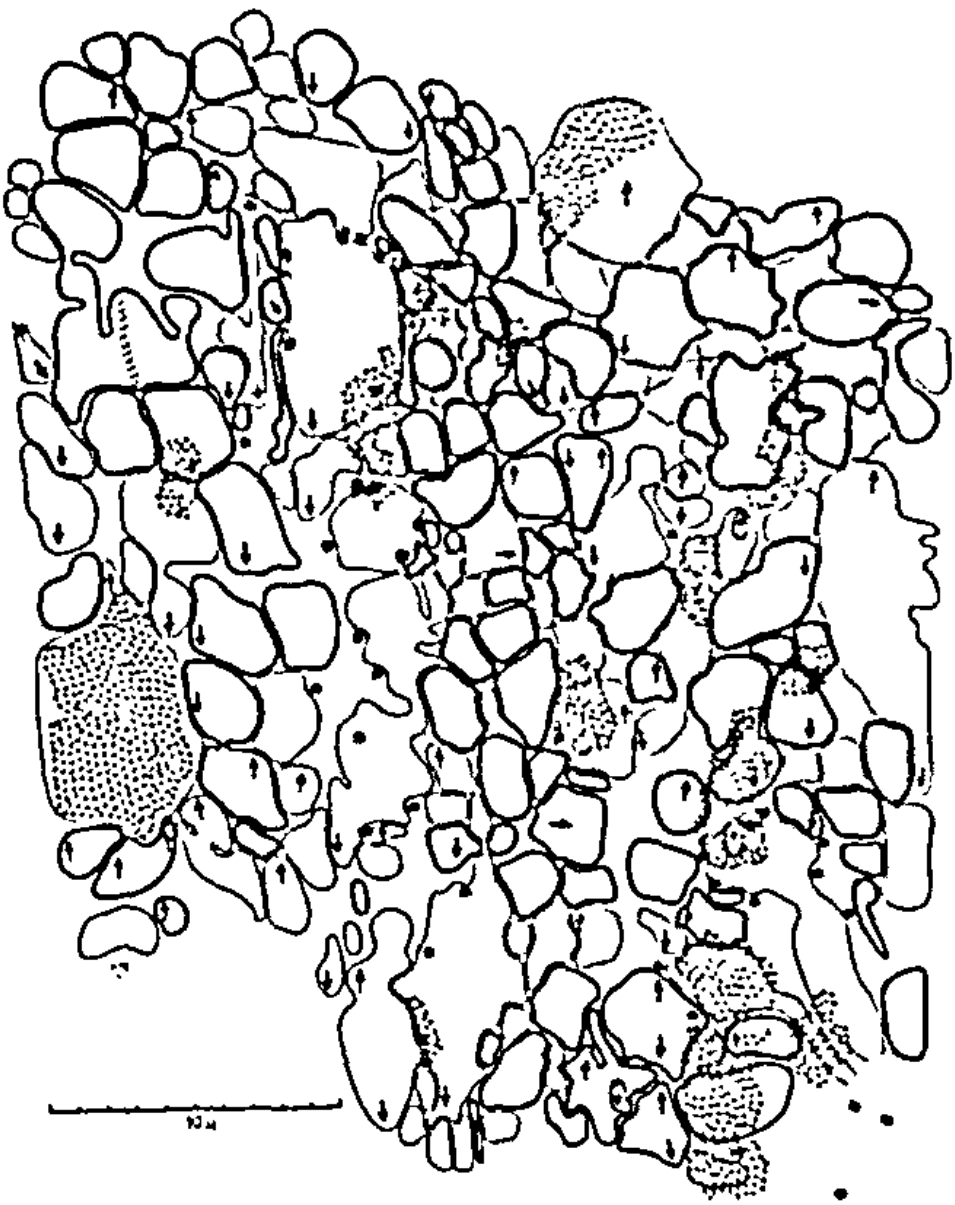
5


PROXIMAL
A
DISTAL
BOUTONS
= F (and P)
= M
= DENDRITE, ASTROCYTIC LAMELLAE INTERPOSED
= FD
= C
= MYELIN, ASTROCYTIC LAMELLAE
= S

6


PROXIMAL
B
114
114
190
180
160
140
120
100
90
100
120
140
160
180
190
190
180
160
140
120
100
90
100
120
140
160
180
190
114
114
DISTAL

7



BOUTONS

= S, C, M

= F (and P)

= "DOUBLE SYNAPSE"

= PRETERMINAL AXON, UNMYELINATED

= PRETERMINAL AXON, MYELINATED

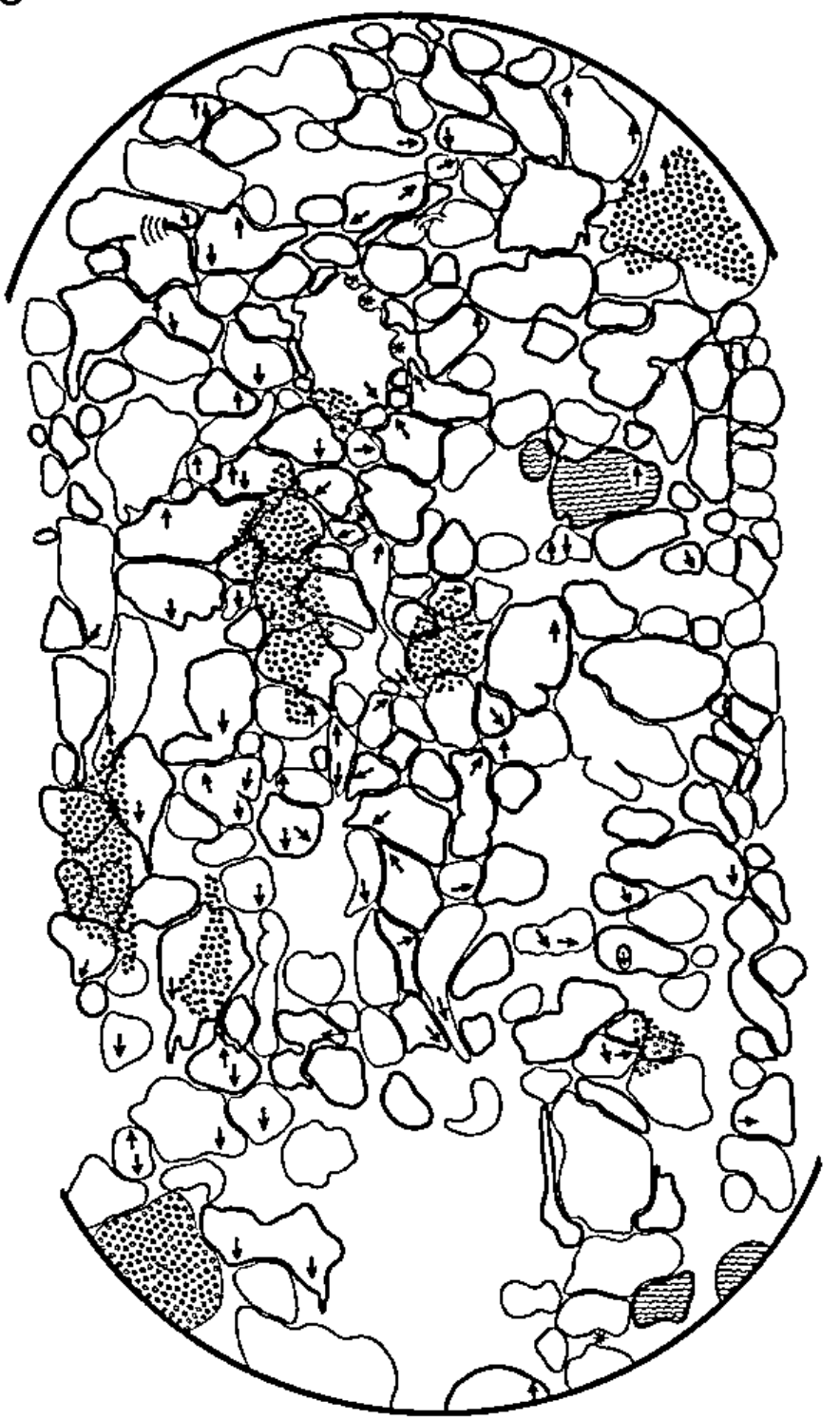
= SUBMEMBRANEOUS, GRANULAR ENDOPLASMIC RETICULUM

= DENDRITIC SPINE

8

PROXIMAL

B

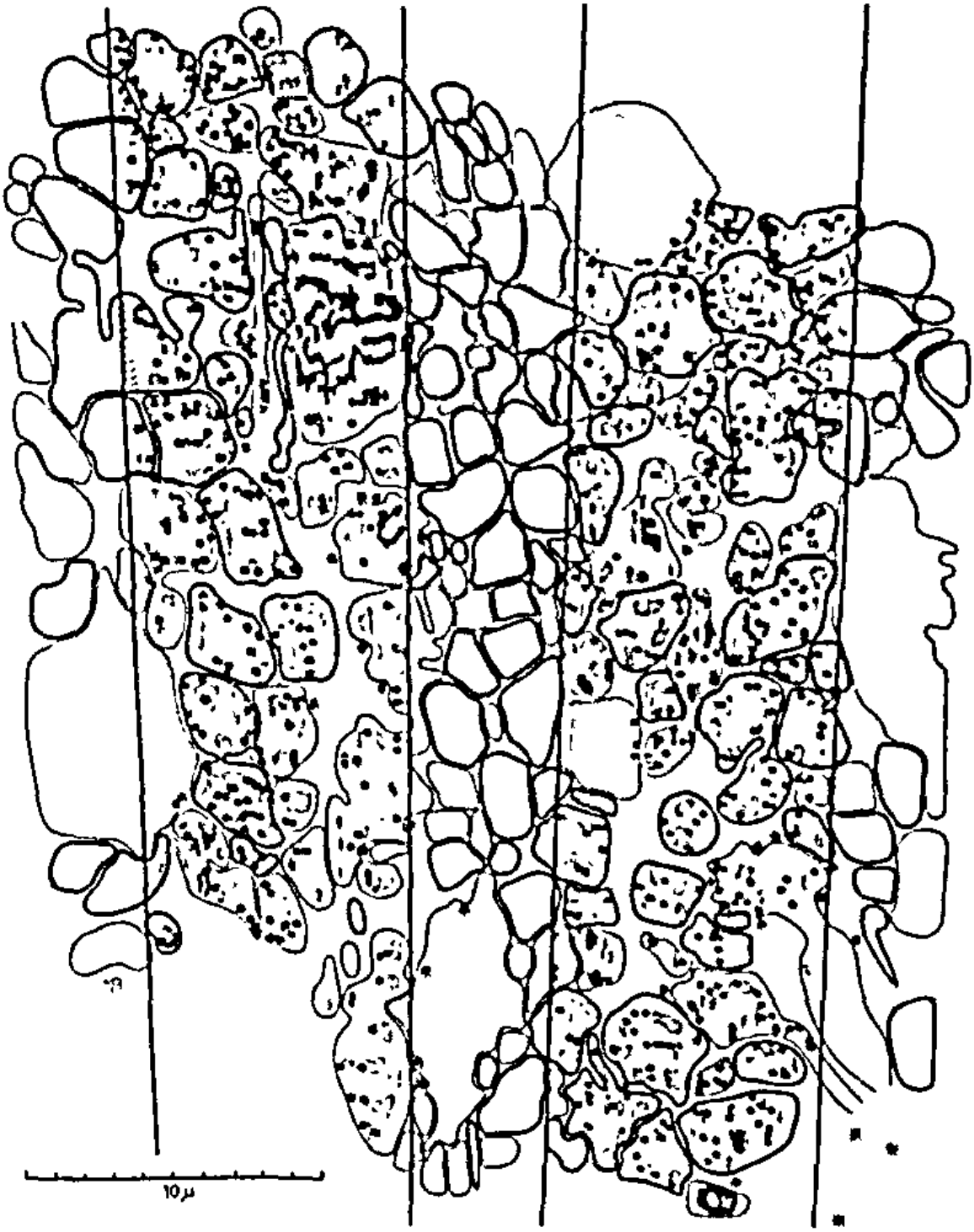


DISTAL

9

PROXIMAL

A



DISTAL

BOUTONS:

= S, C, M

= F (and P)

= SYNAPTIC COMPLEX

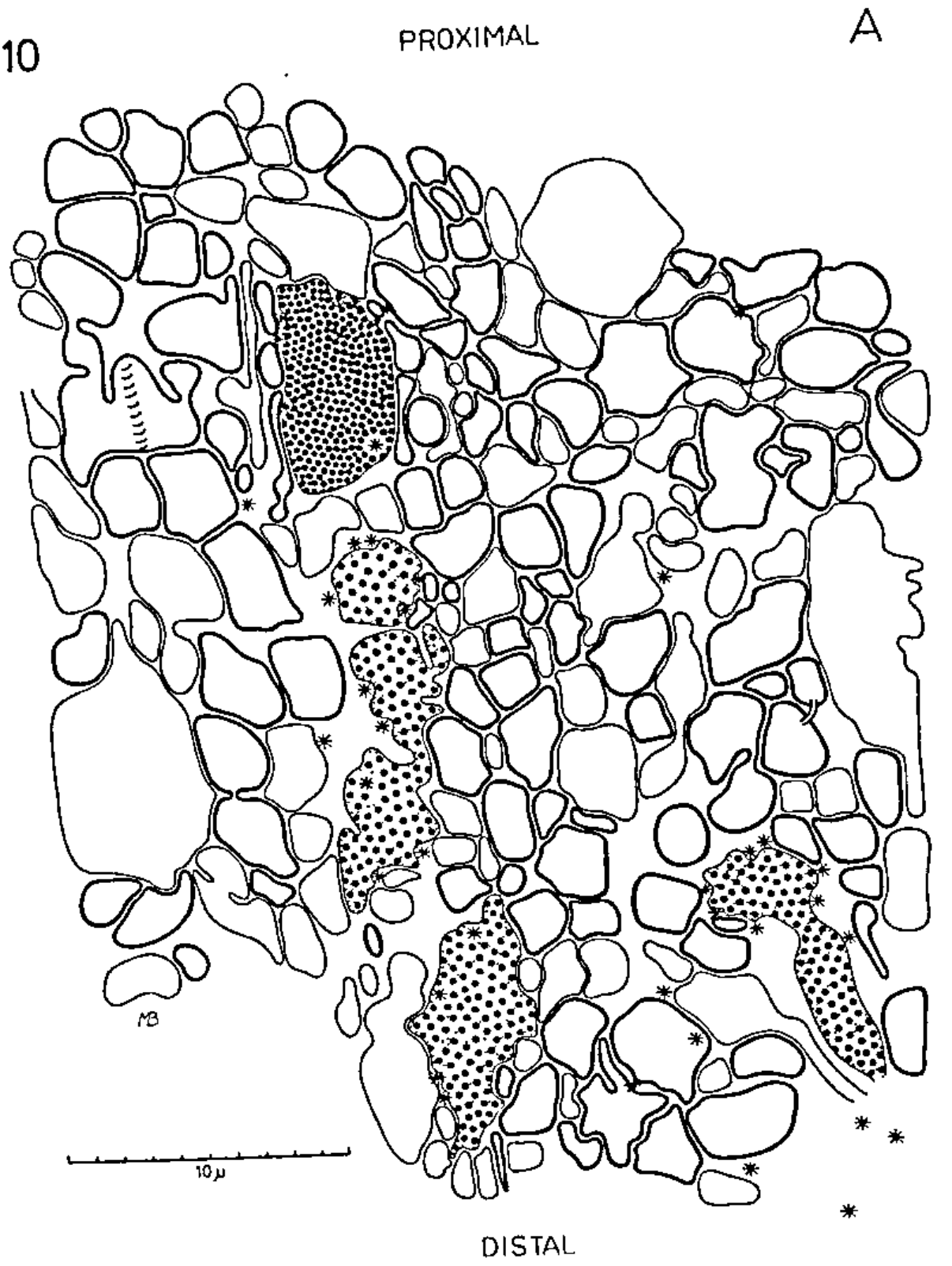
= SYNAPTIC COMPLEX WITH POSTSYNAPTIC DENSE BODIES

= DESMOSOME-LIKE JUNCTION

= SUBSYNAPTIC CISTERNA

= DENDRITIC SPINE

10


PROXIMAL
A
MB
10 µ
DISTAL
BOUTONS
= S, C
= F (and P)
= M
= S with AXO-AXONIC SYNAPSES
* = DENDRITIC SPINE

Observations on the ultrastructure of the axon hillock and initial axon segment of lumbosacral motoneurons in the cat

by

SEBASTIAN CONRADI

INTRODUCTION

From physiologic experiments with the intracellular recording technique there is evidence that activation of a motoneuron, synaptically or antidromically, is initiated in the first, unmyelinated part of the motoraxon, or sometimes in the first node of Ranvier (Fuortes *et al.* 1957, Coombs *et al.* 1957). At these two places, the threshold for impulse-initiation is considered to be lower than at the cell body and dendrites of the motoneuron (Eccles 1957). From the site of origin, the active impulse is propagated downward, along the myelinated motoraxon and upward, invading the cell body and dendrites.

The ultrastructure of the initial axon segment of various types of central neurons other than the spinal motoneurons has been described earlier (Green 1961, Herndon 1963, Kohno 1964, Johnston and Roots 1966, Westrum 1966, Ehrenbrand 1967), but the ultrastructure of the relationships of the neuron cell membrane at the initial segment had not been dealt with when the present results were presented in preliminary form (Conradi 1966). Recently, however, an ultrastructural specialization of the cell membrane at the initial axon segment has been observed in several types of neurons (Campos-Ortega *et al.* 1968, Westman 1968, Palay *et al.* 1968, Peters *et al.* 1968). The results presented therein are similar to the ones found in the spinal motoneurons.

The ultrastructure of the central nodes of Ranvier has been described earlier, mostly on rather thin fibres (Pease 1955, Uzman and Villegas 1960,

Peters 1960, Maturana 1960, Bunge *et al.* 1961, Bunge *et al.* 1965, Meturals 1965; see also Bunge 1968) on which the ultrastructure of the axon membrane after aldehyde fixation has also been described (Andres 1965, Peters 1966, Karlsson 1967) or on large axons in the fish brain (Robertson *et al.* 1963). There are, however, no previous reports on nodes belonging to large mammalian central myelinated fibres.

The present work describes the ultrastructure of the motoneuron at the initial axon segment and at the transition zone between this and the cell body; this zone is usually called the axon hillock. Attention is focused on the ultrastructure of the motoneuron cell membrane and the immediate environment at these two places. Some observations on the relationships of the motoraxon cell membrane at the central motoraxon nodes are included.

Earlier light-microscopic studies on the motoneurons show that boutons seem to be located at the axon hillock (Lorente de No 1938, Barr 1939, Haggar and Barr 1950) but the relationship to the initial axon segment has not been ascertained. In the present study, special care was taken to determine whether boutons are situated on these two parts of the motoneuron. It is concluded that boutons are found only on the axon hillock, and the distribution of the boutons of different types in this zone is compared with that on the motoneuron cell bodies and dendrites, an analysis of which was the subject of the two preceding studies (Conradi 1969 a, b).

MATERIAL AND METHODS

The same material as used in the preceding papers on the relationships of the motoneuron cell membrane (Conradi 1969 a, b) was used here. Some of the observations were also made on animals which had been subjected to unilateral dorsal root section, 1—14 days before fixation (see Conradi 1969 d). The material thus comprises a total of about 60 cats. The description refers to observations on about 30 initial segments and roughly the same number of nodes from a total of about 20 animals.

Semithin transverse sections of the spinal cord in the segments, L 6, L 7 and S 1 were stained with toluidine blue and examined in the light microscope. Some observations on the initial segment and nodes of the motoraxons were made on these sections. The lateral part of the ventral horn was trimmed out and cut into ultrathin sections. For the studies on the motoraxon nodes, the ventral part of the gray substance, just dorsal to the region where the motoraxons enter the white substance, was trimmed out in transversely-cut specimens. Some profiles of big axons, quite similar to those studied in the ventral part of the gray substance, were also found at random around the motoneurons.

Ultrathin sections were collected on one-hole grids, usually 5—15 sections per grid, according to the method of Galey and Nilsson (1966). One longer series of ultrathin transverse sections through the lateral part of the ventral horn was also used for the

study of the initial axon segment. In order to include complete initial segments, the series had to start at random at a region where several neighbouring motoneurons were found in the lateral motoneuron nucleus. Through consecutive light-microscopic sections before the start of the ultrathin sectioning, it was possible to determine that the majority of the actual motoneurons had thus far only been cut through the periphery. In order to include as many motoneurons as possible, the ultrathin sections had the same width as the hole in the grid and were so high that only 7—9 sections could be collected on each grid. Series made in this way, if continued long enough, will sooner or later include whole initial segments. In the present case, actually 2 initial segments were included in the same series at nearly the same level. The series consisted of 200 consecutive ultrathin sections, of which 10 single sections were lost. The mean section thickness, 960 A, was determined from the number of sections required to pass longitudinally-cut dendrites, which were assumed to be cylindrical and the maximal diameter of which was measured on the photographs. At every secon section, the two initial segments were photographed at a primary magnification of 3,300×. The initial segments were reconstructed from paper copies with wax-plates, 1 mm thick. The final magnification, 5,200×, was adjusted to the thickness of the plates, and calculated from the expression: final magnification $= \frac{1 \text{ mm}}{\text{mean section thickness} \times 2}$.

The types of the boutons on the axon hillock were controlled through inspection of the sections at high magnification in the electron microscope and the result was transferred to the copies. The transparent wax-plates were placed on the copies, and the contours of the initial segment, myelin sheath and axon hillock with related boutons and glial extensions were traced with a small knife through the wax. The wax-contours were then glued together and various parts of the reconstruction were painted.

RESULTS

Light microscopy

In toluidine-stained, semithin transverse sections of the spinal cord, longitudinally-sectioned initial axon segments which leave the motoneurons are rather commonly observed (Fig. 1). This gives the impression that many of the initial motoraxon segments run horizontally. Most axons originate at the motoneuron cell body (cf. Strong 1906, Ramon y Cajal 1909, Chu 1954), but axons leaving from the proximal parts of a dendrite were also observed. Most of the initial segments observed were directed ventrally, although some motoneurons, especially in the dorsal part of the ventral horn, send axons medially or dorsally, as has earlier been pointed out (Balthasar 1952).

The myelinated motoraxons in the ventral part of the gray matter usually form a rather dense plexus just proximal to the entrance into the white substance. Only a minority of these fibres run exactly in horizontal direction. In such instances, however, nodes of Ranvier can sometimes be observed. In

a few cases, thin myelinated collaterals were observed to leave the motoraxons at a node (cf. Ramon y Cajal 1909, Scheibel and Scheibel 1966). Outside the myelin sheaths of the motoraxons, rounded compact profiles are often found. They resemble myelin both in staining and outlook (Figs. 1 and 2).

The heavily myelinated large fibres seem difficult to preserve properly with the fixation method used here. Nearly always there was a marked splitting of the myelin (cf. Webster 1964).

Electron microscopy

The axon hillock

The transition between the motoneuron and the initial axon segment, called axon hillock, is cone-shaped. The motoneuron cytoplasm in this region differs from that inside the cell body by lacking granular endoplasmic reticulum (see Palay *et al.* 1968, Peters *et al.* 1968). Instead, there are many neurofilaments and 200 Å neurotubuli, which are mainly longitudinally-oriented (cf. Palay *et al.* 1968, Campos and Ortega *et al.* 1968). Mitochondria and ribosomes are also present (Fig. 2, see also Fig. 8). The motoneuron cell membrane at this region is quite similar to that found at the cell bodies as described earlier (Conradi 1969 a), and it is covered in the same manner by boutons and glial extensions.

The initial segment

At the distal part of the axon hillock, there is a gradual transition into the initial segment (Fig. 2). This is straight and the diameter is nearly constant along its course. In none of the cases was the initial segment observed to branch. The cytoplasm contains many longitudinally-arranged 200 Å neurotubuli, which in longitudinal sections often appear paired (cf. Palay *et al.* 1968, Fig. 3), a phenomenon also observed in the distal part of the axon hillock. In addition, there are some longitudinally-arranged neurofilaments as well as mitochondria, some of which are situated just below the cell membrane. Dense bodies, vesicular profiles and ribosomes are also present, but no granular endoplasmic reticulum is seen.

Dimensions

The dimensions of the axon hillock and initial segment were measured on single longitudinal sections and on the two reconstructions. The findings are presented in Table 1.

Table 1. Dimensions of axon hillock and initial segment

	Axon Hillock		Initial Segment		AH+IS length, μ
	length, μ	diam. base, μ	length, μ	diam., μ	
Model 1	14	8	23	3.3	37
Model 2	13	10	35	3.6	48
Single sections,					
Profile 1	8	9	26	3.3	34
2	8	7	24	3.4	32
3	6	7	23	3.3	29
4	21	15	25	4.2	46
5	13	6	29	2.9	42
6	8	10	28	3.5	36
7	11	11	38	3.7	49
8	12	8	26	3.5	38

It is seen that the initial segment is usually longer than the axon hillock. The diameter of the base of the axon hillock is 2—4 times as large as the diameter of the initial segment. Although the figures for the diameters as obtained from single sections probably do not represent the true values, the diameter of the initial segment seems small, compared to the total diameter of the myelinated motoraxons (about 20μ) in the ventral horn.

The cell membrane of the initial axon segment

The cell membrane of the initial motoraxon segments is specialized in a characteristic way. At the inner side of the membrane, a 200—400 A thick layer of contrast-rich material is attached and extends from the distal end of the axon hillock to the beginning of the myelin sheath (Figs. 4, 5 and 6). It covers the entire circumference of the axon (Figs. 7 and 9) with only a few exceptions (see below). At high magnification the material shows maximum density at a distance of about 100 A from the inner limit of the cell membrane (see Figs. 3 and 4). In this respect, the layer is very similar to the one reported to be present at the axon membrane at the central nodes of Ranvier (see Peters 1966). In longitudinal sections, the thickness of the contrast-rich layer is seen to vary. At regular intervals, there are, namely, dense projections of the material, about 400 A thick, situated about 800 A apart and directed towards the cytoplasm. (Fig. 4). When the axon membrane was cut nearly tangentially, these projections were especially prominent (Fig. 5).

The outside of the axon membrane is covered by a narrow extracellular space, where a finely granular material regularly is observed. The space has a constant thickness of 150—200 A along the initial segment (Fig. 4), but just proximal to the myelin sheath, the layer of granular material sometimes thickens and the material interdigitates with surrounding astrocytic processes (Fig. 6). The granular material is very dense in the best fixations. At many places, the axon membrane shows "coated" invaginations (Fig. 3) (Roth and Porter 1964, Anderson 1964, Andres 1964) which contain the same granular material. A similar finding was made by Campos-Ortega *et al.* (1968). "Coated" vesicles are also sometimes seen in the cytoplasm of the initial segment, and have previously been reported to occur in the axoplasm of central nodes of Ranvier (Andres 1965, Karlsson 1967). Although "coated" invaginations sometimes are observed at the cell membrane of the motoneuron cell body or dendrites, they seem to occur more often at the initial axon segment. At a few places, the axon membrane can show some single, short microvilli-like projections or small flattened-off bulges in which mitochondria are often found. The membrane specialization and extracellular material are usually lacking at these bulges.

As a rule no boutons were observed on the cell membrane of the initial axon segment. Instead, there are many tightly packed glial extensions just outside the extracellular material. Most of the processes were astrocytic-like (Mugnaini and Walberg 1964, Maxwell and Kruger 1965) containing a clear cytoplasm. These processes were usually flattened and oriented parallel to the axon membrane (Figs. 2 and 4). Glial cells with a relatively dark cytoplasm were also found just outside the axon. The nuclei of most of these cells were often chromatin-rich and the cells were frequently flattened and oriented parallel to the surface of the axon (Figs. 2, 5 and 8). In some cases, the initial axon segment seemed encircled by such flattened glial processes (Fig. 7). Most of the dark glial cells around the initial axon segment then looked rather like microgliacytes (Stensaas and Stensaas 1968, Holländer *et al.* 1968, Blinzinger and Kreutzberg 1968, third glial cell type: Vaughn and Peters 1968) than oligodendrocytes (Mugnaini and Walberg 1964, Kruger and Maxwell 1966). Capillaries were often found quite near the initial motoraxon segment.

The boutons on the axon hillock

Boutons are situated on the axon hillock, although not on the initial segment. A few boutons can be observed at the transition between these two regions. The boutons on the axon hillock have much the same characteristics as those

found on other parts of the motoneuron cell membrane (Conradi 1969 a, b). Boutons of the S-, C- and F-types (Conradi 1969 a, b) were regularly present on the axon hillock; M boutons were never observed, and T boutons appeared only rarely. The spectrum of boutons of different types is thus similar to the one found on the motoneuron cell bodies, but different from that found on the dendrites (see Conradi 1969 a, b). Although measurements on the dimensions of the boutons on the axon hillock were not made, the percentage covering of boutons on the cell membrane of the axon hillock seemed rather similar to that found on the motoneuron cell bodies.

The distribution of the boutons on the axon hillock in the two reconstructions is shown in Figs. 9 and 10. No C boutons were present on either one. Some F boutons seem to be clustered together but apart from that, no distinct pattern of localization of the boutons of different types could be distinguished. In the second reconstruction, one adjacent dendrite made contact with the axon hillock. The extension of a dark glial cell, probably a microgliacyte, adjacent to the initial segment, is also shown in the reconstruction.

The first reconstruction shows 32 boutons situated on the axon hillock; two of these were found at the transition between the axon hillock and initial segment. 21 of the boutons were of the F-type, or 66% of the total population; the rest were S-type. In the second reconstruction, 46 boutons were situated on the axon hillock. Of these, 27 boutons, or 59% of the population, were of the F-type, and 19 were S-type.

The myelinated part of the motoraxon

At the beginning of the myelin sheath, the axon is usually somewhat constricted (Fig. 11). The axon is covered by the terminal cytoplasmic pockets (cf. Berthold 1968) of the myelin for 3—4 μ and there is immediate contact between the cell membrane of these structures and the axon membrane, which is not specialized as it is at the initial segment. Usually, not all of the cytoplasmic pockets reach the axon membrane, instead an arrangement similar to the one found at the peripheral motoraxon nodes is observed (cf. Berthold 1968) (Fig. 11). In a few instances the cytoplasmic pockets derived from the most superficial myelin lamellae terminated freely, without making contact with the axon membrane.

Just distal to the beginning of the myelin sheath, the axon diameter increases. The axoplasm contains many neurofilaments and neurotubuli, like other central axons (see Metuzals 1963, Peters 1966, Hirano *et al.* 1967, Bunge 1968). In this region, small figures resembling the granular endoplasmic reticulum in the motoneuron cell bodies were observed in the axo-

plasm in several cases (Fig. 11). Otherwise, the absence of granular endoplasmic reticulum is one of the main criteria for diagnosing profiles of axons in ultrathin sections (Palay 1956).

The myelin sheath of the motoraxons shows some peculiarities as compared to smaller central myelinated axons (Metuzals 1963, Hirano *et al.* 1967, Bunge 1968).

1) Incisures of Schmidt-Lantermann are regularly observed (Figs. 12 and 13). These are relatively abundant and run through the myelin in a zig-zag fashion. The possible existence of the incisures in central myelin has been much debated previously (see Bielschowsky 1928, Lehmann 1959), but they have been demonstrated ultrastructurally in the white substance of the spinal cord of the cat (Bunge et al. 1960).

2) The myelin sheath sometimes shows protrusions, or more frequently compact circular profiles of myelin, 3—6 μ in diameter, are found just outside the myelin sheath, often near incisures and nodes (Fig. 12). Analysis of serial sections revealed that these bodies mostly are spheroid. Usually, they were not continuous with the myelin around the motoraxon, thus, not quite similar to the "redundant" myelin sheaths described by Rosenbluth (1966). In survey articles on the morphology of central myelin, nothing is mentioned about these bodies (Bielschowsky 1928, Lehmann 1959, Sulzmann 1962, Bunge 1968).

Ultrastructure of the axon membrane and surrounding structures at the motoraxon nodes

At the nodes, the motoraxon is usually constricted. The nodal axon membrane faces the node-gap, which is formed between the two adjacent internodal myelin segments (Fig. 14). The length of the node-gap is 0.7—1.1 μ at the axon membrane, as measured on longitudinal sections of 20 profiles. The terminal cytoplasmic pockets of the myelin lamellae are often smaller and filled with a more contrast-rich cytoplasm than the corresponding structures distal to the initial segment (Fig. 15). As in this region and at the peripheral motoraxon nodes (Berthold 1968), not all of the terminal pockets do reach the axon membrane. Instead, in longitudinal sections, several rows of consecutive terminal pockets are seen to project from the axon membrane into the myelin (Fig. 15). Similar structures have not been observed at central nodes of smaller myelinated fibres (Andres 1965, Peters 1966, Karlsson 1967, Hirano *et al.* 1967, Bunge 1968). Here, the terminal cytoplasmic pockets cover 3—4 μ of the axon.

The axoplasm at the node sometimes contains profiles of mitochondria which may be localized just inside the nodal axon membrane. Many mitochondria are also often present in the axoplasm adjacent to the nodes, and in some cases, actual clusters could be observed here (Fig. 16). No aggregation of mitochondria is found just outside the myelin near the nodes, as is the case in peripheral nodes (Williams and Landon 1963, Berthold 1968).

The nodal axon membrane is covered on the inside by a layer of contrast-rich material, quite similar to that found around the initial segment. The node-gap is filled with granular extracellular material (Figs. 17, 18 and 19). This material, which is of the same ultrastructural appearance as that around the initial segment, extends from the axon to the outer margin of the myelin sheath, or even farther. Outside the material, there are many astrocytic extensions, which are also found in the node-gap (Figs. 17, 18 and 19). Few, if any, oligodendrocytic processes are found near the node-gap. Sometimes neuronal profiles were found just outside the extracellular material, but they were usually surrounded by thin astrocytic capsules.

It has to be mentioned that all nodes belonging to small myelinated axons in the ventral horn exhibit only a thin rim of extracellular material, as has previously been found at other places in the CNS (Andres 1965, Peters 1966, Karlsson 1967), after a similar fixation.

In a few instances, the motoraxon was observed to give off a thin, myelinated collateral from the central nodes, near the entrance to the white substance (Fig. 20) (cf. Ramon y Cajal 1909, Scheibel and Scheibel 1966). At these places, the general morphology of the node was similar to that just described. The specialization of the nodal axon membrane and extracellular material extended along the collateral to the myelin sheath.

DISCUSSION

The initial motoraxon segment thus shows a characteristic "undercoating" and is surrounded by dense extracellular material. Since either both or none of the components of the membrane specialization is observed at the same time, they are considered as one entity. Since a similar specialization of the nerve cell membrane seems to be present at the initial segments of other types of neurons (Campos-Ortega *et al.* 1968, Palay *et al.* 1968, Peters *et al.* 1968, Westman 1968) and at central nodes (Andres 1965, Peters 1966, Karlsson 1967) after a similar fixation, the specialization could be a commonly-occurring feature, linked to specific functional properties of the neuron cell membrane.

It might seem controversial to characterize one of the components of the membrane specialization at the initial axon segment as an extracellular material. The presence and appearance of an extracellular compartment in central nervous tissue prepared for electron microscopic studies has, namely, been claimed to be dependant on the preparative procedure. Following permanganate or osmic acid fixation of central nervous tissue, an extracellular space of about 200 A or less is found at most places between the neuronal and glial extension (see for instance Palay *et al.* 1962, Peters 1962, for historic notes, see Robertson *et al.* 1963, Bondareff 1966). It has been claimed that macromolecules can be transported through narrow intercellular channels in the CNS (Brightman 1965, Pappas *et al.* 1966, Torack 1966). Furthermore, there is histochemical evidence that small amounts of an extracellular material are regularly present between the extensions in the CNS (Bondareff 1965, 1967, Pease 1966, Rambourg and Leblond 1967). The material has been suggested to be a mucopolysaccharide, and it has been claimed that such material could serve as a cation exchanger (Bennet 1963). ATPase activity has also been demonstrated at the interfaces between neuronal and glial membranes in the CNS (Torack and Barnett 1963), and physiologic experiments on unmyelinated central axons of amphibia have shown that ionic shifts accompaning impulse-activity, primarily engage small intercellular clefts (Kuffler *et al.* 1966).

After aldehyde fixation, however, central nervous tissue usually exhibits tight apposition of adjacent cell membranes at most places (Karlsson and Schultz 1965, Peters 1966, see also Conradi 1969 a). Some authors claim that this is an effect of the dehydration procedure (Westrum and Lund 1966, Bondareff 1966, Johnston and Roots 1967). From the studies on small central nodes of Ranvier (Andres 1965, Peters 1966, Karlsson 1967) on aldehyde-fixed material and from the present results, it seems, however, clear that aldehyde-fixation, even followed by Vestopal-embedding, is capable of preserving extracellular material. The localization of the material, as described in the present work, could fit well with the above mentioned suggested physiological significance of an extracellular compartment in the CNS.

There is a greater amount of extracellular material around central motoraxon nodes than around nodes belonging to small central fibres. Aggregates of extracellular material, similar to those found around the motoraxon nodes, have only been reported to occur around nodes and boutons belonging to thick myelinated fibres in the fish brain (Robertson *et al.* 1963). Although the significance of this is unclear, it could indicate that the dimension of the axon might in some way be related to the amount of the material.

In this study many "coated" invaginations containing the material were found at the neuron cell membrane at the initial segment, though infrequently at the surrounding glial cell membranes.

Using mostly the same animals as in the present study, Berthold (1968) analyzed the ultrastructure of peripheral motoraxon nodes in the ventral root. Some comparisons can be made: The peripheral nodal axon membrane is like the central one covered at the inside by a layer of contrast-rich material. The dimensions of the node-gap are comparable and just outside the nodal axon membrane, in both the central and peripheral node, there is an extracellular material. At the peripheral part of the motoraxon, there are large accumulations of mitochondria in the Schwann cell cytoplasm in paranodal furrows of the myelin sheath on both sides of the node. At the central nodes, there is no crenation of the myelin sheath paranodally. Compact myelin bodies were, however, often found here. Neither could any accumulations of mitochondria be observed just outside the node. Mitochondria were observed in the axon at the nodes, and in some cases, accumulations of them were found in the axon near the nodes. The latter findings could substantiate Friede's observations (1961) that central white matter histochemically appears to contain little oxidative enzymes; the enzymatic activity was found mainly inside axons, particularly bigger ones, and in some glial cells.

Distribution of boutons on the axon hillock

Due to the nearness to the presumed site of impulse-origin, the boutons on the axon hillock might exert a powerful influence upon the impulse-activity of the motoneurons. Before comparisons with the distribution of boutons on other parts of the motoneurons (see Conradi 1969 a, b) are made, it must be pointed out that the observations in the present work refer to laterally-situated motoneurons in L 6, L 7 and S 1. The quantitative estimations in the preceding papers were performed in a strictly defined small region of L 7; therefore the results may not be comparable. Moreover, the numerical proportion of the different bouton types was investigated on only two reconstructions, which naturally does not permit generalisations. For the most part, however, the general features were the same in both cases.

As already pointed out, the same types of boutons were present on the axon hillock as on the motoneuron cell bodies. The numerical proportion of F boutons, 66% and 59%, respectively, was of the same size-order as, or even higher than the value, corrected for mean length of apposition, obtained for the cell bodies (54%). If the latter is valid, there seems thus to be a continuous decrease in the proportion of F boutons from the axon hillock

towards the distal parts of the dendrites (see Conradi 1969 a). The tendency for the S+T boutons (see Conradi 1969 a) would be the reverse.

If it is assumed that the F boutons are inhibitory (Uchizono 1965, Bodian 1966) and the S+T boutons excitatory, then the inhibitory boutons on the axon hillock seem to outnumber the excitatory ones by about 50%. If the F boutons are slightly bigger than the S+T boutons, as seemed to be the case on the cell bodies, approximately 3/4 of the surface of the axon hillock covered by boutons could be subjected to inhibitory influences. The dorsal root boutons (see Conradi 1969 d) did not seem to be localized at the axon hillock.

ACKNOWLEDGEMENTS

I am much indebted to Professor Sten Skoglund for his encouragement and helpful discussion, and to Miss Christina Trägårdh for skilful technical assistance. The work has been supported by grants from Karolinska Institutet and the Swedish Medical Research Council, projects nr. B69—14X—136—05C and B70—14X—136—06.

SUMMARY

The axon hillock and initial axon segment of the lumbosacral motoneurons in the cat were studied with the electron microscope. The cell membrane of the initial motoraxon segment is covered from the inside by a thin layer of contrast-rich material and it is surrounded by a narrow rim of extracellular material. Adjacent to the initial segment, glial cells containing a dark cytoplasm are often found. Boutons are found on the axon hillock but not on the initial segment. Two wax-models of the axon hillock and initial segment were prepared from ultrathin serial sections. The distribution of boutons of different types on the axon hillock was investigated on these models and it is concluded that the population of boutons is rather similar to that on the motoneuron cell bodies which has previously been described.

Some observations on the motoraxons in the gray substance are included. At the myelinated part of the motoraxon, the myelin sheath exhibits incisures of Schmidt-Lantermann and just outside the myelin sheath there are spheroid, compact bodies of myelin. At the central nodes of Ranvier, the motoraxon is surrounded by a thick layer of extracellular material.

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LEGENDS TO THE FIGURES

Fig. 1. Light microscopic photograph of a motoneuron and the first part of the axon. ×410

Fig. 2. Survey electron micrograph of the axon hillock (AH) and initial segment (IS) of a motoneuron. The beginning of the myelin sheath is also seen. Note the compact myelin bodies (C) outside the myelin. Three glial cells with dark cytoplasm are seen near the initial segment, two are oligodendrocytes (OL); the third, probably a microgliacyte (MG). ×1,900

Fig. 3. Part of an initial segment at higher magnification. The axon is surrounded by flattened astrocytic extensions. The contrast-rich material inside the axon membrane is seen. Note the longitudinal neurotubuli (NT), some of which are grouped together. ×22,400

The inset shows a "coated" invagination of the axon membrane. The contrast-rich material at the inside of the latter is seen to the left (arrow). ×116,500

Fig. 4. Detail of the axon membrane at the initial segment. Just outside the axon membrane a thin rim of extracellular material is seen (×). Outside this, there are astrocytic extensions (AS). Note that the contrast-rich material inside the axon membrane shows maximum density some distance away from the membrane (arrow). The contrast-rich material shows projections, directed inward (DP). ×137,000

Fig. 5. Detail of an initial segment cut obliquely. The dense projections (DP) shown in the preceding figures are prominent. Outside the initial segment, there is a glial cell, probably a microgliacyte (MG). ×13,500

Fig. 6. Detail of an initial segment (IS) just proximal to the myelin sheath (MY). There are large accumulations of extracellular material (×). ×49,400

Fig. 7. Cross section of an initial axon segment (IS). Note the contrast-rich material at the axon membrane. The axon is surrounded by many dark, flattened glial extensions (G). ×17,200

Fig. 8. Survey of the axon hillock (AH) of a motoneuron. A dark glial cell, probably a microgliacyte (MG), is situated at top left. Several boutons are seen to cover the axon hillock; one of these is of the C type (C). ×6,200

Fig. 9. Wax-model of an axon hillock (not painted) and initial segment (stippled with black), reconstructed from serial sections and seen from the front and from behind. Cell body at the bottom of the figure. Note the compact myelin body just outside the beginning of the myelin (arrow). The boutons on the axon hillock are also included. Black=F-type; white stippled=S-type.

Fig. 10. Wax-model of another axon hillock and initial segment (black stippled). Cell body at the bottom of the figure. The boutons are indicated as in the preceding figure. The extension of a flattened glial cell, probably a microgliacyte, is included. Such a cell in similar position was also present at the initial segment in the preceding reconstruction, but was not included. There is apposition between the axon hillock and an adjacent dendrite, part of which is included in the model (arrow).

Fig. 11. The beginning of the myelin sheath distal to the initial segment (IS) is shown. The terminal cytoplasmic pockets of the myelin (TP), not all of which reach the axon membrane, are seen. In the axoplasm inside the myelin, some profiles of granular endoplasmic reticulum (arrow) are observed. ×9,700

Fig. 12. Myelinated part of the motoraxon. Two incisures of Schmidt-Lantermann (SL) are observed. Outside one of these, there is a compact myelin body (arrow). ×4,800

Fig. 13. Detail of an incisure of Schmidt-Lantermann. ×22,600

Fig. 14. A central node of a motoraxon in the gray substance. The axon is seen to be constricted at the node. Some profiles of mitochondria (arrow) are seen in the axon near the node. ×9,600

Fig. 15. Detail, showing the terminal cytoplasmic pockets of the myelin. Some of the pockets show immediate apposition at the axon cell membrane (arrow), but the majority of them do not reach the axon membrane. ×68,500

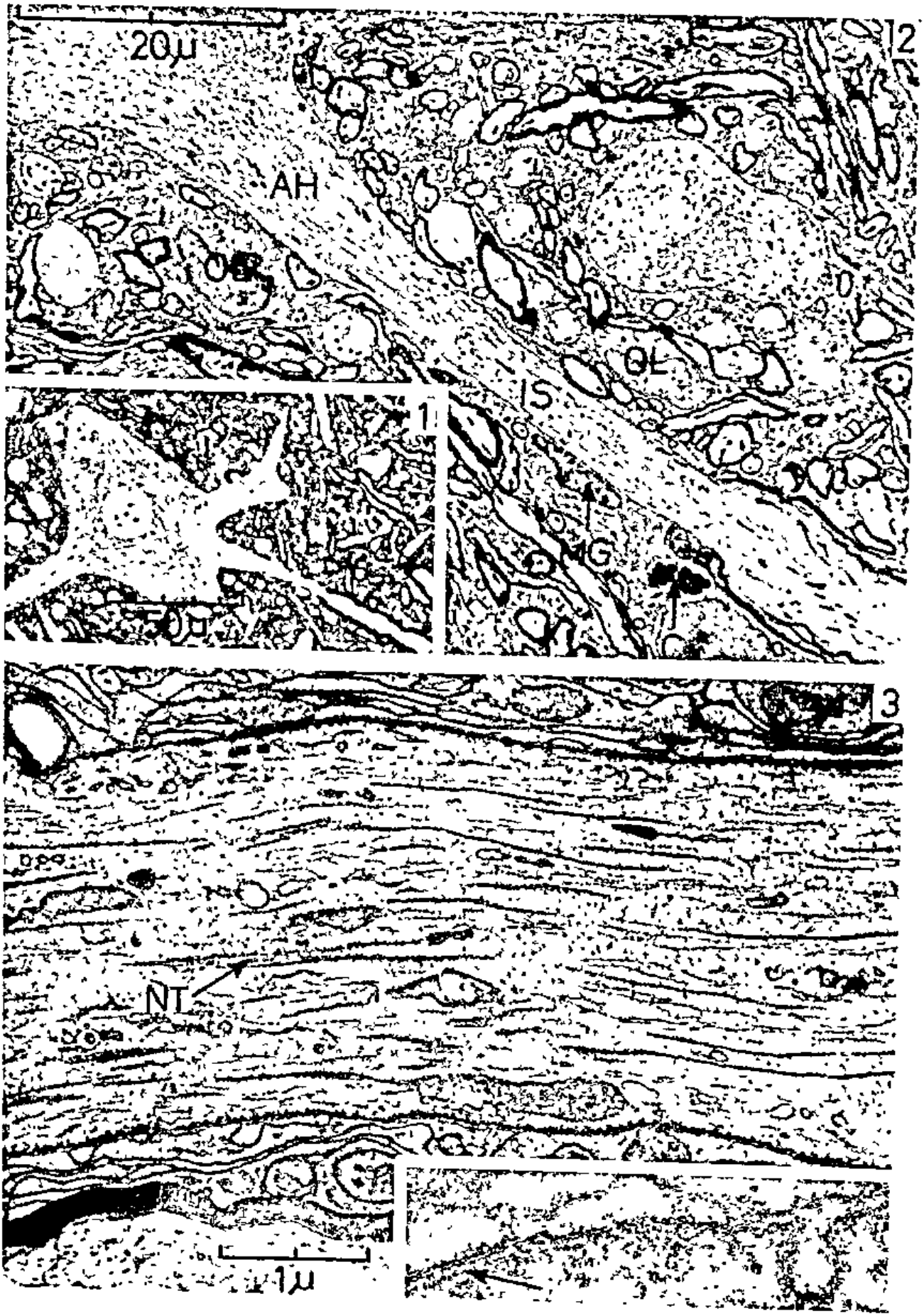
Fig. 16. In this motoraxon, there is an accumulation of mitochondria (arrow) beside the node. The extracellular material (×) in the node-gap is clearly seen. ×7,900

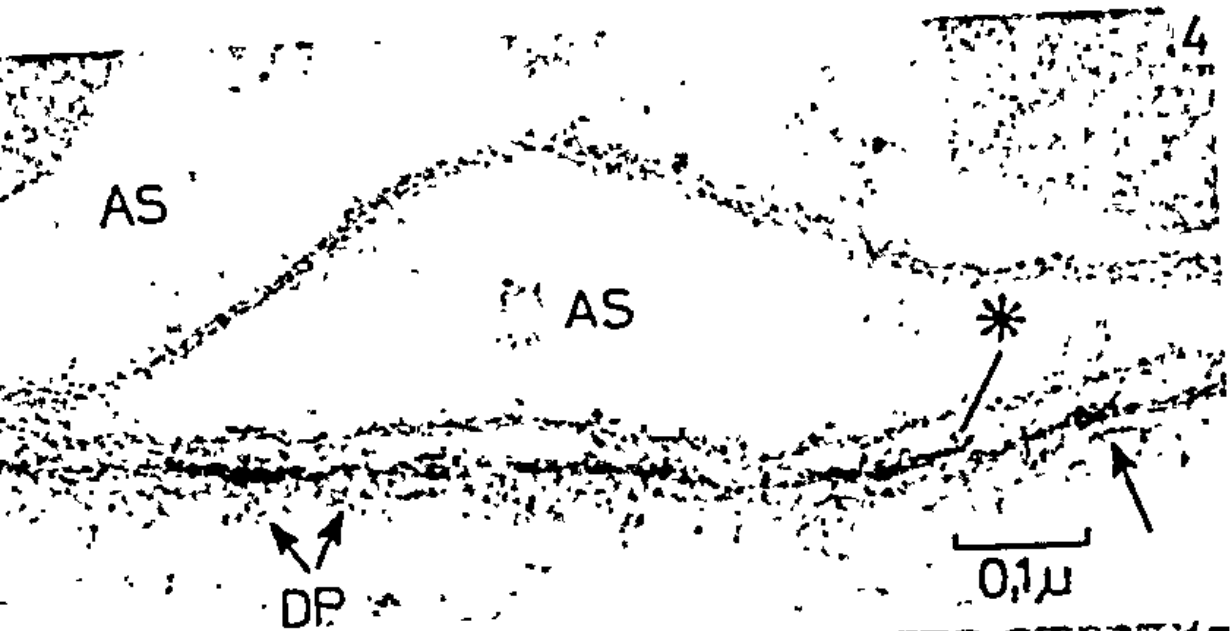
Fig. 17. Detail of a central node of Ranvier on a motoraxon. At the node, the axon (AX) is covered by extracellular material (×) and astrocytic extensions (AS). The inside of the nodal axon membrane is covered by a layer of contrast-rich material (arrow), showing the same characteristics as at the initial segment. ×74,500

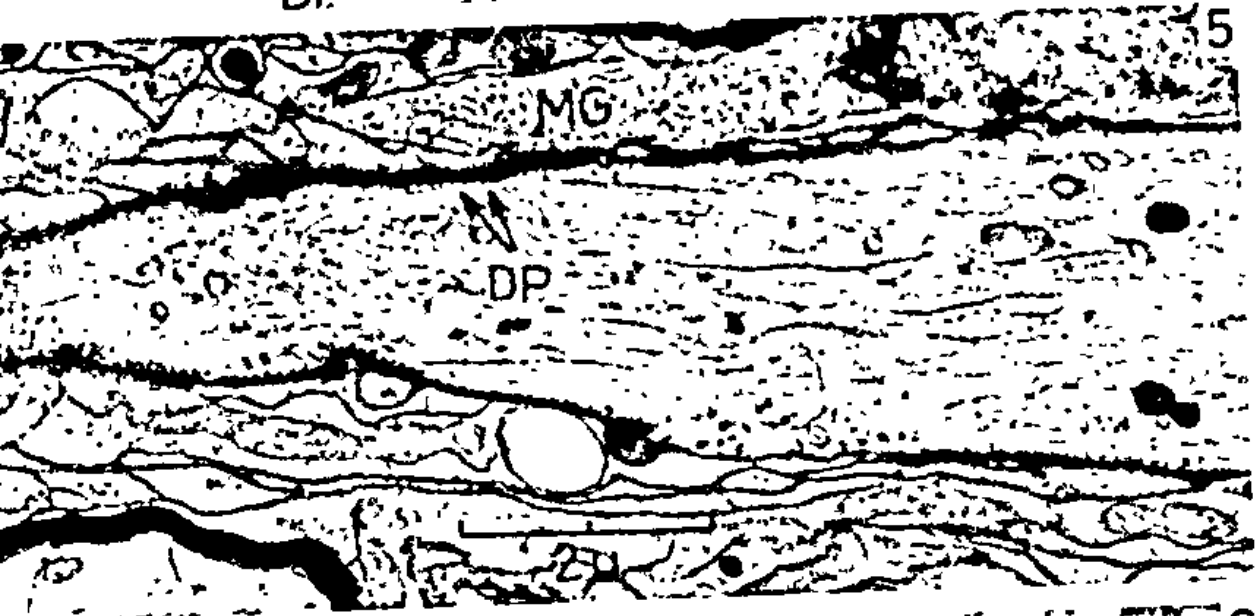
Fig. 18. At this node, the lateral border of the node-gap is formed by terminal cytoplasmic pockets of the myelin (arrow). The astrocytic extensions (AS) in the node-gap are clearly seen. ×26,700

Fig. 19. The figure shows that the extracellular material (×) in the node-gap has a very dense appearance in some fixations. ×48,500

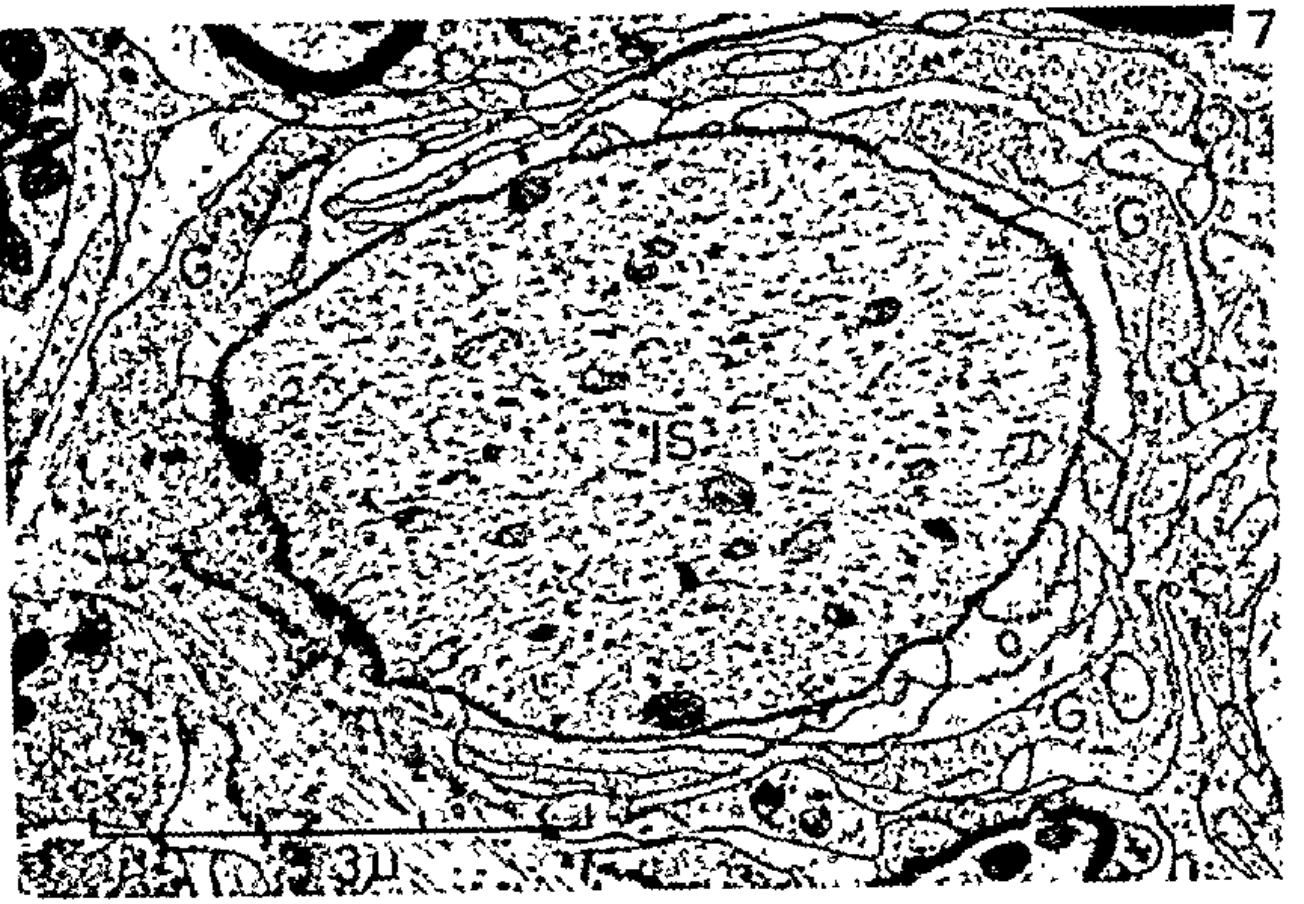
Fig. 20. From this motoraxon node, a myelinated collateral (C), much thinner than the parent axon (AX), is sent out. ×28,800


20µ
AH
12
OL
IS
1
3
NT
1µ

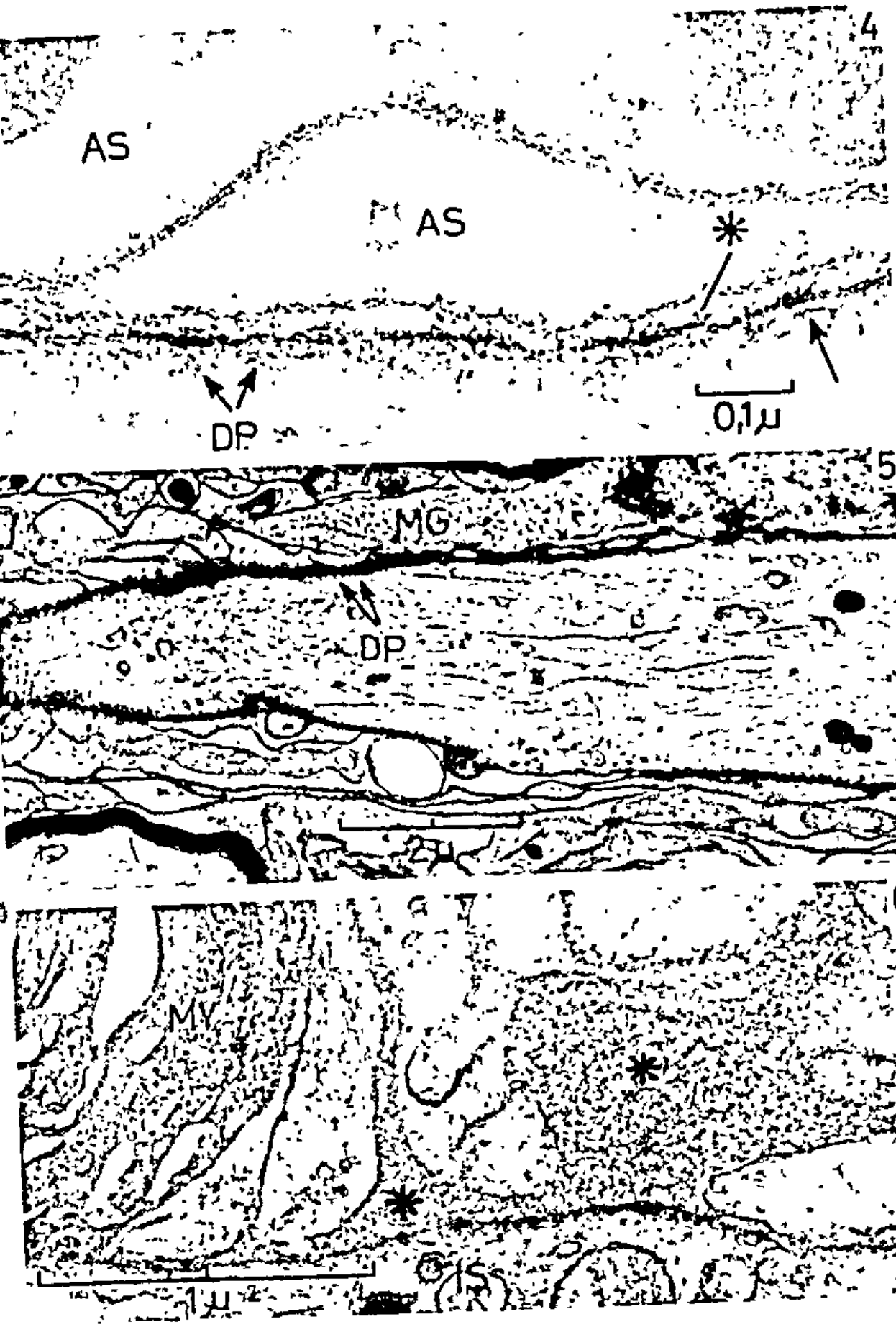

4
AS
AS
DP
0,1μ

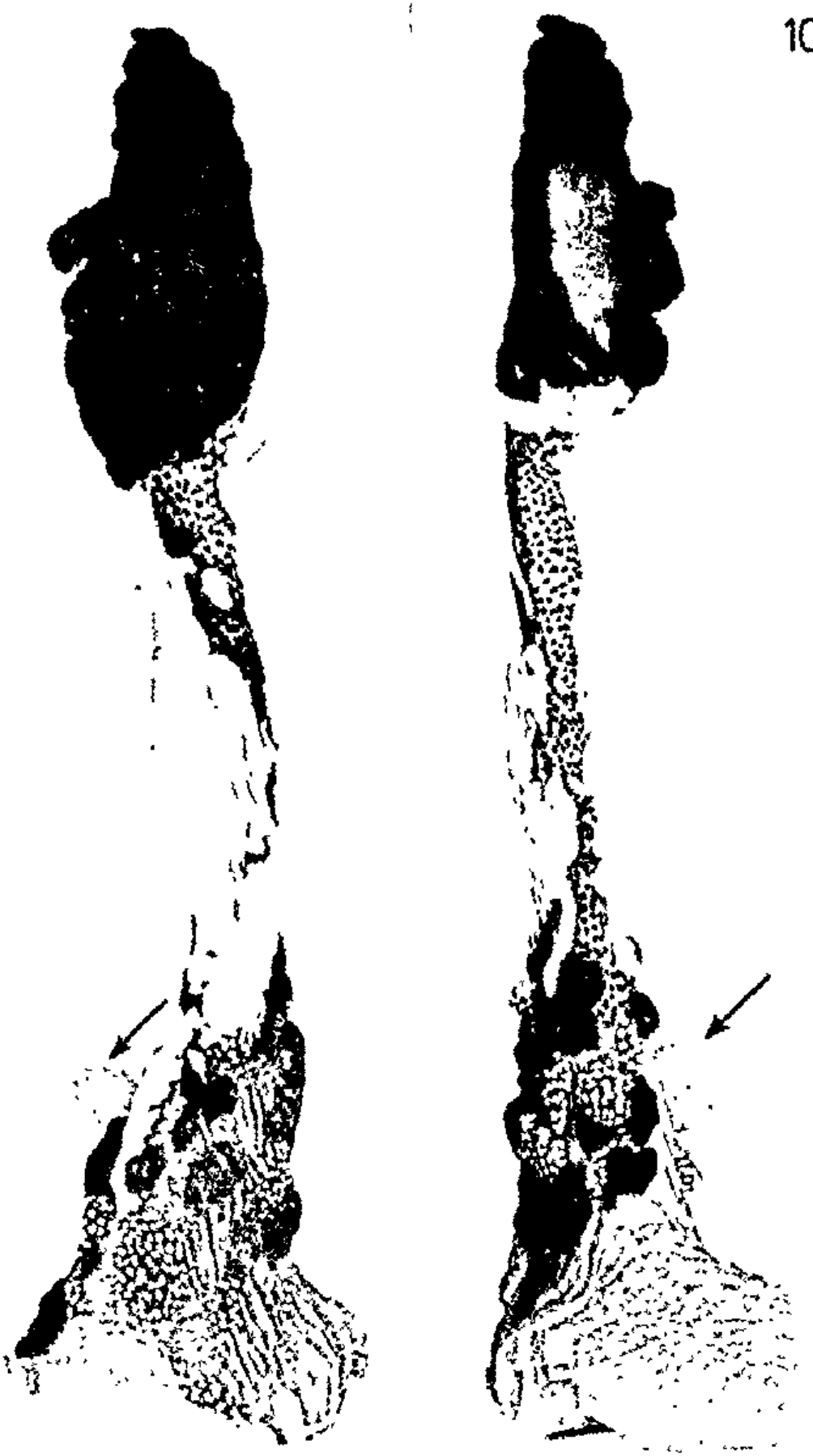

5
MG
DP
2μ


6
MV
1μ

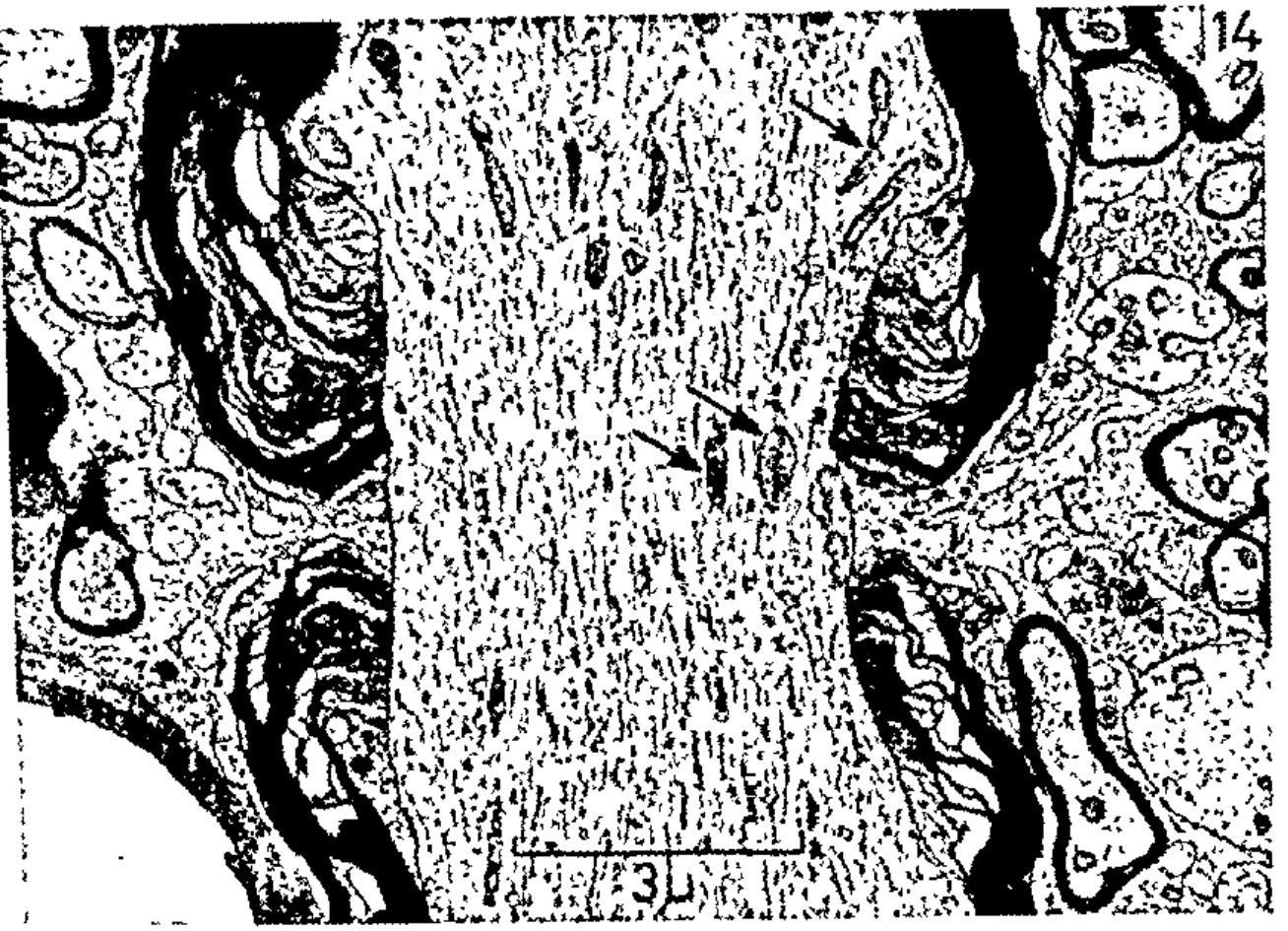

7
G
G
IS
G
3μ


8
MG
AH
5μ


4
AS
AS
DP
0,1µ
5
MG
DP
2µ
6
MY
1µ




11
TP
IS
3μ
12
SL
SL
5μ
13
1μ


14
3μ


15
3μ


16
3μ


17
AS
AS
AX
0,3μ


18
AS
0,5μ


19
0,5μ


20
C
AX
0,5μ

Ultrastructure of dorsal root boutons on lumbosacral motoneurons of the adult cat, as revealed by dorsal root section

by

SEBASTIAN CONRADI

INTRODUCTION

The aim of the present study was to obtain information on the ultrastructure and location of the dorsal root boutons on the motoneurons in the adult cat, which is a prerequisite for studies on the postnatal development of these boutons (see Conradi and Skoglund 1969 b). As pointed out earlier (Conradi and Skoglund 1968), the evaluation of changes in boutons during the first days after dorsal root section requires information on the ultrastructure of the boutons on the motoneurons in the normal state. This has been presented in two earlier communications (Conradi 1969 a, b). For calculations on the loss of boutons several weeks after dorsal root section, knowledge of the frequency and distribution on the motoneurons of these boutons in the normal state (Conradi 1969 a, b) is also needed.

It is of great interest to study in detail the morphology of the dorsal root boutons on the motoneurons.

1) Due to the favourable anatomical conditions, the dorsal root fibres are more readily available for transection than any other of the fibre systems contacting the motoneurons. For the same reason, the conditions for stimulating and recording are also favourable, which explains why the physiologic properties of the monosynaptic pathway are more fully understood than those of other fibre systems projecting on the motoneurons.

2) Presynaptic inhibition was first described in the monosynaptic pathway (Frank and Fuortes 1957). It has been suggested that the phenomenon is

caused by the activity of small boutons, making synaptic contact with the dorsal root boutons on the motoneurons (Eccles 1964, see also Gray 1962). It has not hitherto been possible to prove this, and the present morphological knowledge of the connexions between the dorsal root collaterals and the motoneurons emanates from light microscopic studies (see for example Sprague 1958, Szentágothai 1958, Mikeladze 1966, Illis 1967, Sterling and Kuypers 1967, Carpenter *et al.* 1968, Scheibel and Scheibel 1969). Axo-axonic synapses, however, have been described ultrastructurally in several regions of the brain and spinal cord (see Kidd 1962, Colonnier and Guillery 1964, Walberg 1965, Ralston 1965, 1968 a, Peters and Palay 1966, Szentágothai *et al.* 1966, Karlsson 1967, Pecci Saavedra *et al.* 1968).

The method of mapping fibre connexions by means of degeneration after axon section has been frequently used in neuroanatomic work. At the ultrastructural scale, three main types of degenerative bouton changes following axon section have hitherto been described to occur in the CNS:

a) The boutons are filled with a dense material (see Colonnier and Gray 1962, Walberg 1963, 1964, Mugnaini *et al.* 1967, McMahan 1967, for references, see Aiksne *et al.* 1966).
b) The boutons are filled with neurofilaments (Gray and Hamlyn 1962, Colonnier and Guillery 1964, Dowling and Cowan 1966, Pecci Saavedra *et al.* 1969, for references, see Mugnaini and Walberg 1967).
c) The boutons are engulfed by glial cells (see for example Colonnier 1964, Smith *et al.* 1966).

Boutons showing any of these changes were searched for, when trying to identify the degenerating dorsal root boutons during the first postoperative week. There is light microscopic evidence (Szentágothai 1958) that rather few of the boutons on the motoneurons are of dorsal root origin. Due to this and to the fact the acute ultrastructural changes in boutons were slight, it was decided first to investigate the loss of boutons a comparatively long time after the operation.

Two boutons types, the M type and a variety of the S-type (see Conradi 1969 a, b), both of which have small boutons (P-type) apposed at the convex side, were found to have disappeared already during the first postoperative week. The acute changes in these boutons during the first postoperative days were then studied. The normal anatomy of the two bouton types was studied in detail through serial sections. Although the zone of apposition between the P boutons and these big bouton types does not clearly fulfil conventional criteria for a synaptic complex (see Gray and Guillery 1966), as stated earlier (Conradi and Skoglund 1968), this is the only neuroneuronal contact of the

P boutons which is similar to a synaptic complex and accordingly, it is concluded that dorsal root boutons on homolateral motoneurons are postsynaptic in axo-axonic synaptic complexes.

Some of the results have been published earlier in preliminary form (Conradi and Skoglund 1968, Conradi 1968). In the ensuing description, the normal ultrastructure of the M and big S boutons will be dealt with first, then the changes after dorsal root section will follow.

MATERIAL AND METHODS

24 adult cats of both sexes were subjected to unilateral dorsal root section proximal to the spinal ganglion, under aseptic conditions. The animals were anaesthetized intraperitoneally with Nembutal® (Abbott) 20—40 mg/kg of body weight. A wide laminectomy, comprising the three lowest lumbar vertebrae was performed in most cases. The dura was incised longitudinally for 3—5 cm, and the left dorsal roots L 6, L 7 and S 1 were cut. The degeneration was studied in the segment L 7, but the adjacent roots were also cut in order to obtain maximal degeneration, since it has been shown (Sprague 1958) that every dorsal root sends collaterals to 1—3 segments above and below the site of entrance. In a few cases, only the left 7th lumbar dorsal root was cut just proximal to the spinal ganglion, thus allowing the opening in the dural sac to be restricted. The only disadvantage with the wide incision was that at sacrifice, a herniation of the spinal cord was sometimes found.

An attempt was made to cut all the dorsal root fibres at an equal distance from the spinal cord, and in order to avoid damage to the cord, the roots were cut as distally as possible. Often, the dorsal root arteries could be saved, but the arrangement of these vessels varied greatly. After the operation, the dorsal muscles, fascia and skin were sutured separately, and in most cases, Polymyxin B® (Pfizer) unguent was left in the wound. The operations usually lasted for one-half to one hour. The postoperative care included penicillin, Suspenin® (Kabi) 300,000 IE/day i.m. and cleaning of trophic ulcers in a few cases. The mortality was about one out of ten animals, death occurring during the operation. After a survival time of 1—42 days (Table 1), the animals were subjected to the same fixation procedure as already described (see Conradi 1969 a). Nearly the whole material was perfused at a discontinuous perfusion pressure and 2.7% Rheomacrodex® was added to the perfusates.

For comparison, 12 adult rats were also operated upon and subjected to perfusion fixation in the same way as the cats at survival times of 1—7 days. This material was not analyzed in detail, but some of the main findings were the same in both animals. Fig. 19 is taken from the rat material.

After the fixation, the lumbosacral spinal cord was removed and the 7th lumbar segment was cut into 0.5—1 mm thick transverse sections. The operated and unoperated sides of the cord were postfixed and embedded in the same way as the normal material.

Semithin transverse sections of half the spinal cord were cut, stained with toluidine blue and examined in the light microscope. On these large sections, it was possible to judge the axonal degeneration on the operated side fairly well. The lateral part of the

Table 1. Survival times of the operated animals

Survival Time, days	Number of cats	Survival Time, days	Number of cats
1	1	8—14	4
2	1	15—30	3
3	5	42	2
4	3		
5	3		
6	1		
7	1		

ventral horn was then trimmed out and cut into ultrathin transverse sections, which were collected on one-hole grids. Single sections, as well as consecutive series comprising 20—100 sections, in which only a few single sections were lost, were investigated. Sections from the normal material and the unoperated side served as control.

In those cases, in which the cord was herniated after the operation, the profiles of the motoneurons were sometimes elongated in the anteroposterior direction. In a few of the animals with a short survival time, there seemed to be accidental degeneration, probably an affect of the operation. A few obviously degenerated axons could then be seen in the white substance. This did not, however, interfere with the findings in general.

The number and proportion of the remaining boutons of different types on the motoneurons 42 days after homolateral dorsal root section was estimated on two cats with the same fixation and sampling methods as were used for the normal material (see Conradi 1969 a). Briefly, the boutons on profiles of 20 motoneuron cell bodies (SO) cut through the mid-nucleus plane and adjoining proximal parts of the motoneuron dendrites (DA, DB), as well as on 30 thin, longitudinally-sectioned dendritic profiles in the neuropile (d), were counted on single transverse sections through the lateral motoneuron nucleus in the most cranial millimetre of L 7 in prefixed (Sherrington 1892) animals. The only difference from the procedure used in the normal material was that the dimensions of the bouton profiles were not measured. Due to this, a low final magnification of the montages (× 1,860) could be used, thus markedly reducing the number of photographs that were used.

Two M boutons, including the apposing P boutons from the normal material, were reconstructed with 1 mm wax-plates from consecutive ultrathin sections, according to a technique, presented earlier (Conradi 1969 c). In both cases, none of the serial sections was lost. One of the boutons was taken from the series through the proximal part of a motoneuron dendrite belonging to a lateral motoneuron in the uppermost mm of L 7, the surface relationships of which were analyzed in a preceding communication (Conradi 1969 b). (The M bouton in reconstruction A). The second bouton was taken from segment L 7 of another animal, fixed in the same way. In both cases every section was used. The mean section-thickness was calculated from the number of sections required to pass longitudinally cut dendrites in the tissue. The dendrites were assumed to be cylindrical and the diameter of the dendrite was measured from the photographs. In the first reconstruction, the mean section-thickness was 880 Å, and the final magnification of the reconstruction was × 11,200. The bouton was passed in about 60 sections. A schematic drawing of the reconstruction of this bouton was also

made (Figs. 1 and 2). The second reconstruction (Figs. 3 and 4) shows an M bouton, located at the border between the motoneuron cell body and the proximal part of a dendrite. Here the mean section-thickness was about 560 Å, and the magnification of the wax-reconstruction ×17,800. The bouton was passed within 100 sections.

RESULTS

Evidence showed (see below) that the big bouton types, the M-type and a variety of the S-type, which have P boutons apposed at the convex side, disappear from the motoneuron surface during the first week after homolateral dorsal root section. Due to this, the normal ultrastructure of these boutons was studied in more detail. Serial ultrathin sections were used for this purpose. Special emphasis was placed on determining whether or not the contact between the P boutons and the bigger boutons fulfil the criteria for a synaptic complex. It was thought to be of further interest to investigate the number and distribution of P boutons on the convex side of the bigger boutons.

Normal material

The M boutons

Several M boutons were wholly or partially investigated through serial sections. In two cases, wax-models of such a bouton and the apposing P boutons were prepared (Figs. 1—4). A constant feature seemed to be that several P boutons could be apposed at one M bouton. Also, the P boutons were often situated along the lateral border of the M boutons, thus being apposed both at this and at the postsynaptic extension, which was usually the proximal part of a motoneuron dendrite.

In Figs. 1 and 2, one of the reconstructed M boutons is shown. In the adjoining schematic drawing, eleven P boutons are found to be apposed at this M bouton and nine of them are also apposed to the motoneuron cell membrane. Two big P boutons are apposed only at the convexity of the M bouton. From the schematic drawing, seven dendritic spines are seen to be situated along the lateral border of the M bouton. This and the extension of the synaptic complex of the M bouton are also shown in the reconstruction of the entire adjacent dendritic surface (Conradi 1969 b, Fig. 9).

The second reconstruction is shown in Figs. 3 and 4. This M bouton, which is somewhat bigger than the first one described, was situated at the soma-dendritic junction of a motoneuron. The preterminal axon, which is unmyelinated the last distance before the bouton, shows an ovoid expansion

filled with synaptic vesicles. The expansion is not, however, presynaptic to any other neuronal extension. Seven P boutons are apposed at the lateral margin of the M bouton and at the motoneuron cell membrane, and an additional four P boutons, somewhat bigger than the foregoing, are situated around the expansion. The extension of the synaptic cleft of the M bouton is depicted in Fig. 4 d. A few dendritic spines were situated adjacent to this bouton.

Thus, in both the reconstructions, some of the P boutons were apposed both at the motoneuron cell membrane and at the M boutons, others only at the M boutons. It was not, however, possible to distinguish different types of P boutons. Although the thin, unmyelinated preterminal axons of the P boutons could not be traced for longer distances through the series, it seemed, nevertheless, as if at least some of these axons were running on or very near to the motoneuron cell membrane.

The contact area between P and M boutons. In single sections, the contact area between P and M boutons was often difficult to resolve. When the plane of the section was ideal, the following features could be observed. At most places of the contact region, the cell membranes of the boutons showed immediate apposition, quite like the situation found at the interface between other neuronal cell membranes in the neuropile (see Conradi 1969 a) (Figs. 1 b and 3 c). Not infrequently, desmosome-like junctions were found at the cell membranes of the two boutons (Fig. 6). At low magnification, these were easily mistaken for synaptic complexes. At some places, a narrow zone of granular extracellular material about 100 A thick and only about 0.1 μ wide, was found between the cell membranes of the boutons (Fig. 5). The synaptic vesicles of the P boutons were apposed at the cell membrane here, but no corresponding arrangement was found inside the M boutons. There was a slight rim of contrast-rich material attached at the cell membranes of the boutons on both sides of the extracellular material (Fig. 5). Since these regions did not clearly fulfil the criteria for a synaptic cleft (cf. Conradi and Skoglund 1966) because of the small extracellular space, serial sections were required to judge whether the P boutons establish additional structures similar to synaptic complexes with any other neuronal extensions. From investigation of serial sections on about 25 P boutons it seemed clear that this was not the case. In those instancies, in which the P boutons were apposed also at the motoneuron cell membrane, no structure similar to a synaptic complex was found at the region of apposition. Assuming that every bouton makes at least one synaptic complex—and this seemed to hold true for nearly all the boutons on the proximal part of the motoneuron dendrite, analyzed in a preceding

paper (Conradi 1969 b)—the contact regions between the P and M boutons must then be interpreted as a synaptic complex.

The big S boutons with apposed P boutons

As pointed out earlier (Conradi 1969 a, b) it was sometimes observed on single sections from the normal material, that P boutons in a few cases are apposed at the convex side of big boutons on proximal parts of the motoneuron dendrites (Fig. 7). Since these profiles in some cases might have been derived from M boutons, sectioned in a plane outside the synaptic cleft, these profiles were included together with the M boutons in the quantitative studies on single sections. As mentioned earlier (Conradi 1969 b), the big S and M boutons differed most markedly in size and shape of the synaptic complex, the big S-type mostly establishing synaptic complexes indistinguishable from those of other S boutons. The contact region between P and the big S boutons seemed to be of a character similar to that between P and M boutons. Usually only one P bouton was observed on the analyzed big S boutons.

Distribution and dimensions of M and big S boutons on the motoneurons

As stated in connexion with the quantitative estimation on normal material (Conradi 1969 a, see also Table 2), the M and big S boutons seem to be located mainly on the proximal parts of the motoneuron dendrites.

Most of the boutons were situated in region DA (within that region of the dendrite covered at one side by the first 20 boutons in one section, counting from the cell body). In this region, about 1% of the bouton profiles were of these two types, as seen in the single sections, although the proportion varied between the different dendrites. From observations on single sections, several adjacent M boutons were often found on one and the same dendrite. This could be in keeping with the recent observations on the terminal axonal ramifications of the dorsal root fibres by Scheibel and Scheibel (1969). A few M boutons were also observed on the motoneuron cell bodies or on dendritic profiles in the neuropile. The preterminal axons of the boutons of these types were always noted to be unmyelinated for the last distance before the bouton. No bouton *de passage* was found. In only a few cases were M boutons found to make synaptic contact with more than one extension. No boutons of these types were observed to contain neurofilaments to a marked degree in the normal material. The mean length of the above-mentioned types seemed to be 3.5—5 μ, judging from single sections. The mean height was about 1.5 μ.

Operated material

Axon degeneration

In the light microscope, profiles of obviously degenerating axons were observed on the operated side of the spinal cord in segment L 7, from the second postoperative day on. In most cases, the axoplasm was dark and appeared shrunken. Collapsed myelin sheath or distended profiles of myelin, in which no axoplasm was visible, were also encountered. The degeneration was most apparent at the entrance zone of the dorsal root and in the dorsal funiculi. Degenerated axons were observed in large numbers in the ipsilateral dorsal horn and to a lesser extent in the ventral horn. On the contralateral side, there were few, if any, such profiles. The axon degeneration seemed to be maximal after about one week, but many degenerated axons could still be seen 6 weeks postoperatively.

In the electron microscope, profiles of degenerated axons were observed in the neuropile of the ventral horn from the third postoperative day. The number of those profiles seemed to increase until the 5—7th postoperative day. Then, several such profiles were always present on every ultrathin section, containing two to five motoneurons. At many places, profiles of dark degenerated axons were situated quite near the motoneurons, and in some instancies, the outline made them look very similar to big dark boutons at low magnification. At this stage a few degenerated axon profiles could also be seen in the contralateral ventral horn.

The degenerated axons nearly always had a dark axoplasm, which at high magnification appeared granular. Profiles of distended mitochondria and dense bodies could often be distinguished in the axoplasm (Fig. 8). This type of axon degeneration has been described earlier in several reports on Wallerian degeneration in the CNS (see for example Ralston 1965, McMahan 1967, Lampert 1967). Often, the myelin formed big loops outside the dark axoplasm.

At a few places, distended myelin sheaths lacking axoplasm were observed. The region inside the myelin appeared clear and in it, profiles of highly distended mitochondria and some floccular material could sometimes be observed (Fig. 9). In a few cases, highly distended profiles, often surrounded by myelin were observed on the operated side. At times, the interior of such profiles contained many neurofilaments (Fig. 11): at other times, many small mitochondria and membrane bound dense bodies could be seen to be distributed in a somewhat fibrillar ground substance (Fig. 12). Similar findings have been reported in earlier studies on the distal stump following axon transection in the central and peripheral nervous system (Webster 1962,

Blümcke *et al.* 1966, Schlote 1966, Lampert 1967, Pellegrino de Iraldi and de Robertis 1968, Zeléna *et al.* 1968).

Often the profiles of degenerated axons were surrounded by astrocytic extensions. The process of removal of the axons was not studied in detail. No degenerating unmyelinated axons were observed with certainty. The morphology of degenerating unmyelinated axons has been difficult to settle, even in regions where they ought to be abundant (see for example Hamori *et al.* 1968).

Bouton degeneration

When the neuropile in the ventral horn on the operated side is studied during the first postoperative week, the most apparent feature is the paucity of degenerating boutons, as judged by conventional criteria. No boutons were observed to show the dense type of degenerative change seen in other regions of the CNS (see Alksne *et al.* 1966, McMahan 1967), although such boutons have been described to be abundant in the dorsal horn after dorsal root section (Ralston 1965, 1968 b). It was also very difficult to distinguish any boutons, showing the neurofilamentous type of degeneration (see Mugnaini and Walberg 1967), partly because some boutons in the normal material contained neurofilaments (cf. Gray 1963). At only a few places, often near degenerated axons, membrane-bound profiles were observed inside glial cells. These structures were similar in size and appearance to degenerating boutons phagocyted by glial processes, as described earlier (Colonnier 1964). One such a profile is seen in Fig. 15.

Since no information regarding the type or types of degenerating boutons could be gained from these observations, it was decided to investigate whether the number of any of the bouton types seemed to decrease by one week postoperatively. It was then thought possible to evaluate the acute postoperative changes in the actual boutons prior to disappearance.

The number of the M boutons was found to be markedly reduced from about the third postoperative day. By about one week postoperatively, they had disappeared completely. The same seemed true for the profiles, looking like be big S boutons with apposed P boutons. These findings were based on observations on more than one hundred sections from the about twenty animals with a survival time of three days or more. In the normal material or on the unoperated side, usually one to three M boutons could be observed in a section comprising two to four profiles of motoneuron cell bodies.

Table 2. Quantitative estimation on operated animals

normal material (Conradi 1969 a)

	CAT 1				CAT 2				CATS 1+2				CATS 1+2			
	SO	DA	DB	d	SO	DA	DB	d	SO	DA	DB	d	SO	DA	DB	d
Mean diameter, μ	43.0	8.1	6.2	3.9	39.9	9.8	7.3	3.8	41.5	8.9	6.9	3.8	49.0	11.7	9.0	4.8
Mean membrane length, μ	148.8	121.5	86.5	117.5	158.1	169.5	75.3	118.5	153.5	143.8	78.6	118.0	152.5	143.5	106.5	115.2
Boutons:																
S mean number	10.7	18.6	13.5	11.5	16.8	30.4	13.9	14.7	13.8	25.3	13.8	13.1	11.7	18.8	14.7	14.3
proportion of all bouton profiles, %	31.4	40.7	39.1	43.9	36.7	42.8	44.1	44.6	34.4	41.9	42.6	44.3	34.3	37.7	38.6	46.0
T mean number	0.2	0.5	0.5	2.8	0.8	2.3	1.4	3.5	0.5	1.4	1.1	3.1	0.4	0.9	1.3	1.8
proportion of all bouton profiles, %	0.6	1.2	1.4	10.7	1.7	3.3	4.4	10.5	1.3	2.4	3.5	10.6	1.2	1.9	3.4	5.9
F mean number	21.6	24.5	20.3	11.8	25.3	35.9	15.8	14.8	23.5	30.0	17.1	13.3	19.7	27.6	20.9	14.8
proportion of all bouton profiles, %	63.3	53.0	58.7	44.9	55.2	50.6	50.2	44.8	58.7	51.8	52.8	44.9	57.5	55.3	54.9	47.5
C mean number	1.6	2.0	0.3	0.1	2.9	2.3	0.4		2.3	2.2	0.4	0 07	2.4	1.9	0.9	0.1
proportion of all bouton profiles, %	4.7	4.4	0.7	0.5	6.3	3.3	1.3		5.6	3.7	1.1	0.2	6.9	3.9	2.4	0.4
M and big S mean number													0 05	0.6	0.3	0.07
proportion of all bouton profiles, %													0.1	1.2	0.7	0.2
Mean total number of boutons	34.1	45.7	34.5	26.3	45.8	71.1	31.5	33.0	40.0	57.8	32.3	29.6	34.2	49.8	38.1	31.1
Mean membrane length, μ / Mean total number of boutons	4.4	2.7	2.5	4.5	3.5	2.5	2.4	4 0	3.8	2.5	2.4	4.0	4.5	2.9	2.8	3.7

Quantitative estimation

In order to ascertain the findings and to try to evaluate whether boutons of other types also disappear, a quantitative study of the boutons at a long time after the operation was performed in the same way as in the normal material (Conradi 1969 a). A survival time of six weeks was chosen, since according to quantitative studies on axon degeneration in the pyramidal tract, the degeneration of the biggest axons was not complete until about this time (v. Crevel and Verhaart 1963). The result is presented in Table 2 and the values from the normal material (Conradi 1969 a) are included. As was the case in the normal material, the M boutons and the big S boutons with P boutons apposed at the convex side are included under the same heading.

It can be seen that the mean minor diameter of the motoneuron cell bodies and dendrites is somewhat less in the operated than in the normal material. In regard to the boutons, both the number and relative porportion of the different types on different regions of the motoneurons are quite similar in the operated and normal animals. The boutons under the M heading were not present at all in the operated animals, but the number of T boutons was rather similar in the two materials. It was concluded that if boutons of any types other than the M- and big S-types disappear, the number of them is too small to be revealed by this type of investigation.

Bouton changes during the first postoperative days

As pointed out earlier, profiles similar to phagocyted boutons which were previously described (see for example Colonnier 1964, Smith *et al.* 1966), and boutons containing neurofilaments (Gray and Hamlyn 1962, Colonnier and Guillery 1964, Dowling and Cowan 1966, Mugnaini and Walberg 1967, Pecci Saavedra *et al.* 1969) were observed during the first postoperative week. These profiles were investigated in further detail, with special attention paid to, whether any profiles could be shown to arise from M boutons.

Phagocytosis. Circular, membrane-bound inclusion bodies, 1—2 μ in diameter, were observed inside glial processes, both just outside proximal parts of the motoneuron dendrites and in the neuropile between the 3rd and 7th postoperative day. The glial extensions were principally of two different types.

The inclusion bodies were most often found inside extensions of the astrocytic type (see Mugnaini and Walberg 1964, Maxwell and Kruger 1966). The

bodies were rather constant in appearance, with a dark or somewhat vacuolized ground subsubstance. This can be seen in Fig. 16 h.

At high magnification, profiles of distended mitochondria could sometimes be observed inside the inclusion bodies. Neither a synaptic complex nor part of a postsynaptic extension could be observed inside the glial cytoplasm, which structures were observed by Colonnier (1964).

At many places, inclusion bodies similar to phagocyted boutons were observed inside rather small glial cells containing a chromatin-rich nucleus. The cytoplasm of these cells was amorphous and somewhat more contrast-rich than the astrocytic cytoplasm, and can be seen in Fig. 12. This type of glial extension could be observed both just outside the proximal parts of the motoneuron dendrites and in the neuropile. Some cells of this type were also seen in the normal material and similar glial cells have been observed in other parts of the CNS, especially during degeneration (third neuroglial type: Vaughn and Peters 1969, microglia: Blinzinger and Kreutzberg 1968, Holländer *et al.* 1969). The inclusion bodies inside these cells were either similar to those found inside astrocytic extensions, or, most frequently, were very vacuolized. Often the glial cell membrane was intricately invaginated around these inclusions. (Fig. 12).

Postsynaptic material could sometimes be observed at the inside of the motoneuron cell membrane just adjacent to the glial extensions containing these inclusion bodies. Postsynaptic dense bodies were found immediately inside the postsynaptic material and quite long rows of the postsynaptic dense bodies could be seen (Figs. 12 and 13). This was observed frequently at proximal parts of the motoneuron dendrites. Intact postsynaptic dense bodies have been reported to occur below phagocyted degenerating boutons in sympathetic ganglia after section of the preganglionic axons (Taxi 1962, Sotelo 1968). In the present material, long rows of postsynaptic dense bodies, attached at a membrane fragment were also sometimes encountered in the cytoplasm of proximal parts of the dendrite, just inside the cell membrane (Figs. 12 and 13). The distance between the remaining boutons at both sides of the region of the motoneuron cell membrane occupied by the glial cell, was usually 5—7 μ. The boutons just adjacent to the glia often exhibited postsynaptic dense bodies.

Due to the presence of the long rows of remaining postsynaptic dense bodies and to the localization of these complexes on the proximal parts of the motoneuron dendrites, it was thought that these phenomena represented a disintegrating M bouton. In one instance, seven days postoperatively, a big bouton showing a synaptic complex with a long row of postsynaptic dense

bodies, thus probably of the M-type, was observed on the proximal part of a motoneuron dendrite. The bouton covered, however, only half the synaptic cleft. On the rest of the cleft, there was apposed a big extension, which contained tubular and vesicular profiles—most likely of glial origin (Fig. 14). This configuration might represent an intermediate stage between the normal state and the phenomena described above.

One of the dense membrane-bound inclusion bodies just outside the proximal part of motoneuron dendrite was analyzed through consecutive sections (Figs. 15 and 16). The body was found to be completely surrounded by glial extensions. Adjacent to the inclusion body, there were two small, apparently normal boutons, containing irregularly-shaped synaptic vesicles and embedded in glial extensions. These boutons were very similar to normal P boutons and did not establish synaptic contact with any neuronal extension in the series. The area occupied by the remaining postsynaptic dense material was large and completely covered by postsynaptic dense bodies; it was slightly invaginated at one place and a membrane-fragment with attached postsynaptic dense bodies was found inside the cytoplasm of the dendrite at the site of invagination.

The further studies included the cytoplasm of the motoneuron dendrites, since long rows of postsynaptic dense bodies attached to membrane-fragments had been observed inside the motoneuron cell membrane. At proximal parts of the motoneuron dendrites, a characteristic complex was found in several instances in different animals, from the 3rd to 5th postoperative day. In these cases, circular, membrane-bound bodies, about 0.5 μ in diameter, were situated in the motoneuron cytoplasm immediately inside the cell membrane (Figs. 17 and 18). Usually 1—3 profiles of similar appearance were grouped together. The interior of these bodies was mostly dark and somewhat granular or vacuolized. Profiles similar to distended mitochondria could also be observed. Immediately outside the limiting membrane of these bodies, there was a thin rim, about 150 A in width, filled with a granular material. Bordering this was another membrane parallel to the first, at the outside of which were attached a layer of contrast-rich material, about 300 A thick and long rows of circular bodies. The dense bodies were exactly alike the postsynaptic bodies of Taxi even to forming rows in the same way.

The inclusion bodies appeared as if part of the boutons, including the synaptic complex, had been invaginated into the motoneuron cytoplasm. Walberg (1963) has suggested that normal dendrites may play a role in the removal of degenerating boutons. The localization of the above-mentioned configurations at the proximal parts of the motoneuron dendrites and the

presence of the long rows of postsynaptic dense bodies were interpreted to indicate that the structures emanated from M boutons. In order to study the relationships of these profiles, analysis of serial sections was performed in one case (Fig. 17). It was found that at least one of the membrane-bound bodies—the only one completely covered by the series—seemed to be completely surrounded by motoneurons cytoplasm; and nearly the entire surface was covered by circular dense bodies apposed exactly as the postsynaptic bodies of Taxi (1961).

An attempt was also made to find an example, in which a bouton of the M-type was only partly invaginated into the motoneuron. Despite extensive searching, this could not be found in the material on the cat, but was present at one instance in the material on the rat (Fig. 19).

Neurofilaments. From the third postoperative day, some big boutons, containing spherical synaptic vesicles and to which P boutons were apposed at the convex side, were observed to be filled with neurofilaments. These boutons were observed both on proximal parts of the motoneuron dendrites and in the neuropile (Fig. 20). Postsynaptic dense bodies were usually not observed below these boutons, which is a reason why some of these boutons may have originally been big S boutons with axo-axonic synapses. Smaller boutons containing neurofilaments were also observed both on motoneuron cell bodies and dendrites and in the neuropile. The relative number of these boutons compared to those in the normal material was, however, difficult to evaluate. Generally, it seemed as if the proportion of boutons containing neurofilaments varied among the different animals. After the first postoperative week big, seemingly distended profiles, packed with neurofilaments, were observed in the neuropile (see Fig. 9).

Ultrastructural bouton changes inside intact M boutons during the first postoperative days

In order to study whether changes in the boutons occur prior to disintegration, the morphology of M boutons remaining during the first postoperative week was studied. During the third to seventh postoperative days, some M boutons were observed only a short distance away from obviously disintegrating boutons. These M boutons could exhibit rather numerous granular vesicles (Fig. 21), an increase in the number of which has been reported to occur inside degenerating boutons in sympathetic ganglia (Taxi 1962, Hunt and Nelson 1965, Hamori *et al.* 1968) and in the cerebellum (Smith *et al.* 1966). In some cases, the remaining M boutons were also observed to contain

distended mitochondria or membrane-bound dense bodies. The bouton depicted in Fig. 21 was also slightly invaginated into the postsynaptic dendrite. As a whole, however, the early postoperative changes inside M boutons seemed too slight and too varying to permit any general conclusions.

Other bouton changes during the first postoperative week

During the first postoperative week, some boutons containing membrane-bound, dense lysosome-like bodies were observed in the ventral horn (Fig. 22). Such bodies have earlier been observed in boutons and myelinated axons after axon transection (Webster 1962, Schlote 1966, Blümke *et al.* 1966, Lampert 1967, Hamori *et al.* 1968, Pellegrino de Iraldi and de Robertis 1968). In the present material the dense bodies were observed inside both small and bigger boutons, but whether any particular bouton type was affected could not be ascertained. Membrane-bound dense bodies were even present inside boutons in the normal material, particularly in the FD-type (Conradi 1969 a).

During the first postoperative week, some boutons were observed, which contained spherical synaptic vesicles, and in which the ground substance showed a slightly increased density. Below these or other boutons, containing spherical vesicles, postsynaptic dense bodies seemed to be clumped (Fig. 22). In a few cases, profiles, probably of neuronal origin, were observed to contain highly distended mitochondria and granular vesicles dispersed in a ground substance of a somewhat increased density (Fig. 23). These extensions seemed to show one of the degenerative bouton changes, described in the cochlea after transection of the olivocochlear bundle (Smith and Rasmussen 1965). Thus, there was some evidence that changes other than phagocytosis and an increase in the number of neurofilaments might occur in the boutons during the first postoperative week. However, it could not be definitely shown whether boutons other than those of the M- or the big S-types with axo-axonic contacts were regularly affected.

Structures in the operated material, considered to be indepentent of degeneration of the dorsal root terminals

Some dark, membrane-bound profiles, of about the same size as boutons and surrounded by astrocytic extensions, were observed outside the motoneuron cell membrane. Boutons were often apposed at these structures (Fig. 24). At high magnification no vesicles could be observed in these dense profiles. These profiles are obviously dendrites, which either shrank during

the preparative procedure or are in the process of degeneration (see Grant and Westman 1969). They can also be found in the normal material.

Sometimes, lamellae of granular endoplasmic reticulum, arranged as below the C boutons (see Conradi 1969 a), were observed just inside the motoneuron cell membrane, at a region covered on the outside by astrocytic extensions (Fig. 25). It has been argued that such figures represent degeneration of C boutons following dorsal root section (Uchizono 1968), although this arrangement was also observed in the normal material.

DISCUSSION

Ultrastructure and distribution of dorsal root boutons in the normal material

The findings in the operated material were interpreted as evidence for the M and big S bouton types being of dorsal root origin. The normal ultrastructure of the M boutons, which is the easier to recognize and possibly the one occurring more frequently of these two, has been described in two previous communications (Conradi and Skoglund 1968, Conradi 1968). In the present report, the ultrastructural characteristics of M boutons as described above, were defined from investigations of serial ultrathin sections. For practical reasons, as for example, in statistical evaluation or studies after experimental procedures, it is also useful to define the different bouton types so that they can be recognized in single sections (see Conradi 1969 a). In these the M-type is usually recognized by the big size of the boutons, the shape of the synaptic vesicles and the large number of postsynaptic dense bodies. There are almost no S boutons of the same size as M boutons on the motoneuron cell membrane. Some boutons, containing spherical synaptic vesicles and often localized at small dendritic profiles in the neuropile, can sometimes exhibit one or two large profiles of synaptic complexes, in which 6—9 postsynaptic dense bodies can be seen in one section (Conradi 1969 a). These boutons are smaller than those of the M-type; moreover, they are never observed to be postsynaptic to other boutons.

On the dendritic portion from the normal material, analyzed through serial sections (Conradi 1969 b), the M and big S boutons with axo-axonic contacts were surrounded by dendritic spines. It has recently been suggested (Szentágothai 1968) that the presence of spines adjacent to boutons is linked to the functional activity of these boutons; that is, the spines might be part of an arrangement, with which the size of the contact area between the bouton and postsynaptic membrane can be increased.

The axo-axonic synaptic complex. Axo-axonic synapses have been demonstrated ultrastructurally in several regions of the brain and spinal cord (see for example Kidd 1962, Gray 1962, Colonnier and Guillery 1964, Walberg 1965, Ralston 1965, 1968, Szentágothai *et al.* 1966, Peters and Palay 1966, Karlsson 1967, Pecci Saavedra *et al.* 1968). Axo-axonic synapses have been claimed to occur on cat spinal motoneurons (Khattab 1968), although the picture presented in that report shows no similarity to the structures described in the present paper. At most places, the above observations seem to fulfil conventional criteria for a synaptic complex (Gray and Guillery 1966). In the axo-axonic contact described in the present paper to be a synaptic complex, the thickness and extension of the synaptic cleft were less than those usually occurring at synaptic complexes. An apposition of the synaptic vesicles towards the presynaptic membrane, as found here, might be a more useful criterion for a synaptic complex than the existence of a synaptic cleft of certain dimensions.

Distribution of dorsal root boutons on the motoneurons. The majority of the M and big S boutons were situated on the proximal parts of the motoneuron dendrites, often very close to the cell body. In this region they seemed to form about 0.5% of the population (the values corrected for size of the boutons, see Conradi 1969 a). This comparatively low proportion seems to confirm the light microscopic findings of Szentágothai (1958), that there are relatively few but big dorsal root boutons on every motoneuron, the boutons being predominantly situated at proximal parts of the dendrites. The present findings do not, however, support some others from light microscopic studies (Illis 1967) in which the dorsal root boutons were found all over the motoneuron dendrites, where they were claimed to form about half the total population of boutons.

Several M boutons seemed often to be grouped on the same, horizontal dendrite. The investigation was not concerned with whether the M boutons might be predominantly localized at dendrites, projecting in a certain direction from the motoneurons. From light microscopic studies, it has been argued (Sterling and Kuypers 1967) that the dorsal root axons run in a vertical direction for the last distance before the motoneurons, consequently focusing the interest on the occurrence of dorsal root boutons on the vertical motoneuron dendrites.

Some M boutons were found in the neuropile surrounding the motoneuron cell bodies. The distance from the cell body at such instancies could, naturally not, be estimated. Since a few M boutons were observed to establish more than one synaptic contact, those apposed at small dendritic profiles in single

sections could thus make contact with the proximal part of a motoneuron dendrite, which was out of the plane of section.

As mentioned, it was difficult to judge whether there are dorsal root boutons of types other than those described here and localized for instance, at distal portions of the dendrites. If at all present, they ought, according to the quantitative estimation, to be relatively few. However, it has to be pointed out that the number of the M and the big S boutons is also so small, that if these types had not been recognized as separate entities, the disappearance of them a long time after the operation would not have been recognized in a quantitative estimation.

Physiologic considerations on the normal anatomy and distribution of the dorsal root boutons

Physiologically, the dorsal root synapses on ipsilateral motoneurons are excitatory (Lloyd 1943). The fact that these boutons contains spherical synaptic vesicles supports the hypothesis that there is a correlation between the shape of the synaptic vesicles and the effects on the postsynaptic membrane (Uchizono 1965, Bodian 1966). The finding that all M boutons seem to be of dorsal origin also indicates, that the morphologic typing of boutons can be of physiologic significance, which encourages further investigation along these lines. Based on investigations with the intracellular recording technique (Rall *et al.* 1967) it has been suggested, that the dorsal root synapses are predominantly situated at the motoneuron dendrites, but that the distance to the cell body varies. Results in the present study might confirm this suggestion although it was as mentioned difficult to judge the distance to the cell body of the relatively few M boutons located at small dendrites in the neuropile. Another suggestion has been that "tonic" and "phasic" motoneurons, respectively, receive dorsal root boutons varying in number and possibly also in localization (Burke 1968). If this is true, then such an arrangement could be important for the sampling procedure underlying quantitative estimations like those performed in this study.

Presynaptic inhibition. The physiological theory on presynaptic inhibition postulated the existence of excitatory synapses on the dorsal root terminals (Frank and Fuortes 1967, see Eccles 1964). The presynaptic boutons were thought to depolarize the dorsal root synapses. Most of the physiological evidence for this arrangement was obtained from experiments on extensor motoneurons. In the present study, however, the most lateral motoneurons in L 7 were investigated, and these neurons probably innervate physiologic

flexors (Romanes 1951). The correlations between the present findings and the physiological results, are thus somewhat restricted.

The synaptic vesicles of the P boutons are small and irregular in shape. Such a shape of excitatory synaptic vesicles contrasts to the general theory regarding the significance of the shape of synaptic vesicles (Uchizono 1965, Bodian 1966). However, the theory was based on observations of boutons on the cell bodies and dendrites of motoneurons and Purkinje cells, and might thus not be valid for the P boutons in this material.

In both of the reconstructions several P boutons, some of which belonged to the same preterminal axon, were apposed at the M bouton. Although the significance of this is unclear at present, it cannot be excluded that there are separate systems, with possibly different sites of origin, of presynaptic boutons on the same dorsal root terminal. No P boutons seemed to be located on the preterminal axons of the M boutons.

Some workers (Green and Kellerth 1966, Terzuolo and Llinas 1966) have suggested that much of the inhibition of the motoneurons, which has hitherto been denoted as presynaptic with regard to the dorsal root terminals, in reality is accomplished by boutons localized on the motoneuron dendrites, possibly those remote from the cell body. The present findings favour the hypothesis of Eccles (1964), but the above-mentioned suggestions might still be valid.

Operated material

The observation that the boutons, which in this study are claimed to be of dorsal root origin, disappear from the third postoperative day on, agrees well with findings from physiologic experiments. It has been reported that following stimulation of the central stump of the dorsal roots after dorsal root section, transmission through the monosynaptic pathway has nearly disappeared from the third postoperative day (Savoskina 1966). There are hitherto no ultrastructural quantitative reports on the loss of boutons after long-time degeneration. Therefore, neither the optimal survival time for such investigations nor the risk for errors due to functional inactivity or volume changes in the tissue (cf. Westman 1969), was known. The mean minor diameter of the motoneuron profiles seemed to be somewhat smaller in the operated material. At least as regards the cell bodies, this could have been caused by an elongation following herniation of the cord. Despite this, the number of boutons per motoneuron or dendrite profile seemed to be roughly similar in the normal and long-time degenerated materials.

It has, however, again to be pointed out, that the material in the quantitative study was very small, due to the rather complicated handling procedure, and therefore the conclusions must be drawn with considerable caution.

Ultrastructural bouton changes. Boutons, showing the dense appearance, described as a criterion for degeneration (Colonnier and Gray 1962, Walberg 1963, 1964, Aiksne *et al.* 1966, McMahan 1967, Mugnaini *et al.* 1967), were not observed in the present material, although such boutons are present in the dorsal horn during the first week after dorsal root section (Ralston 1965, 1968 b). The difference might be due to the use of different fixation methods or to local factors, for example the nearness of phagocyting cells. Furthermore, the boutons investigated here seem much bigger than those found by Ralston, which may be of importance. Profiles, similar to bouton fragments, as shown in earlier studies on degeneration in other regions of the CNS (Colonnier 1964, Smith *et al.* 1966), were, however, observed inside glial cells and the motoneurons during the first postoperative week in this material. Neurofilaments and other bouton changes, earlier reported to be signs of degeneration, were also observed inside boutons during the first postoperative week. Thus, many different types of early bouton changes after axon section could exist in this system. In the present material, some M boutons, lying only a short distance from disintegrating boutons exhibited an almost normal morphology during the first postoperative week. In view of these data and the fact that all possible types of early degenerative bouton changes in the CNS have probably not yet been recognized, it seems clear that almost no rigid statement on the normal structure, number or localization of boutons belonging to a certain fibre system, can be made from observations only a short time after axon section.

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SUMMARY

In order to study the ultrastructure of the dorsal root boutons on the motoneurons, the ventral horn was studied in the material of normal adult cats, presented earlier (Conradi 1969 a) and on 24 adult cats, which had been subjected to unilateral dorsal root section 1—42 days before fixation. The loss of boutons on the motoneurons after the operation was estimated against the background of the ultrastructure and distribution of boutons of different types on the motoneurons in the normal state, as described in two preceding communications (Conradi 1969 a, b). Big boutons, M-type, containing spherical synaptic vesicles and predominantly located on the proximal parts of the motoneuron dendrites were found to disappear during the first week after homolateral dorsal root section. Some evidence is also presented that a few big boutons of the S-type disappear. On the convex side of the boutons of these two types, there are regularly apposed small boutons (P-type) containing synaptic vesicles of irregular shape. From analysis of serial sections the P boutons were found to establish a synaptic complex with these big boutons.

Changes in boutons during the first week after homolateral dorsal root section were also studied. Evidence is presented to verify that M boutons are engulfed by glial cells or by the motoneurons from the third postoperative day on.

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FIGURES

Figs. 1 and 2. Reconstruction from serial sections of an M bouton on the proximal part of a motoneuron dendrite (the bouton is the M bouton in region A of the dendrite analyzed a preceding paper, Conradi 1969 b). In Fig. 2 the model is seen from the convex side of the bouton and below the photograph a schematic drawing is included. On the latter it is seen that the M bouton is surrounded by nine P boutons (black), some of which are covered by the M bouton when seen from above (stippled). Two P boutons are also localized on the convexity of the M boutons (striped). There are seven dendritic spines (concentric circles) along the lateral border of the M bouton, four of these (notched) are covered by the latter when seen from above. The extension of the synaptic complex of the M bouton is illustrated in Conradi 1969 b, Fig. 9.

In Fig. 1 a—e, five of the sections through the bouton are shown. The location of the sections is illustrated in the schematic drawing. The sections are directed along the longitudinal axis of the bouton as seen from the wax-plates composing the model.

In *Fig. 1 a* one of the sections along the upper edge of the schematic drawing is shown. Three P boutons are attached at the lower end of the M bouton. In b, one of the dendritic spines is seen to the left. One P bouton is situated below the spine, and one just above the spine. In c, there are still two P boutons cut through at the left side of the photograph. In d, the big black boutons at the lower side in the drawing is visible on the M bouton, the axon of which also is cut through. In e, all four P boutons at the lower end of the M bouton in the schematic drawing are cut through. (The two black boutons at the lower right are not quite correctly drawn, in comparison to the model).

Figs. 3 and 4 show the reconstruction of another M bouton at the soma-dendritic junction of a motoneuron. As seen from Fig. 4 b, this M bouton showed an expansion filled with synaptic vesicles, just proximal to the M bouton. The expansion is covered by four P boutons (black and stippled in Figs. 4 a and c), whereas the M bouton proper is surrounded by seven P boutons (black in Figs. 4 b and c). The extension of the synaptic cleft of the M bouton is shown in Fig. 4 d (black clefts in the M bouton, the black P boutons surround the lateral border of the M bouton).

The sections in Fig. 3 pass in order from the rear towards the reader in Fig. 4 a and the plane of section is seen from the contours of the wax-plates.

In Fig. 3 a, the M bouton is cut before the expansion was visible. Two P boutons are present at both sides of the M bouton.

In Fig. 3 b, the expansion (E) is seen and is covered by the four P boutons. At the right of the M bouton, another P bouton is seen.

Fig. 3 c shows the M bouton, cut through the connexion (arrow) with the expansion (E). Two of the P boutons on the latter are still visible and an additional P bouton is seen at the left side of the M bouton.

In Fig. 3 d, the expansion is passed. One P bouton is still visible at the convex side of the M bouton. At the right side of the M bouton, a dendritic spine is seen. ×17,400

Fig. 5. Part of the region of apposition between a P and an M bouton. It is seen that the synaptic vesicles of the P bouton are smaller than those of the M bouton and are of a somewhat irregular shape. A small extracellular space is present between the boutons and the synaptic vesicles of the P bouton are apposed at the membrane at this place. ×126,500

Fig. 6. Part of the region of apposition between a P and an M bouton. A desmosome-like junction (arrow) is present between the boutons. ×119,500

Fig. 7. Profile of a big bouton (S) containing spherical vesicles and to which two P boutons are apposed at the convex side. The big bouton was followed for about one micron through serial sections, but no postsynaptic dense bodies were observed. The bouton might have been of the big S variety described in the text. ×26,100

Fig. 8. Axon profile, containing a dark ground substance. The myelin sheath is somewhat wrinkled. Seven days postoperatively. ×16,100

Fig. 9. Profile of a distended myelin sheath, surrounding a clear area, containing some flocculer material and distended mitochondria. Four days postoperatively. ×10,500

Fig. 10. Profile of an axon, containing packed neurofilaments. Four days postoperatively. ×7,900

Fig. 11. Profile containing numerous mitochondria, dense bodies and neurofilaments. Seven days postoperatively. ×11,000

Fig. 12. On the proximal part of a motoneuron dendrite (M) there is a dark glial cell, probably a microgliacyte (MG) which contains inclusions, some of which are vacuolized. Postsynaptic dense bodies are situated at the inside of the motoneuron cell membrane and there are two membrane fragments (double arrows) with postsynaptic dense bodies inside the motoneuron cytoplasm. These are shown in higher magnification in Fig. 13. Three days postoperatively. ×24,300

Fig. 13. Detail of the square in Fig. 12. The postsynaptic dense bodies are indicated by arrows. ×71,500

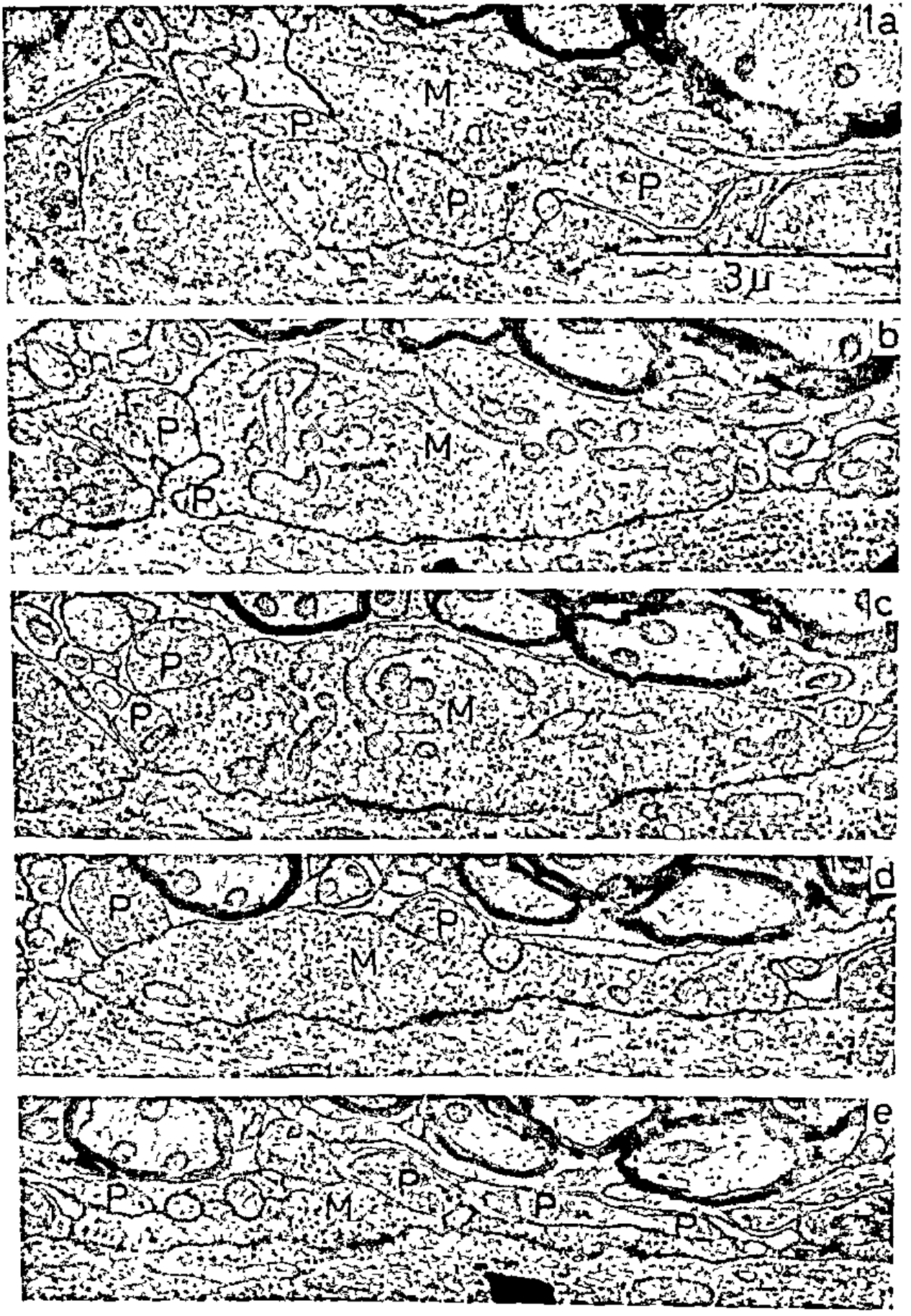
Fig. 14. On the proximal part of a motoneuron dendrite (M), a profile of a bouton (B) with spherical vesicles is surrounded by a large process (G), probably of glial origin. There is a long extent of apposition of postsynaptic dense bodies (arrows) at the inside of the motoneuron cell membrane, which is partly covered by the bouton, partly by the big glial process. Seven days postoperatively. ×31,400

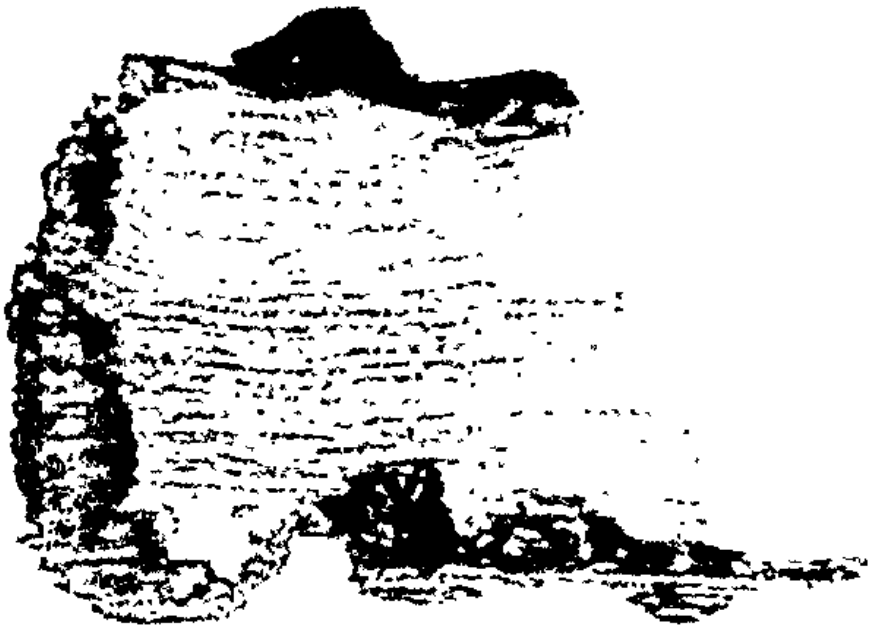
Fig. 15. Survey photograph at the proximal part of a motoneuron dendrite (M). A dark inclusion body is situated inside a glial process with dark cytoplasm, probably microgliacytic (MG). The region inside the square is followed through serial sections in the next figure. Three days postoperatively. ×13,100

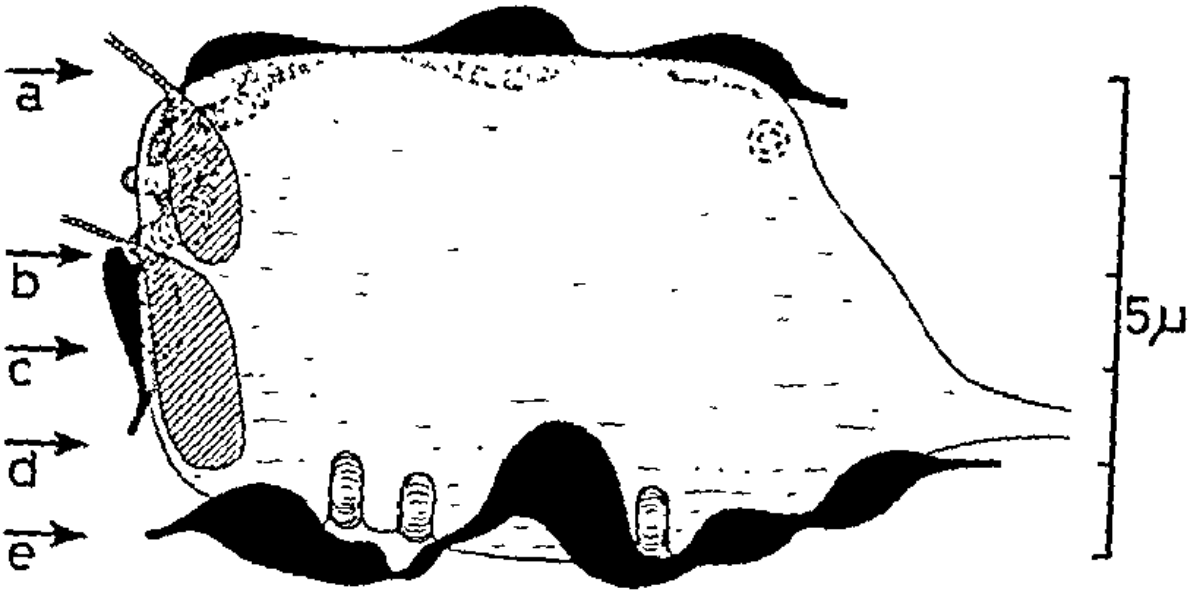
Fig. 16 a—h. Series through the inclusion body, seen in Fig. 15 and the surroundings.
a, section 1. The same as in Fig. 15. ×28,000

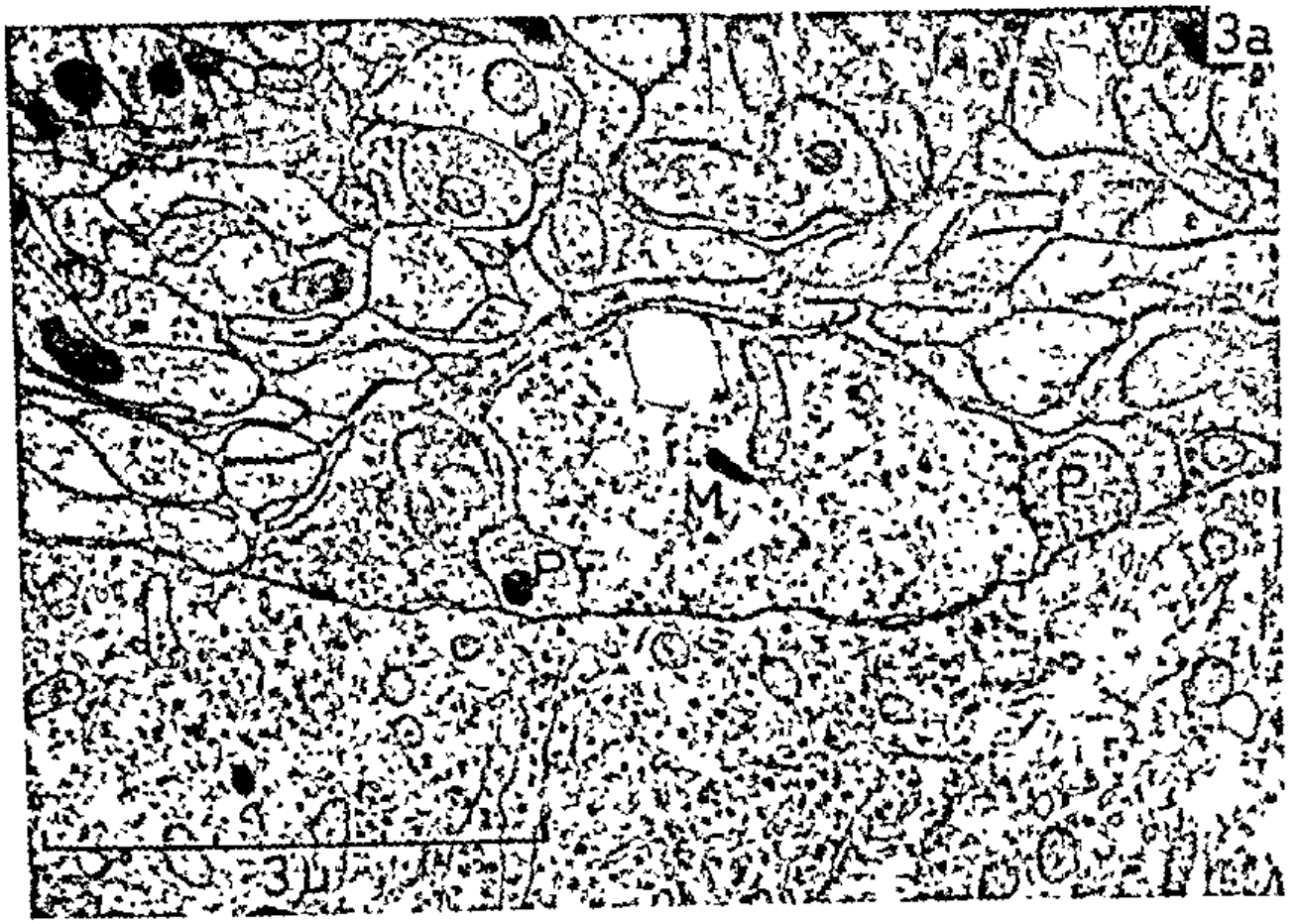
b, section 6. Contrast-rich material is found at part of the apposition region between the glial cell and the dendrite.

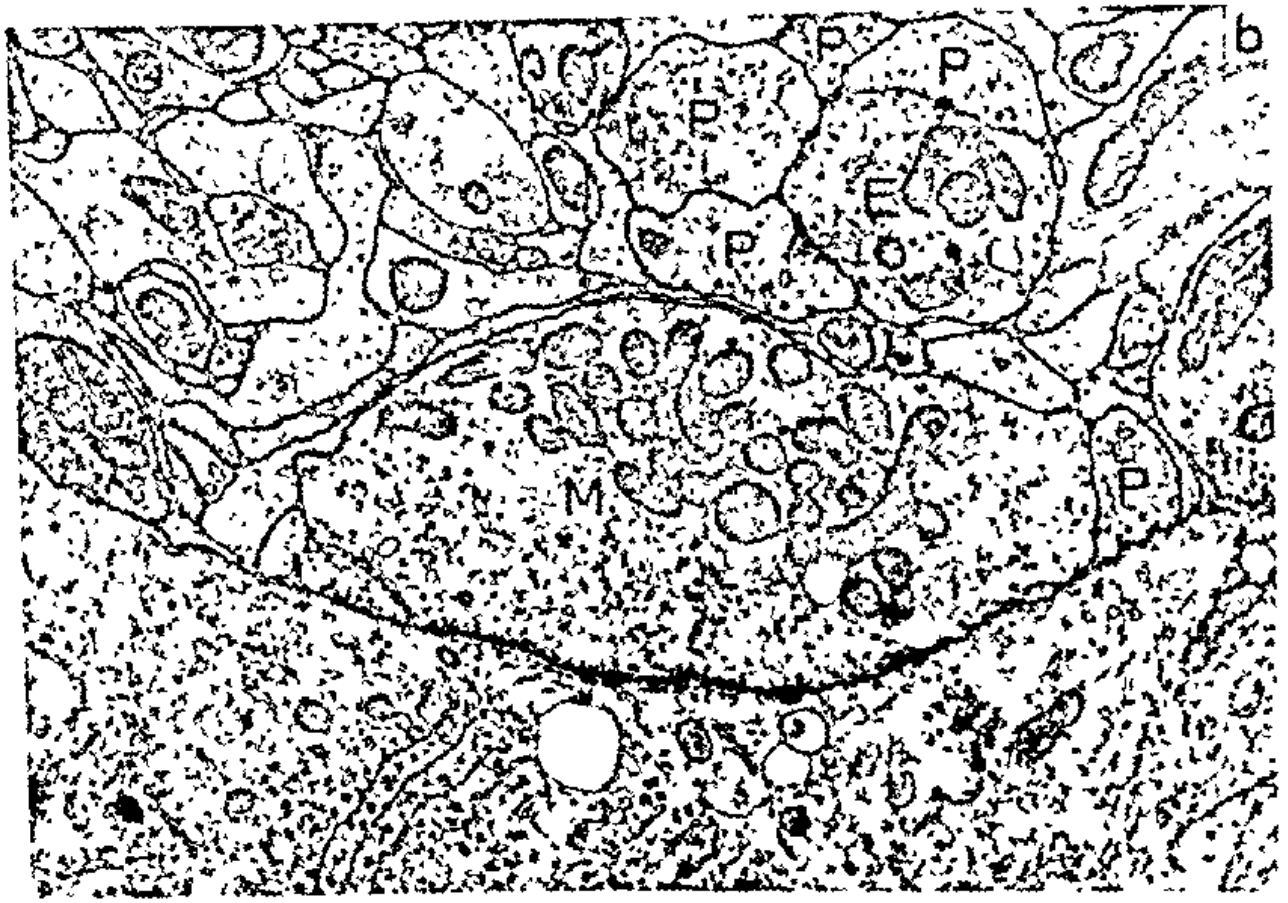
c, section 12. The inclusion body has disappeared. Postsynaptic dense bodies (arrow) are apposed at part of the motoneuron cell membrane facing the glial process. The glial process to the left has a clear cytoplasm of the astrocytic type (AS). Inside this, a bouton profile (B) is seen in both this and the next figure. The bouton does not establish any synaptic complex in the series, and it is probably of the P type.


1a
M
P
P
P
3µ
b
P
P
M
c
P
P
M
d
P
P
M
e
P
P
M
P
P
P




a
b
c
d
e
5µ


3a
M
P
P
3µ


b
P
P
P
P
P
E
M
P


P
P
P
P
M
3μ

4a



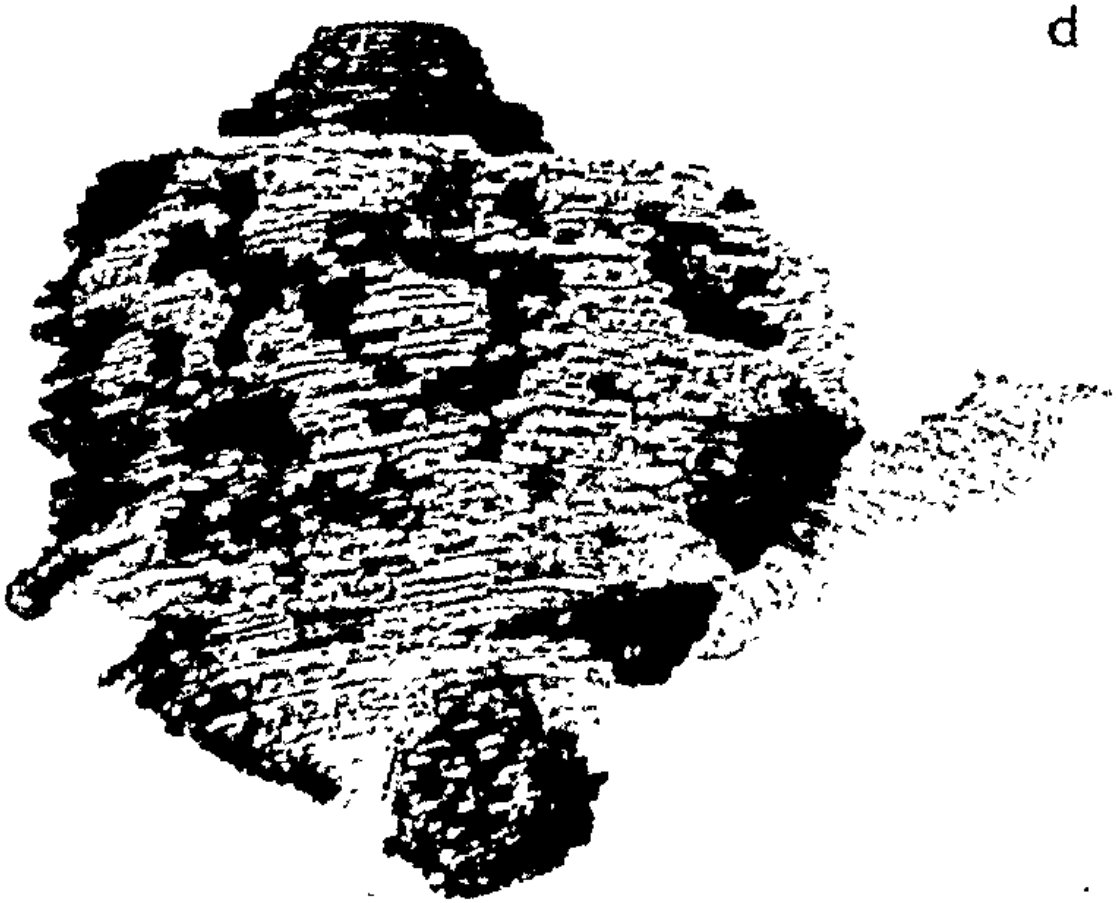
b



4c



d



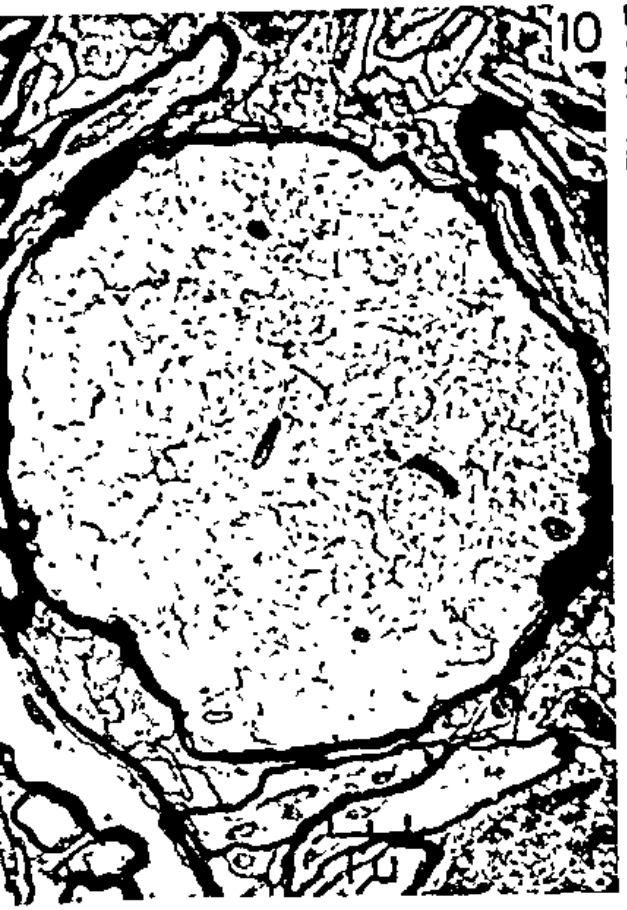

5
P
M
0,1 μ


6
P
M
0,1 μ


7
P
P
P
S


8
1μ

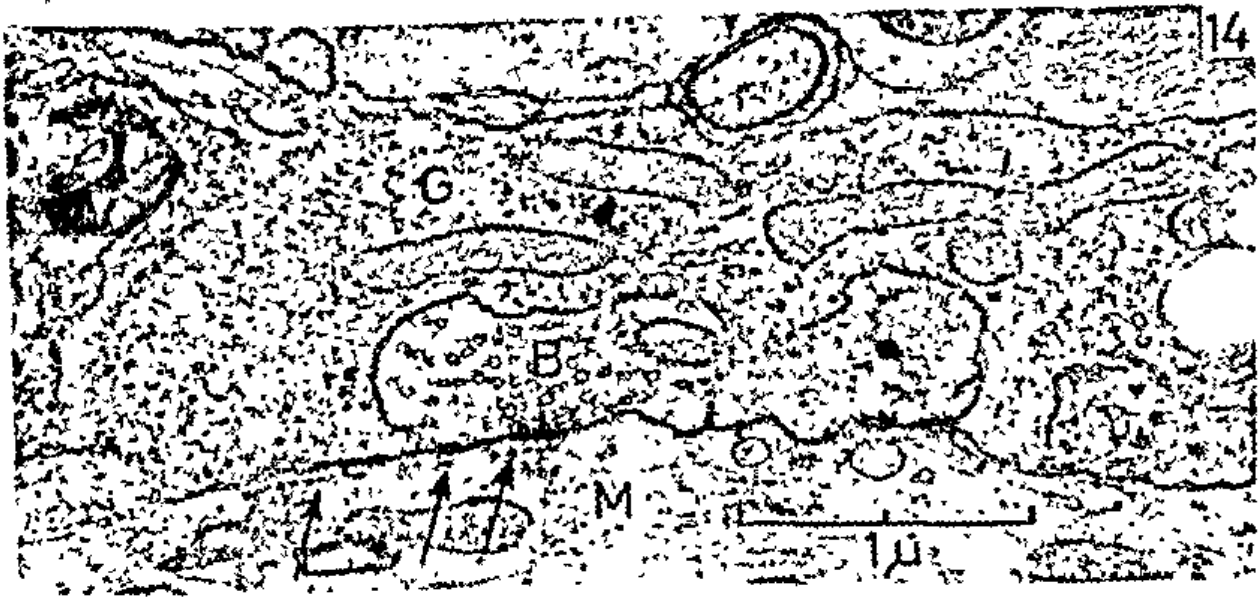

9
1μ

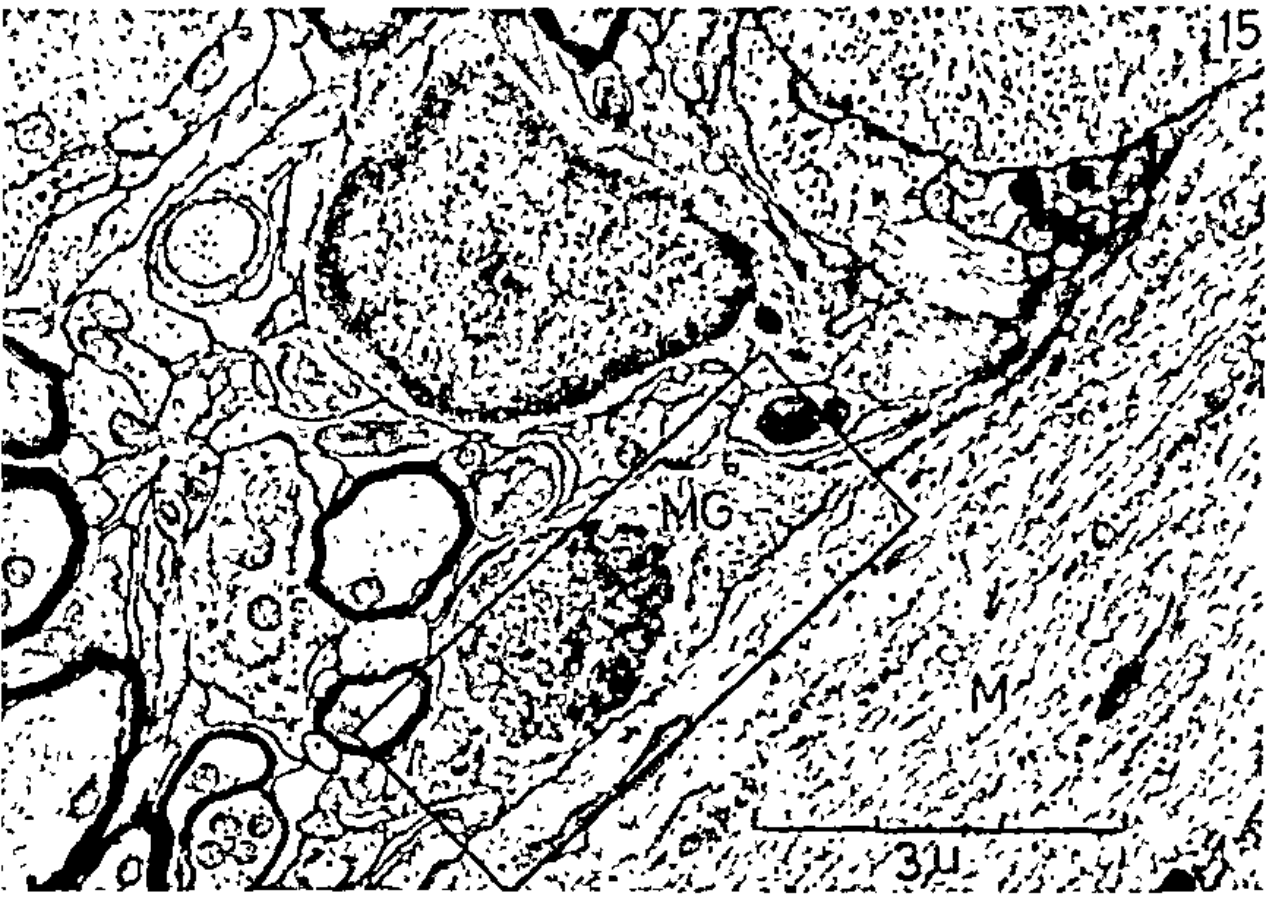

10
1μ


11
1μ

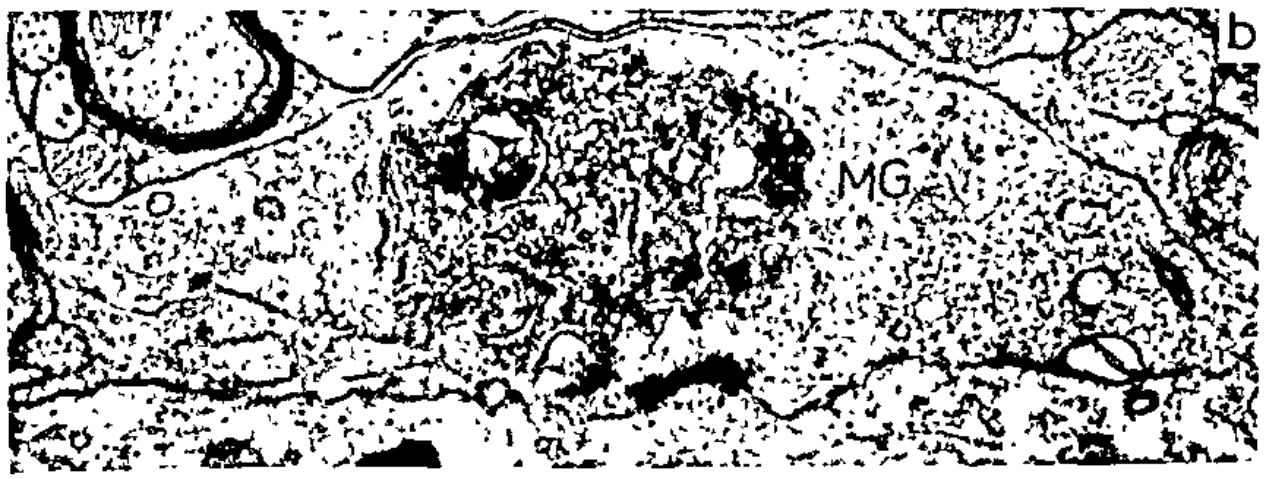

12
MG
M
1μ


13
0,5μ


14
G
B
M
1μ





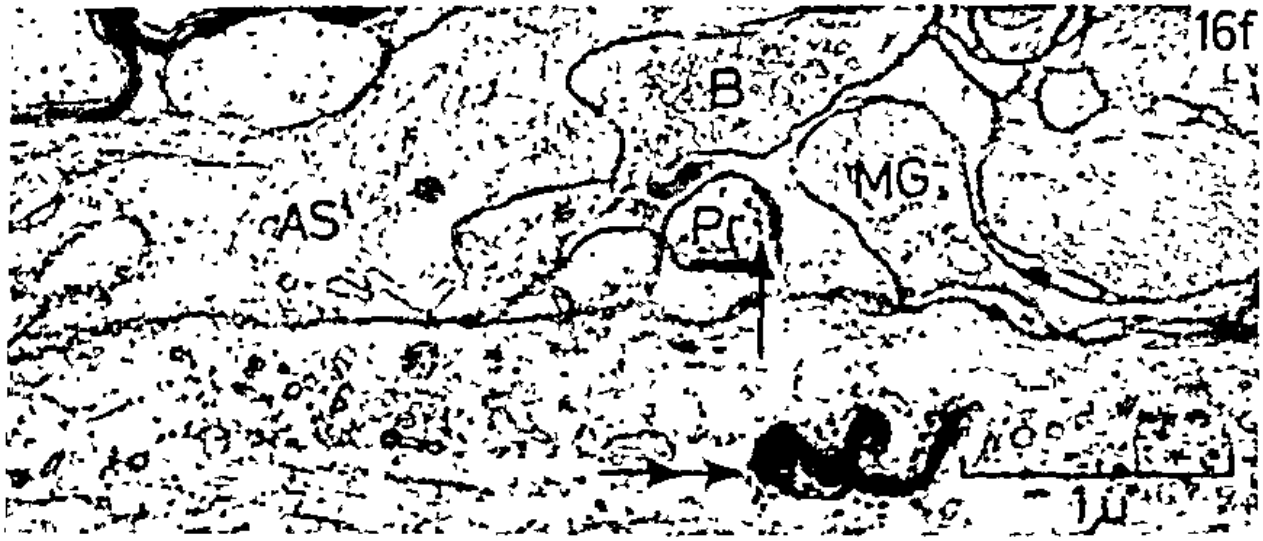


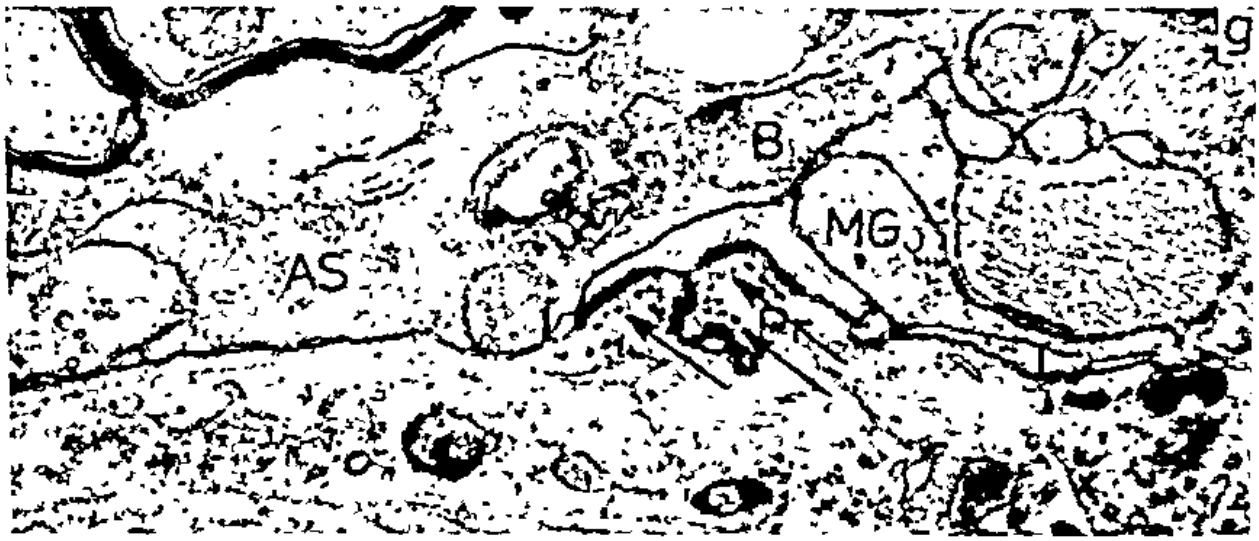
x

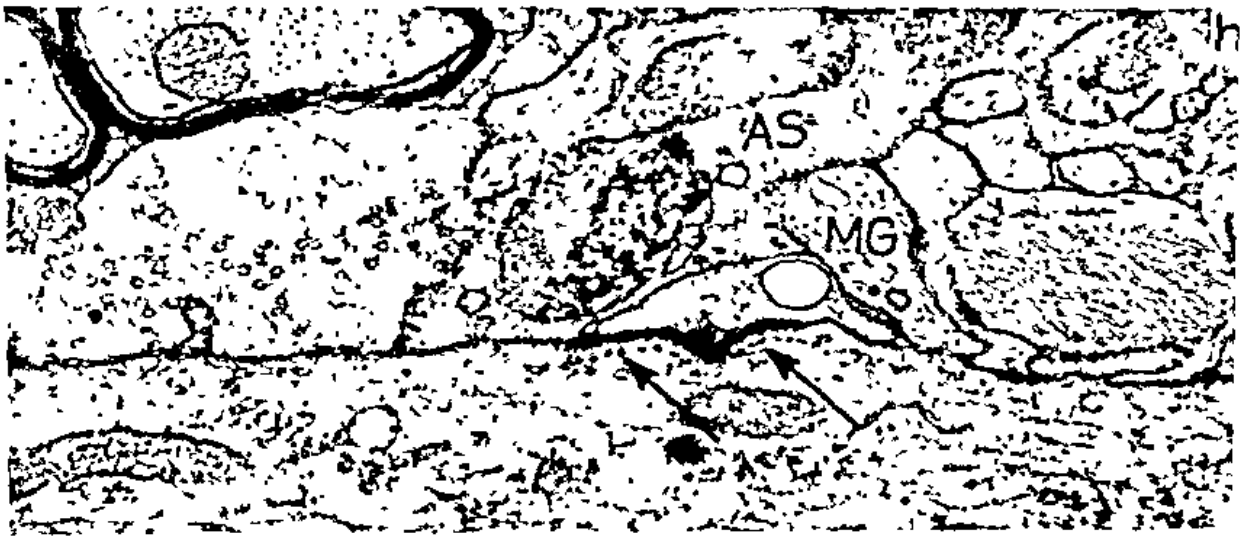

16c
B
MG
AS
JU


d
B
B
MG
AS

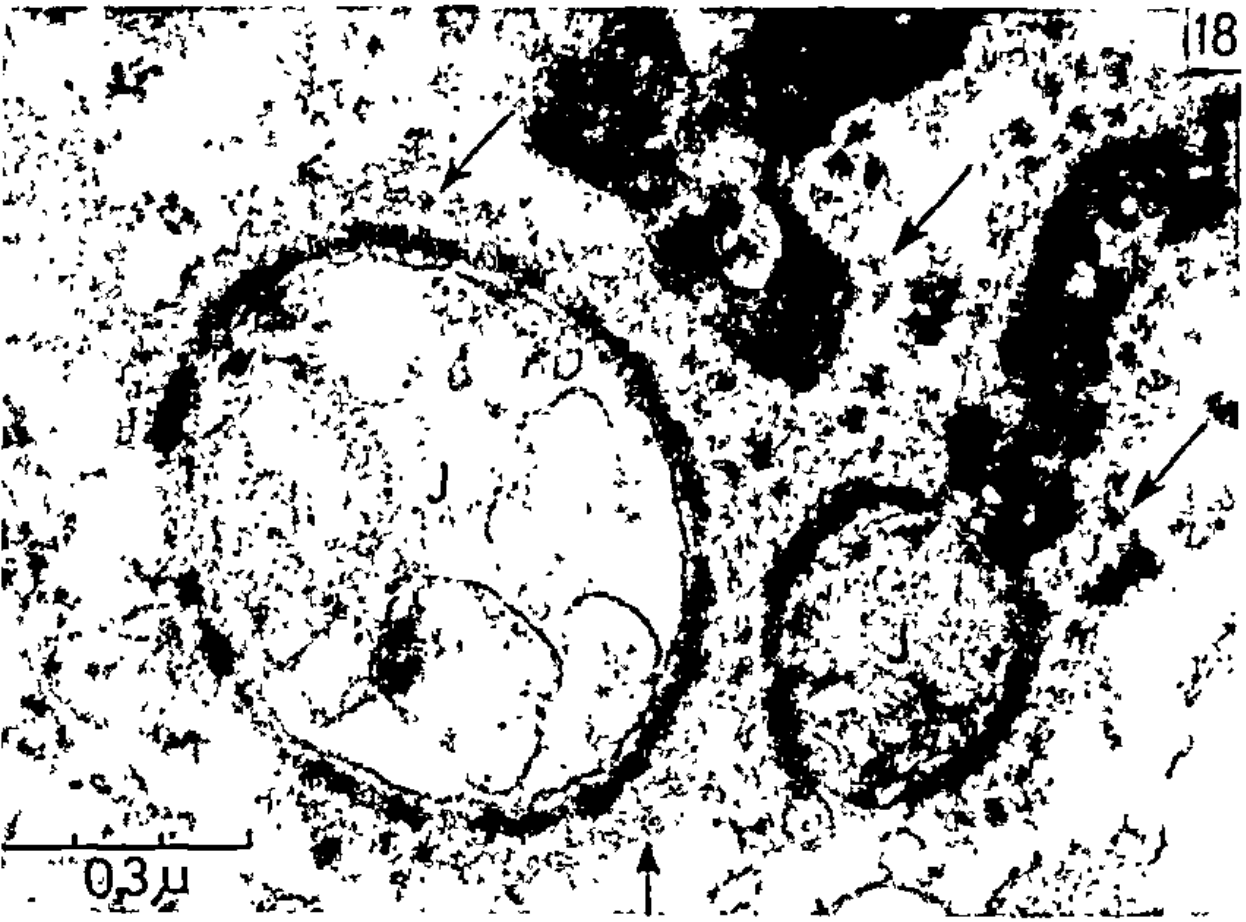

e
B
MG
AS

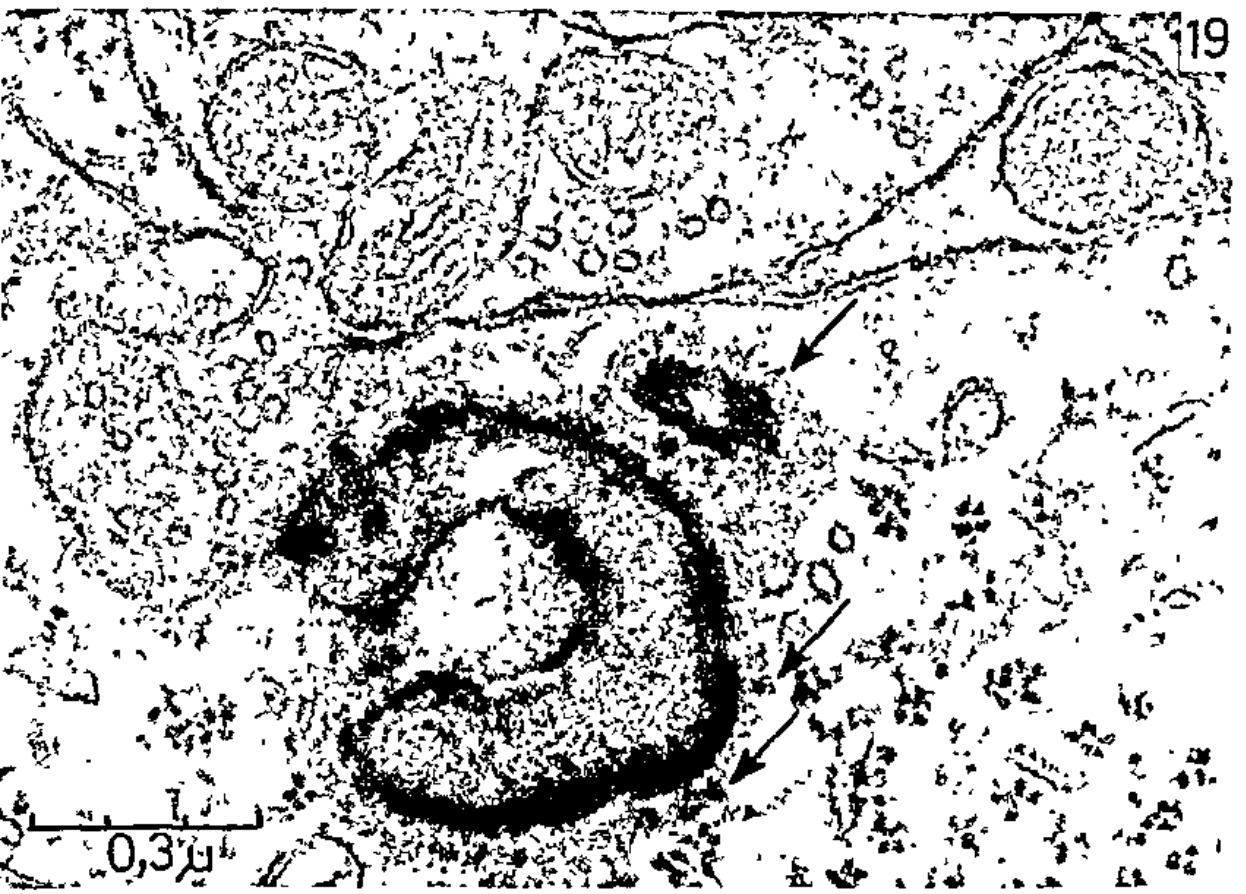

16f
B
MG
AS
Pr
1µ


g
B
MG
AS
Pr


h
AS
MG


17a
G
P
1μ
b
c
d
e
f
g


18
0,3 μ


19
0,3 μ


20
P
P
1μ


21
2μ


22
1μ


23
1μ


24
0,5μ


25
0,5μ

ACTA PHYSIOLOGICA SCANDINAVICA
SUPPLEMENTUM 333

ON MOTONEURON SYNAPTOLOGY IN KITTENS

An electron microscopic study
of the structure and location of neuronal
and glial elements on cat lumbosacral motoneurons
in the normal state and after
dorsal root section

BY

SEBASTIAN CONRADI and STEN SKOGLUND

STOCKHOLM 1969

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FROM THE DEPARTMENT OF ANATOMY, KAROLINSKA INSTITUTET, STOCKHOLM, SWEDEN

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Observations on the ultrastruture and distribution of neuronal and glial elements on the motoneuron surface in the lumbosacral spinal cord of the cat during postnatal development

by

SEBASTIAN CONRADI and STEN SKOGLUND

INTRODUCTION

Against the background of the knowledge of the ultrastructure of the adult spinal motoneurons and their surface relationships, (Conradi 1969 a, b, c, d) an investigation has been performed on the postnatal development of these structures. So far, the developmental material is rather limited, but since it contains a number of new data, some even rather unexpected, the observations hitherto made are presented in two papers. The first one deals with the relationships of the motoneuron cell membrane during normal development, the second one describes the development of the initial motoraxon segment and degenerative changes after dorsal root section.

Studies of the developmental spinal physiology has revealed that great changes take place postnatally, especially during the first three weeks (for review, see Skoglund 1966). Some of these changes can be attributed to changes in the peripheral afferent pathway of spinal reflexes. Studies of the monosynaptic pathway, however, have shown that important changes with regard to excitation and inhibition take place centrally (see Skoglund 1966). Part of these changes might be due to postnatal modifications in the number and location of the boutons on the motoneurons.

The problem of the location on the motoneurons of boutons having different functions has long attracted considerable attention from Eccles and collaborators (Eccles 1964). Recently, a series of paper dealing with this very same subject appeared from Frank's laboratory (see Rall 1967). In this series,

the assumption was made that changes of the motoneuron membrane impedance during synaptically-induced activity point to a specific localization of the involved boutons. The actual localization of such boutons, however, is unknown.

A thorough knowledge of the distribution of boutons on the motoneurons at early developmental stages, compared to the situation in the adult, might thus make possible correlations with physiologic differences between these stages. The simultaneous changes in morphology and function during development offers a unique situation for correlative studies.

There is no earlier ultrastructural report on the central connexions of the motoneurons in the kitten. From light microscopic studies, quantitative information on the postnatal growth of the motoneurons and other parameters in the spinal cord of the kitten has, however, recently been obtained (Mellström and Skoglund 1969). Studies on the mouse have provided evidence of a postnatal growth and branching of the motoneuron dendrites (Sakla 1959). A postnatal increase in the number of glial cells in the gray substance has also been reported (Sakla 1965). In the earlier light microscopic studies, it was reported that the motoraxons are myelinated already at birth (Langworthy 1929, Windle 1930), whereas myelination of the fine fibres in the gray substance is not developed until the 4—5th postnatal week in the kitten (Windle 1930).

Although the ultrastructure and distribution of neuronal and glial extensions on the motoneurons during the postnatal development are not known, several aspects of the development of neurons, glial cells, boutons and axons have been described in ultrastructural studies on other parts of the CNS (for references, see the Discussion).

In regard to the function of the developing nervous connexions, the formation of the first synaptic complexes has been shown to be accompanied by changes in the electrical activity of the tissue. The statement results from studies of growing tissue cultures (see Bunge *et al.* 1967) and experiments with developing neocortex and hippocampus in the cat (Voeller *et al.* 1963, Purpura *et al. 1965*, Schwartz *et al.* 1968). In the latter investigations, the first synaptic complexes were situated at the proximal parts of the dendrites. Both excitatory and inhibitory influences were present in the earliest stages. Furthermore, in an ultrastructural and functional study of the spinal cord of monkey fetuses (Bodian 1966 b, Bodian *et al.* 1968) it has been shown that the appearance of segmental reflexes is coincidental with the appearanace of the first synaptic complexes, likewise situated on proximal parts of the motoneuron dendrites. Axo-somatic synaptic complexes appear at the time

of onset of more complex reflex patterns, when there is also a rapid increase in the number of boutons with flattened vesicles.

The aim in the present paper was to obtain knowledge of the ultrastructure of the individual glial and neuronal elements, particularly boutons, on the motoneuron surface, as well as of the distribution of these structures from birth. The quantitative estimation deals with chiefly the same parameters as were investigated in the adult (Conradi 1969 a, b), namely:

a) A rough estimation of the dimensions of the motoneuron cell bodies and dendrites.
b) The localization of the different types of boutons on the motoneuron surface.
c) Size of the profiles of boutons of different types.
d) Percentage bouton covering on the motoneuron cell membrane, both total and for each of the different types of boutons.
e) The numerical proportion of the boutons of different types on various parts of the motoneuron.
f) The packing density of the boutons.

At first, the situation in the newborn is described, in addition to observations from random sections, a quantitative study on single sections, performed in the same manner as in the adult (Conradi 1969 a), is undertaken. The proximal part of a motoneuron dendrite is also investigated in serial sections, as was done in the adult (Conradi 1969 b). Then some features of the postnatal changes of the membrane relationships are described. Some quantitative estimations at the 3-weeks stage are also included.

It is found that the motoneurons are covered by a large number of boutons already in the newborn stage. The boutons are much smaller than in the adult, but the general appearance of them and the morphology of the synaptic complex are nearly the same. All the bouton types found in the adult are present at birth, except for the M-type (Conradi 1969 a, d) which cannot be recognized with certainty until from the third postnatal week. The percentage bouton covering of the motoneuron surface in the newborn is generally somewhat lower than in the adult except on the cell bodies, where the values are about the same or somewhat higher than in the adult. In the newborn, the percentage covering of boutons with spherical synaptic vesicles on the motoneurons, particularly the cell bodies, is larger than in the adult. The findings are discussed against the background of the functional changes described earlier (Skoglund 1966).

MATERIAL AND METHODS

In all, 49 normal kittens, 0—100 days old, were used. Some of the observations were also made on the unoperated side in an additional 28 kittens, which had been subjected to unilateral dorsal root section, 1—7 days before the fixation (Conradi and Skoglund 1969 b, Table 1).

The kittens were fixed in the same way as has previously been described from the adult cats (Conradi 1969 a). For details of the fixation methods, the reader is referred to the aforementioned investigation and the study of Berthold and Skoglund (1968). Some brief remarks will be given.

Fixation method

In the earlier part of the investigation, a total of 30 kittens was perfused with prerinse and fixative from 10—50 ml glass syringes. The rinsing fluid was Ringer's solution. The fixative consisted of 5% glutar aldehyde, usually purified with active charcoal (Fahimi and Drochmans 1965) and dissolved in a phosphate buffer, giving a buffer tonicity of 360 mOsm in the fixative. Following tracheotomy and artificial respiration, a wide thoracotomy was made and the left lung was removed. The aortic arch was clamped, a cannula or teflon catheter was inserted downward into the descending aorta and the right auricula was opened. 10—30 ml prerinse at 37°C, immediately followed by 10—25 ml fixative at 37° and 50—200 ml icecold fixative were perfused for 10—15 min.

In the later stages of the investigation, and including all the operated animals, this method was modified and used in 19 normal animals. Five percent purified glutar aldehyde was then dissolved in Millonig buffer (Karlsson and Schultz 1965), which had been diluted with distilled water to give a tonicity of 250—300 mOsm in the fixative, which is isotonic to the serum of the cat (Handbook of Biological Data 1956). Usually 8.1% or 2.7% Rheomacrodex was added to the rinsing fluid and fixative. After removal of the left lung, as in the foregoing method, a cannula or sharpened teflon catheter was inserted downward directly into the descending aorta, without previous clamping. Then 10—30 ml rinse at 37°, 25—50 ml fixative at 37° and 50—500 ml icecold fixative were perfused from three infusion sets, connected to the perfusion cannula with a 3-way stopcock. Each infusion set was provided with a blood-pressure balloon and a manometer. Through this arrangement, the perfusion pressure could be changed and continuously controlled. When the perfusion catheter had been inserted downward into the aorta, the inferior v. cava was cut. Rinsing

Table 1. Age of the animals

Age, days	No. of animals	Age, days	No. of animals
0—3	10	22—28	4
4—7	8	29—42	4
8—14	8	43—56	3
15—21	9	56—100	3

fluid was perfused at 60—100 mm Hg for 10—20 sec., warm fixative for 20—30 sec. and cold fixative 10—15 min. The fixative was perfused either at a continuous pressure of 60—100 mm Hg in a few animals, or at a discontinuous pressure, varying periodically between about 50 mm Hg and zero, 2—3 times per minutes, in the majority of the animals.

After the perfusion, the spinal canal was opened and the lumbosacral cord removed. Thin transverse sections of segment L 7 were cut and divided into symmetrical halves. These were rinsed in either veronal acetate buffer (Cedergren 1959) or in 300 mOsm Millonig buffer, postfixed with 2% OsO_4, dissolved in either of these buffers, for 4 hours. After another rinse, the specimens were dehydrated in acetone and embedded in Vestopal W. Semithin transverse sections of half the spinal cord were cut with an LKB 4801 ultrotome and stained with toluidine blue. The most lateral part of the ventral horn was trimmed out and cut into ultrathin sections. The sections were collected on one-hole grids, according to the method of Galey and Nilsson (1966). Shorter series of 20—50 consecutive sections and longer series, comprising 250—400 sections, in which only a few single sections were lost, were also investigated. The sections were stained with uranyl acetate and lead citrate. A Philips EM 300 was used.

Fixation quality

The quality of fixation could be controlled by mere macroscopical examination. After successful perfusions the spinal cord was hard and yellow. At inspection in the electron microscope, especially the preservation of the mitochondria seemed sensitive to the quality of the fixation. The mitochondria were often vacuolized, particularly after poor perfusions. The best fixation was obtained after perfusions at a discontinuous pressure from the infusion sets. With kittens less than 14 days old, however, the fixation was rather good, regardless of whether a continuous or a discontinuous perfusion pressure was used. Moreover in these animals the cervical cord and brain were well perfused, despite the location of the perfusion catheter in the descending aorta. When the kittens were older than 14 days, the best results were obtained with a discontinuous perfusion pressure (cf. Berthold 1968).

Quantitative studies

A quantitative estimation on the membrane relationships of the motoneuron cell membrane from single sections was performed in the newborn stage (2 days old) and at 3 weeks. The method was again the same as used in the adult material (Conradi 1969 a). Briefly, the procedure was the following: Two kittens from two different litters were used at each age and equal halves of the material were taken from each animal. The animals, which according to macroscopical examination of the spinal roots seemed to have a prefixed lumbosacral plexus (Sherrington 1892), were perfused with 5% glutar aldehyde in 200 mOsm Millonig buffer from the infusion sets. Rheomacrodex was added to the rinse and to the fixative to give a concentration of 2.7%. The lateral motoneuron nucleus in the most cranial millimetre of L 7 was cut into semithin transverse sections in a cranio-caudal direction. When motoneurons were cut through the nucleus, 2—3 grids of ultrathin sections, 1—3 μ apart, were prepared. From these sections, the profiles of 20 motoneuron cell bodies, cut through the mid-nucleus level, and the profiles of proximal parts of horizontal dendrites projecting

from these motoneurons, were photographed. Six additional long profiles of horizontal proximal dendritic segments, belonging to motoneurons which were not ideally sectioned, as well as profiles of 30 thin, horizontal dendrites from the neuropile, were also analyzed. As pointed out earlier (Conradi 1969 a) nothing is known about the origin of these thin dendritic branches. As will be shown, several features of the membrane relationships of these profiles are, however, common in most of the animals. At least some of the dendrites might belong to motoneurons.

In the newborn animals, photographs were taken at a magnification of 3,250×. Four to six photographs were required for every motoneuron cell body. Paper copies with a final magnification of about 16 000 × were prepared and taped together to montages, on which the membrane length of the motoneuron cell bodies (SO) and dendritic profiles were measured. The minor diameter of the cell bodies and average diameter of the dendritic profiles were also measured. The dendritic profiles were divided into three categories: The proximal parts of motoneuron dendrites, covered at one side in the section by the first 20 boutons, counted from the cell body, were treated separately (DA), as were the parts of the profiles distal to this, (DB). The thin dendritic profiles in the neuropile were denoted d. The type of every profile of boutons or glial extensions on the motoneuron cell bodies and dendrites was judged from inspection of the actual section, at high magnification in the electron microscope, and the result was transferred to the paper. The length of apposition and height of every bouton profile was measured on the montages. These values and the number of boutons of the different types were set up in table form with separate headings for the four categories of motoneuron and dendritic profiles. The mean length of the profiles of the different bouton types was calculated in every column. In addition, the percentage bouton covering of the membrane, both total and for each bouton type was calculated. The numerical proportion of bouton profiles of the different types, as well as the mean distance along the cell membrane between the centres of adjacent boutons, were also calculated. Some of the results from the quantitative estimation in the newborn were studied statistically.

The membrane relationships of the motoneurons on the 3-week old kittens was investigated quantitatively in a somewhat simplified way. The length of apposition of the bouton profiles was not measured, allowing use of a lower final magnification (×1,800) and thus reducing markedly the number of photographs. The number and types of the boutons apposed to the profiles were likewise estimated on single sections in the above manner.

Statistical control

The bouton covering, both total and for each bouton type separately, on each of the four regions (SO, DA, DB, d), were compared, both to each other, using an F-test, and to the corresponding values from the adult (Conradi 1969 a) using a t-test.

The mean length of the profiles of the S+T and F bouton types (see Conradi 1969 a) was studied on the cell bodies and the thin dendrites. The values for the two types on the same region were compared using a t-test, as were the values for the same bouton type on the two regions. In each case, comparisons with the adult were made, likewise with a t-test. The variance of the values for length of apposition of these two bouton types on the cell bodies and thin dendrites was also calculated and compared to the corresponding values from the adult.

Reconstructions from serial sections

The membrane relationships of the proximal part of a motoneuron dendrite in the newborn stage were analyzed through serial transverse ultrathin sections, cut in a cranio-caudal order. The dendrite belonged to a motoneuron from one of the specimens on which the quantitative estimation in the newborn was performed. The reconstructed part of the dendrite was completely cut through within about 80 sections; one single section was lost. Assuming a cylindrical shape of the dendrite, the mean section-thickness was 600 A, as calculated from measurements of the diameter of the dendrite at several places. The dendrite, which was directed ventromedially from the motoneuron cell body, left the latter in a nearly horizontal direction after a short initial curve upward. The diameter of the dendrite in the most proximal part was about 3 μ, which seemed somewhat less than average for similar processes. About 20 μ from the cell body, the dendrite curved slightly medially and upward and became somewhat thicker, 4 μ. Again growing thinner, it bent slowly downward, then upward and finally disappeared out of the sections (see Fig. 13 a). In each section the dendrite was photographed with a primary magnification of 6,400×. Usually 3—4 photographs were required for each section and the copies were taped together to montages of the dendrite, where the final magnification was 18,000×.

The types of the different boutons on the montages were judged from mere inspection at high magnification in the electron microscope. Every 5—7th section was examined and the result transferred to the copies. The membrane relationships of the dendrite were illustrated in a drawing, where the cell membrane was depicted unfolded (see Fig. 13). The detailed technique has been presented earlier (Conradi 1969 b). Briefly, it was the following: the mantle surface of the dendrite at the magnification used is calculated and depicted on a transparent paper, on which the consecutive sections are represented as lines. The transparent paper is placed on the montages in successive order and the outline of the dendrite is aligned with the line representing the actual section. The type and length of apposition of the structure making contact with the cell membrane of the dendrite are then transferred to the corresponding line on the paper. If the analyzed dendrite has a cylindrical shape and is cut longitudinally, the mantle surface is a rectangular area, where the sections form straight parallel lines. The distance between these lines is thus greatest at those parts of the paper representing the upper and lower margins of the mantle surface.

In the present study, an attempt was made to apply the method to a curved dendrite, in which the diameter was not constant. Here, the mantle surface was imagined as consisting of the mantle surfaces of several, consecutive flat cylindres of different diameters, not placed concentrically For the sake of simplicity, the uppermost margin of the dendrite was represented on the paper as a straight line in the middle of the mantle surface area, and the sections as symmetrical curved lines on both sides (See Fig. 13 b). This representation of the sections gives rise to a considerable distortion of the membrane relationships at some places, particularly along the lower part of the mantle surface However, the major aim of this study was to judge the topography of the membrane relationships and some general features of the boutons at birth, and to give an appreciation of the packing density of the boutons. No calculations of the size and shape of the apposition areas of the boutons were made. The following parameters of the membrane relationships of the dendrite were analyzed: location,

from these motoneurons, were photographed. Six additional long profiles of horizontal proximal dendritic segments, belonging to motoneurons which were not ideally sectioned, as well as profiles of 30 thin, horizontal dendrites from the neuropile, were also analyzed. As pointed out earlier (Conradi 1969 a) nothing is known about the origin of these thin dendritic branches. As will be shown, several features of the membrane relationships of these profiles are, however, common in most of the animals. At least some of the dendrites might belong to motoneurons.

In the newborn animals, photographs were taken at a magnification of 3,250×. Four to six photographs were required for every motoneuron cell body. Paper copies with a final magnification of about 16 000 × were prepared and taped together to montages, on which the membrane length of the motoneuron cell bodies (SO) and dendritic profiles were measured. The minor diameter of the cell bodies and average diameter of the dendritic profiles were also measured. The dendritic profiles were divided into three categories: The proximal parts of motoneuron dendrites, covered at one side in the section by the first 20 boutons, counted from the cell body, were treated separately (DA), as were the parts of the profiles distal to this, (DB). The thin dendritic profiles in the neuropile were denoted d. The type of every profile of boutons or glial extensions on the motoneuron cell bodies and dendrites was judged from inspection of the actual section, at high magnification in the electron microscope, and the result was transferred to the paper. The length of apposition and height of every bouton profile was measured on the montages. These values and the number of boutons of the different types were set up in table form with separate headings for the four categories of motoneuron and dendritic profiles. The mean length of the profiles of the different bouton types was calculated in every column. In addition, the percentage bouton covering of the membrane, both total and for each bouton type was calculated. The numerical proportion of bouton profiles of the different types, as well as the mean distance along the cell membrane between the centres of adjacent boutons, were also calculated. Some of the results from the quantitative estimation in the newborn were studied statistically.

The membrane relationships of the motoneurons on the 3-week old kittens was investigated quantitatively in a somewhat simplified way. The length of apposition of the bouton profiles was not measured, allowing use of a lower final magnification (×1,800) and thus reducing markedly the number of photographs. The number and types of the boutons apposed to the profiles were likewise estimated on single sections in the above manner.

Statistical control

The bouton covering, both total and for each bouton type separately, on each of the four regions (SO, DA, DB, d), were compared, both to each other, using an F-test, and to the corresponding values from the adult (Conradi 1969 a) using a t-test.

The mean length of the profiles of the S+T and F bouton types (see Conradi 1969 a) was studied on the cell bodies and the thin dendrites. The values for the two types on the same region were compared using a t-test, as were the values for the same bouton type on the two regions. In each case, comparisons with the adult were made, likewise with a t-test. The variance of the values for length of apposition of these two bouton types on the cell bodies and thin dendrites was also calculated and compared to the corresponding values from the adult.

sometimes found. Rather thick astrocytic processes with a clear cytoplasm were often situated just outside the motoneuron cell membrane at the cell body/ and proximal parts of the dendrites, as well as at small dendritic profiles. It was often difficult to distinguish these from other profiles of possible neuronal origin.

b) *Boutons.* Boutons were found to make contact with the motoneuron cell bodies, proximal parts of the dendrites and small profiles of dendrites in the neuropile. The boutons seemed rather evenly distributed throughout these regions and were as a rule tightly packed (Fig. 5) and much smaller than in the adult. Synaptic complexes and desmosome-like junctions, quite alike corresponding structures in the adult, were regularly observed (see below). Usually, the synaptic complex, as in the adult, did not reach the lateral margin of the zone of apposition of the boutons. In contrast to the situation in the adult, the boutons on the motoneuron cell bodies and proximal parts of the dendrites often established synaptic contact with yet another neuronal profile ("double synapse"). For the most part, this second synaptic complex was made with very thin dendritic profiles, which were found in a large number just outside the motoneurons (Fig. 5).

In addition to the synaptic vesicles, the boutons contained a few small mitochondria, but in single sections mitochondrion profiles were not seen in all boutons, in contrast to the situation in the adult (Fig. 5). A few boutons were observed to contain granular vesicles. Often, glycogen-like particles were present in the boutons (cf. Konishi 1966) (Fig. 6), but neurofilaments and membrane-bound dense bodies were seldom encountered. At some places, boutons containing tubular profiles were observed during the neonatal period (Fig. 7). These boutons are very similar to those previously reported in various developmental disturbances in human neocortex (Gonatas *et al.* 1967). Similar boutons have been observed during normal ontogeny in the chick cerebellum (Mugnaini and Forströnen 1967).

Boutons were rather frequently apposed at the spinous projections from the motoneurons. Sometimes a contrast-rich material, 100—300 A thick, was attached at the inner side of the cell membranes of the spines, which at low magnification gave the impression of a dark interior of the spines (Fig. 8). When recognized, the preterminal axons of the boutons were always unmyelinated. Flattened astrocytic capsules were found around some of the boutons (Figs. 7 and 8) but not to the same extent as in the adult. Instead, the boutons were often covered by thicker astrocytic processes, adjacent boutons or unmyelinated axons.

c) *Formation of new synaptic complexes.* From some developmental ultrastructural studies (Glees and Sheppard 1964, Bodian 1966, Wechsler 1966, see discussion by Bunge *et al.* 1967), there is evidence that the first step in the formation of synaptic contacts is the establishment of a desmosome-like structure between an axon or bouton and a postsynaptic extension. Secondary to this, synaptic vesicles are observed on the presynaptic side. Some findings (Bodian 1966 b, del Cerro and Snider 1968) also indicate that the growth cones of neuronal extensions contain several profiles of large vesicles.

In the present material, it was rather difficult to distinguish between profiles of unmyelinated axons or axonal swellings on one hand and astrocytic extensions on the other; some boutons were, namely, very small and contained only few synaptic vesicles. Vesicle-like profiles could also be seen inside processes obviously of astrocytic origin. Desmosome-like junctions of a size comparable to that of a synaptic cleft, were seldom observed between the motoneuron cell membrane and small extensions containing only a few synaptic vesicles. Profiles, similar to the growth cones, mentioned above, were not encountered.

Different types of boutons

The boutons on the motoneuron cell membrane can be divided into morphologic types, similar to those found in the adult (see Conradi 1969 a).

S-type. Small boutons containing spherical synaptic vesicles (S-type) were regularly observed in the tissue. The diameter of the vesicles was 350—500 A. The synaptic cleft of these boutons was about 150 A thick, and the lateral extension of the cleft was 0.2—0.5 μ. A thin, contrast-rich material was attached at the presynaptic membrane and the layer of postsynaptic material was about 50—200 A thick (Fig. 9 a).

T-type. Boutons, containing spherical synaptic vesicles, in which the postsynaptic dense bodies of Taxi (T-type) could be observed below the postsynaptic membrane, were present to a lesser extent than in the adult. At many places, the postsynaptic dense bodies were very small and could not clearly be seen in the newborn, but this type of synaptic complex could often be recognized through the thick and somewhat irregular layer of contrast-rich material, 200—300 A thick (Fig. 9 b). When clearcut postsynaptic dense bodies were at hand, a band of contrast-rich material, described earlier by Gray (1963) in the adult spinal cord, was sometimes present just inside these bodies. (This is seen in Fig. 11.)

F-type. Boutons containing flattened synaptic vesicles (F-type) were also observed on the motoneurons in the newborn. (Fig. 9 c. These boutons are also seen in Fig. 6). The diameter of the vesicles is 200—300×400—600 A. As in the adult, the synaptic cleft is rather narrow, 90—130 A and the layer of postsynaptic dense material is thin, 50—150 A. Again as in the adult, there seemed to be F boutons with either large or small synaptic clefts, but this difference was not accounted for in the quantitative estimation. No F boutons with a dense ground substance (FD-type in the adult) were found in the newborn. There was sometimes a granular precipitate, especially prominent on flattened synaptic vesicles, in some of the specimens, in which veronal acetate buffer had been used in the postosmification procedure; this too resembled the situation in the adult animals.

C-type. Comparatively big bouton profiles containing spherical synaptic vesicles 350—500 A in diameter, where a large subsynaptic cistern is situated just below the whole postsynaptic membrane (C-type), were rather frequently observed on the motoneuron cell bodies and proximal parts of the dendrites in the newborn (Fig. 10). The synaptic cleft was very narrow, 30—80 A, and as in the adult, extended below nearly the entire bouton. No contrast-rich material was present at the pre- and postsynaptic membranes and the synaptic vesicles were usually not apposed at the whole extent of the presynaptic membrane. The C boutons were often seen to be partly invaginated into the motoneuron cell bodies (Fig. 10). As in the adult, these boutons were not observed on the smaller neurons in the ventral horn.

M-type. In the adult material (Conradi 1969 d), big boutons, obviously of two types and both containing spherical synaptic vesicles, were found to have small boutons, (P-type) containing small synaptic vesicles of an irregular shape, apposed at the convex side. One of these bouton types (M-type) showed a big, irregularly shaped synaptic complex, covered with postsynaptic dense bodies. The other type of bouton was quite similar to the boutons of the S-type. Clearcut M boutons were not observed in the newborn animals.

In some rare instances, profiles of rather big boutons containing spherical synaptic vesicles and having another bouton apposed at the convex side were observed (Fig. 12). One or more profiles of a synaptic complex between these big boutons and the motoneurons could be seen in the sections. Postsynaptic dense bodies were present in some cases. The synaptic vesicles of the small apposing boutons seemed to be of the same size and shape as at P boutons in the adult. These boutons are further dealt with in a forthcoming paper (Conradi and Skoglund 1969 b). In the quantitative study on single sections

in the newborn stage (see below), two types of bouton profiles were included below the heading "M- and big S-type", they are:

a) Boutons with spherical vesicles having another bouton profile apposed at the convex side.
b) Big boutons with spherical synaptic vesicles, showing one large or several smaller profiles of a synaptic complex, covered by a thick layer of postsynaptic material or several postsynaptic dense bodies.

It must be pointed out that these profiles *do not* completely fulfil the criteria for the M and big S boutons with axo-axonic contacts, established in the adult (Conradi 1969 a, d).

Quantitative estimation in the newborn stage

The size and distribution of bouton profiles of the different types on the motoneuron cell bodies and dendrites in the newborn were studied on single transverse sections through the lateral motor column in the most cranial millimetre of L 7 (see Material and Methods). The aim of this estimation and the one at the three-weeks stage (see below) was to compare the bouton population with that in the adult (Conradi 1969 a). As already mentioned, the material (a total of about 50 profiles from two kittens, taken at each stage) is rather small, because of the complicated handling procedure. Despite this, there are several quite consistent general features in regard to the parameters which will be dealt with. The results from the newborn animals are presented in Tables 2—5. The mean values will be used in the following description, although it seems clear that there are some general differences between the two animals; the value for the mean minor diameter of the motoneuron cell bodies is, for example, somewhat bigger in kitten no. 2.

a) *Profiles of motoneuron cell bodies and dendrites.* The mean minor diameter of the motoneuron cell bodies in the newborn was 29 μ. This is about 60% of the corresponding value in the adult (49 μ) (cf. Mellström and Skoglund 1969). The mean diameter of the various dendritic profiles were usually less than half the size of the corresponding values in the adult. The values were 5.9 μ (DA), 3.7 μ (DB), 2.0 μ (d) in the newborn and 11.7 μ (DA), 8.4 μ (DB), 4.8 μ (d) in the adult.

Spinous projections from the cell membrane were rather frequently encountered on both cell bodies and dendrites (Table 2) and seemed to be most common at proximal parts of the motoneuron dendrites. In the adult material,

Table 2. Quantitative estimation, newborn kitten

Cat 1=1—10, D1—3, d1—15

Cat 2=11—20, D4—6, d16—30

		1			2			3			4			5			6		
		SO	DA	DB	SO	DA	DB	SO	DA	DB	SO	DA	DB	SO	DA	DB	SO	DA	DB
Diameter, μ	1	23.0	8.6	2.6	23.6	4.4		25.7	8.6	3.9	27.0	5.7	7.4	25.0	9.5	4.6	24.3	2.8	5.7
Membrane length, μ	2	87.2	156 4	73.6	54.5	91.2		91.1	45.6	76.0	98.0	65.9	54.9	55.4	120.0	28.0	81.4	110.8	18.8
Boutons																			
S, number	3	18	53	29	15	28		25	16	27	17	14	23	17	37	13	12	25	5
F number	4		2	4		1		3	2	6		6	2	1	3	1	1	3	2
F, number	5	25	62	35	17	45		32	21	30	33	26	15	25	44	10	28	55	7
C, number	6	1						4	1	2	2	2			4			1	1
M and big S, number	7		1	1		2				2	1	1	1		1				
Spines, number	8		3	2		2			1	3					4	1			
Apposing dendrites																			
number	9	1	1					1		3			1						
length, μ	10	3.4	0.41					0.68		0.63			0.47						
Apposing oligodendrocytes																			
number	11									1							1		
length, μ	12									1.0							4.0		
Boutons, total number	13	44	118	69	32	76		64	40	67	53	49	41	43	89	24	41	84	15
Boutons, total length, μ	14	37.2	98.9	46.9	23.6	55.2		50.7	31.4	50.9	39.6	40.7	30.1	35.5	72.1	19.1	26.1	69.3	9.9
Membrane length, μ / Boutons, total number	15	2.6	1.3	1.1	1.7	1.2		1.4	1.1	1.1	1.8	1.4	1.3	1.3	1.1	1.2	2.0	1.3	1.3
Boutons, total length, μ / Membrane length, μ	16	0.64	0.63	0.64	0.43	0.62		0.67	0.69	0.67	0.40	0.61	0.55	0.64	0.71	0.68	0.32	0.63	0.53

	7			8			9			10			11			12			13		
	SO	DA	DB	SO	DA	DB	SO	DA	DB	SO	DA	DB	SO	DA	DB	SO	DA	DB	SO	DA	DB
1	28.4	6.4	2.7	29.7	8.8	3.7	27.0	5.4		23.6	4.7		35.1	9.1	3.4	33.8	10.1		37.2	5.7	
2	73.1	140.6	30.4	89 2	109.5	42.9	78.7	88.2		110.8	27.0		94.9	118.6	99.3	89.5	80.3		120.6	32.4	
3	16	61	15	15	31	12	14	26		29	16		33	36	44	14	25		28	20	
4	2	6		1	2		2	2					5	2	1		2		3		
5	21	47	13	32	44	21	17	32		40	13		35	46	39	36	29		44	9	
6	1	3				1	3			1			2	4		4	3		3	1	
7		1															1				
8		4		2	1			1					3	3	3	1	2			1	
9		1													3						
10		0.74													0.61						
11							1			1											
12							6.4			5.9											
13	40	118	28	48	77	34	36	60		70	29		75	88	84	54	60		78	30	
14	38.1	95.9	22.6	36.9	59.1	28.0	29.9	40.7		57.4	16.5		61.8	77.7	63.6	54.7	54.5		75.3	24.7	
	1.8	1.2	1.1	1.9	1.4	1.9	2.2	1.5		1.6	1.6		1.3	1.3	1.2	1.7	1.3		1.5	1.1	
	0.52	0.68	0.74	0.41	0.54	0.65	0.38	0.46		0.52	0 61		0.65	0.66	0.64	0.61	0.68		0.62	0.76	

	14			15			16			17			18			19			20		
	SO	DA	DB	SO	DA	DB	SO	DA	DB	SO	DA	DB	SO	DA	DB	SO	DA	DB	SO	DA	DB
1	27.0	3.4	2.7	25.0	3.4	3.4	31.1	5.4	2.0	34.5	4.5	5.1	32.4	9.0	6.0	33.8	6.8		35.8	6.1	3.4
2	106.4	93.2	50.7	92.6	133.2	10.8	76.4	127.0	10.8	97.3	115.0	29.3	85.8	157.1	48.3	105.7	97.3		92.9	105.1	13.5
3	28	37	22	25	38	2	16	44	5	22	42	9	32	42	16	17	27		25	43	4
4		1	1		2			4		1	5	2	4	7	1	1			1	2	
5	46	53	17	28	51	7	14	48	5	29	38	21	28	61	19	21	22		33	37	6
6	1			6	1		1		1	2				1					3	1	
7		2	2					2		2	6	2	2								
8										1											
										0 68											
										1						1					
	75	91	40	59	92	9	31	96	11	3.5						1.0					
	62 8	65.4	31.1	52.3	77.8	6.6	34.6	78.7	8.9	54	85	32	66	112	37	41	55		62	8	10
	1 4	1.0	1.3	1.6	1.4	1.2	2.5	1.3	1.0	49.7	65.5	26 4	50.7	92.0	29.1	45.7	58.3		58.9	74.3	7 7
	0 59	0.70	0 63	0.56	0 58	0.63	0.45	0 62	0 82	1.8	1.6	1.0	2.6	1.4	1.3	2.4	1.8		1.5	1.3	1.4
										0.51	0.57	0 90	0.59	0.59	0.60	0.43	0 60		0.64	0.71	0.57

Table 2. (cont.)

		D1		D2		D3		D4		D5		D6	
		DA	DB	DA	DB	DA	DB	DA	DB	DA	DB	DA	DB
Diameter, μ	1	5.1		4.4	2.0	4.9		5.3	3.4	5.1	3.4	3.2	1.6
Membrane length, μ	2	64.3		71.6	7.4	54.7		55.4	38.5	76.4	57.4	85.8	18.5
Boutons: S, number	3	18		13	1	27		20	13	21	13	21	9
T, number	4	1		1		2		3	1	3	2	2	
F, number	5	20		27		18		17	12	20	19	21	2
C, number	6	1		2		1			2			3	
M and big S, number	7										1		
mber	8								1	1	1		
lendrites, number	9			1									
length, μ	10			0.68									
oligodendrocytes, number	11							1					
length, μ	12							0.54					
	13	40		43	1	48		40	28	44	35	47	11
	14	*35.8*		*31.6*	*4.4*	*27.9*		*41.6*	*21.2*	*41.6*	*29.0*	*44.1*	*8.2*
	15	1.6		1.7	1.1	1.1		1.4	1.4	1.7	1.6	1.8	1.7
	16	0.56		0.44	0.59	0.51		0.75	0.55	0.55	0.50	0.51	0.45

Table 2. (cont.)

		d1	d2	d3	d4	d5	d6	d7	d8	d9	d10	d11	d12	d13
Diameter, μ	1	1.4	2.2	1.0	2.7	3.9	1.5	2.4	2.5	3.2	3.0	2.0	1.4	1.7
Membrane length, μ	2	45.9	54.9	60.0	89.5	75.5	43.9	42.6	109.5	73.8	44.9	57.4	43.9	53.4
Boutons: S, number	3	9	10	16	33	28	18	17	25	28	12	24	15	14
T, number	4	1		2		5	2	1	6	2	2	2	3	
P, number	5	9	6	11	41	28	12	13	24	32	19	13	22	15
C, number	6					2								
M and big S, number	7													
Spines, number	8		1			2	1		2		1			
Opposing dendrites, number	9													
length, μ	10													
Crossing oligodendrocytes, number	11													
length, μ	12													
Boutons, total number	13	19	16	29	74	63	32	31	55	62	33	39	40	29
Boutons, total length, μ	14	12.5	12.2	23.7	49.7	45.0	26.1	21.8	48.7	45.2	28.1	27.2	26.9	22.0
$\frac{\text{Membrane length, } \mu}{\text{Boutons, total number}}$	15	2.4	3.4	2.1	1.2	1.2	1.4	1.4	2.0	1.2	1.4	1.5	1.1	1.8
$\frac{\text{Boutons, total length, } \mu}{\text{Membrane length, } \mu}$	16	0.27	0.22	0.40	0.55	0.60	0.60	0.51	0.45	0.61	0.63	0.12	0.61	0.41

Table 2. (cont.)

	d14	d15	d16	d17	d18	d19	d20	d21	d22	d23	d24	d25	d26	d27	d28	d29	d30
1	3.0	2.4	1.5	1.5	1.4	1.7	2.0	2.0	3.4	2 0	1.5	2.0	1.4	1.4	1.6	1.7	1.4
2	65.9	60.8	66.9	32.1	71.3	48.3	51.0	46.3	55.4	115.5	81.1	98.6	37.8	52.7	58.8	70.3	63.5
3	17	23	12	8	19	11	7	7	20	33	17	33	9	12	12	14	3
4	1	2	3		1	4		4	4	6	7		1		1		1
5	22	21	14	8	20	11	14	11	17	37	14	36	7	14	10	4	7
6																	
7														1			
8	1		1				1			1	1	1		1		4	1
9									1			1		3	2	1	
10									0.54			0.34		0.50	0.51	0.81	
11																	
12																	
13	40	46	29	16	40	26	21	22	41	76	38	69	17	27	23	18	11
14	31.4	35.3	25.1	11.3	27.7	28.0	16.1	17.8	28.5	55.2	31.2	47.0	12.6	20.6	19.5	15.5	8.9
15	1.6	1.3	2.3	2.0	1.8	1.9	2.7	2.1	1.4	1.5	2.1	1.4	2.2	2.0	2.6	3.9	5.8
16	0.48	0.58	0.38	0.35	0.39	0.58	0.42	0.38	0.51	0.50	0.39	0.48	0.33	0.23	0.33	0.22	0.24

Table 3. Mean values, newborn kittens

	CAT 1				CAT 2				CATS 1+2			
	SOMA	DA	DB	d	SOMA	DA	DB	d	SOMA	DA	DB	d
Mean diameter, μ	25.7	6.1	4.1	2.3	32 6	5.9	3.4	1.8	29.2	6.0	3.7	2.0
Mean membrane length, μ	81.9	86.8	41.5	61.5	96 2	98.2	37.7	63.0	89.1	92.5	39.4	62.3
Boutons: S mean number	17.8	28.1	15.6	19.3	24.0	32.0	13.7	14.5	20.9	30.0	14.6	16.9
proportion of all bouton profiles, %	37.8	42.0	44.8	47.5	40.1	44.8	46.1	45.8	39.1	43.5	45.5	46.8
mean length, μ	0.77	0.74	0 68	0.75	0.81	0.80	0.73	0.74	0.80	0.77	0.71	0.75
mean height, μ	0.39	0.47	0.47	0.43	0.42	0.53	0.51	0.47	0.41	0.51	0.49	0.45
T mean number	1.0	2.4	1.9	1.9	1.5	2.5	0 8	2.1	1.3	2.5	1.3	2.0
proportion of all bouton profiles, %	2.1	3.6	5.4	4.8	2.5	3.6	2.7	6.8	2.3	3.6	4.0	5.6
mean length, μ	1.0	0.79	0.71	0.82	0.99	0.96	0.98	1.1	1.0	0.88	0.80	0.96
mean height, μ	0.50	0.50	0.45	0.57	0.50	0.52	0.48	0.56	0.51	0.51	0.46	0.57
F mean number	27.0	34.9	16.4	19.2	31.7	34.8	14.7	14.9	29.4	34.8	15.4	17.1
proportion of all bouton profiles, %	57.3	52.2	47.0	47.4	53.0	48.7	49.5	47.3	54.9	50.4	48.3	47.3
mean length, μ	0.77	0.76	0.76	0.73	0.92	0.83	0.77	0.75	0.85	0.80	0.77	0.74
mean height, μ	0.43	0.48	0.49	0.44	0.51	0.55	0.61	0.52	0.47	0.52	0.55	0.48
C mean number	1.2	1.0	0.5	0.1	2.4	1.3	0 4		1.8	1.2	0.4	0.07
proportion of all bouton profiles, %	2.5	1.5	1.4	0.3	4.0	1.8	1.3		3.4	1.7	1.4	0.2
mean length, μ	1.6	1.7	1.4	2.1	1.6	1.7	2.3		1.6	1.7	1.8	2.1
mean height, μ	0.50	0.63	0.70	0.57	0.60	0.72	0.50		0.54	0.68	0.60	0.57
M and big S, mean number	0.1	0.5	0.5		0.2	0.8	0.1	0.07	0.2	0.6	0.3	0.03
proportion of all bouton profiles, %	0 2	0.7	1.4		0.3	1.0	0.3	0.2	0.3	0.9	0.9	0.1
mean length, μ	1.1	1.7	1.8		1.7	2.0	1.9	1.8	1.5	1.9	1.8	1.8
mean height, μ	0.50	0.54	0.83		0.70	0.75	0.50	1.1	0.60	0.71	0.62	1.1
Spines, mean number	0.5	1.2	0.4	0.5	0.8	1.8	1.0	0.7	0.7	1.5	0.7	0.6
Apposing dendrites, mean number	0.5	0.2	0.1		0.1	0.2	0.3	0.5	0.3	0.2	0.2	0.3
mean length, μ	1.2	0.6	0.5		0.7	2.0	0.6	0.5	1.1	1.2	0.6	0.5
Apposing oligodendrocytes mean number	0.4				0.2	0.08			0.3	0.04		
mean length, μ	4.3				2.3	0.5			3.6	0.5		
Mean total number of boutons	47.1	66.8	34.9	40.5	59.8	71.4	29.7	31.6	53.5	69.1	32.0	36.1
Mean total length of boutons, μ	37.5	51.8	26.0	30.4	54.2	60.7	23.2	24.3	45.9	56.3	24.5	27.3
$\frac{\text{membrane length, } \mu}{\text{total no. of boutons}}$	1.7	1.3	1.2	1.5	1.6	1.4	1.3	2.0	1.7	1.3	1.2	1.7
$\frac{\text{total length of boutons, } \mu}{\text{membrane length, } \mu}$	0.49	0.60	0.64	0.47	0.57	0.62	0.61	0.38	0.515	0.608	0.622	0.439

only a few spines were encountered and all of them were found on the dendrites.

The dendro-dendritic apposition regions were also included in the quantitative study. A total of 23 observations of such junctions were made in the quantitative study.

b) *Location of the boutons of different types.* Both S and F boutons were present on the motoneuron cell bodies as well as on the dendrites. As in the adult, profiles of F boutons were most frequently encountered on the cell bodies in single sections with the proportion diminishing with increasing distance from the cell bodies.

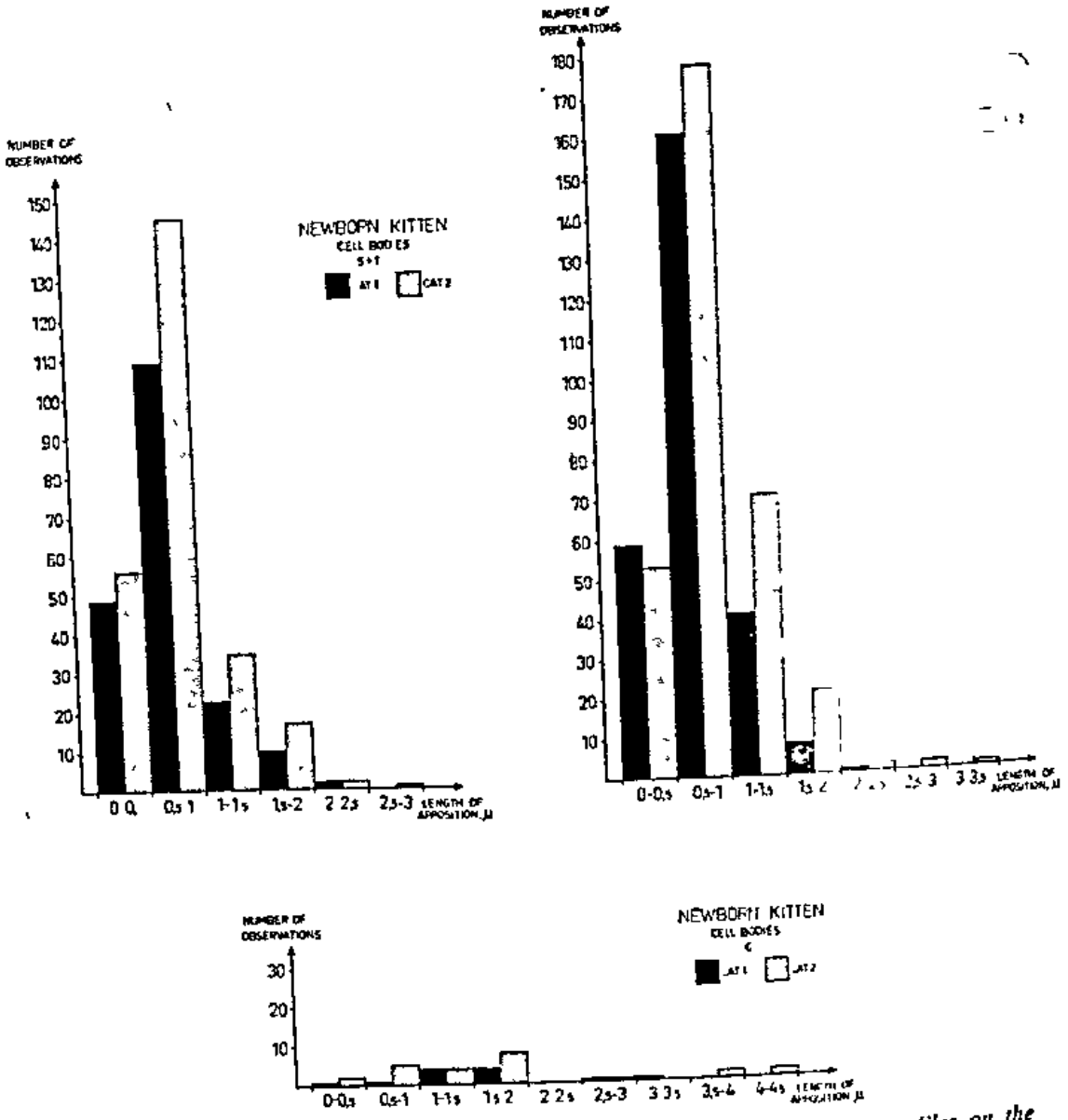
The situation for S and particularly T boutons was the reverse. The C boutons seemed to be restricted to the motoneuron cell bodies and proximal parts of the dendrites. Most of the boutons, included in the M and big S column, were situated on proximal parts of the motoneuron dendrites. As already pointed out, these boutons, however, did not completely fulfil the criteria for the M-type and big S-type with axo-axonic contacts, as defined in the adult.

Thus, the location of the different bouton types was very similar to that in the adult. In order to obtain more accurate information on the spectrum of the boutons on different regions of the motoneurons, the bouton covering has to be studied (see below).

c) *Size of the bouton profiles.* The mean length of apposition of the S and F boutons in the neonate is about 0.7—0.8 μ as compared to 1.7—2.1 μ in the adult (adult bigger than newborn for both types at all regions, $p < 1\%$), which means that the two most commonly occurring bouton types in the newborn are about half the size they attain in the adult. There are no significant differences between the mean values on the cell bodies and thin dendrites for the S- or F-type; neither is there any significant difference between the mean values of these two types.

In the newborn, as in the adult, the mean length of apposition of the T boutons seems slightly larger than for S boutons. The mean length of apposition of the C boutons is about twice that of the S and F boutons, as compared to about 2,5 times that in the adult.

The size-range for the length of apposition of both S and F boutons on the cell bodies are shown in text-Fig. 1. The majority of the observations lies near the mean values, and the size-range is noted to be rather small for both types. The calculation of the variances for the length of apposition showed



Text-Fig.: Diagram showing the length of apposition of all the bouton profiles on the motoneuron cell bodies in the quantitative study on the newborn stage.

roughly equal values for S and F boutons, both on the cell bodies and on the thin dendrites. The variance in all cases was much less than in the adult.

d) *Bouton covering (Tables 3 and 4).* The highest total bouton covering of the motoneurons, roughly 60%, was found on the proximal parts of the dendrites. From here on, the covering decreased towards the cell bodies and towards the distal dendrites. These regional differences are significant at the 10%

Table 4. Newborn kitten. Bouton covering, separated for the different bouton types. The developmental change, expressed as difference between adult and newborn, is included

Bouton type	SOMA		DA		DB		d	
	Covering %	Change %	Covering %	Change %	Covering %	Change %	Covering %	Change %
S + T	20.1	−7.5	27.4	−2.9	28.7	− 2.7	23.3	+0.5
F	27.9	−0.7	10.1	+9.6	30.1	+11.8	20.3	+3.9
C	3.2	+3.8	2.1	+4.0	2.1	+ 2.6	0.2	+0.3
M + big S	0.3	−0.1	1.3	+0.6	1.3	− 0.2	0.1	+0.1

level. In the adult, the highest values for bouton covering were also found on the proximal parts of the motoneuron dendrites, where they are higher than in the newborn. The values for total bouton covering on the cell bodies in the newborn seems equal to, or slightly higher than, in the adult. The differences in total bouton covering between newborn and adult are not significant at any of the four regions.

The values for the covering of the boutons of different types are shown in Table 4; the differences from the adult stage are included. The S and T boutons are included under the same heading, since it was observed in the adult material (Conradi 1969 a, b) that one and the same bouton may establish synaptic complexes of both types.

It must be pointed out at once that the regional differences in the covering of the boutons of different types in the newborn stage are not significant.

Compared to the adult, however, some differences are apparent. The covering of F boutons increases on the dendrites from newborn to adult; on region DA, the rise is significant ($p < 5\%$). On the cell bodies, there is no increase at all for the F boutons. The covering of C boutons seems also to increase, both on the cell bodies and on the dendrites, but the values are not significant. For the S+T boutons, there is a decrease towards the adult stage on the proximal parts of the dendrites and particularly on the cell bodies, where the decrease is significant at the 10% level. Also, a decrease for the ratio between the covering of S+T and F boutons on the cell bodies is significant at the 10% level.

The figures for the mean distance along the cell membrane between the centres of adjacent boutons are included in Table 3. These are further dealt with in connexion with the 3-week stage.

Three-dimensional reconstruction in the newborn

The membrane relationships of the proximal part of a motoneuron dendrite in the newborn kitten were analyzed through serial sections. The mantle surface of the dendrite was illustrated unfolded (Figs. 13—19). The findings were compared with those, obtained from a similar reconstruction from adult animal (Conradi 1969 b).

It can be seen that the largest part of the dendritic cell membrane is covered by boutons (Figs. 15 and 16). Adjacent dendrites and myelin sheaths only small areas, rather narrow intervening astrocytic processes were present at these places. The unlabelled parts of the surface are covered by astrocytic and unidentified processes.

The apposition areas of the boutons seem for the most to be slightly convex. Adjacent boutons are tightly packed at only a few places. Instead, a small space is generally left between the boutons. A large area in Figure 15 is entirely devoid of boutons. All types of boutons were identified, except for F boutons, but three rather big S boutons, obviously exhibiting an axo-axonic synaptic contact, were present (Fig. 15). The size-range of the boutons, both between the different types and within the same type, is not so pronounced as in the adult. S and F boutons seem to be about the same size, while the C boutons are somewhat bigger. No obvious clustering of boutons of the same type was observed.

As shown in Figs. 17 and 18, the preterminal axons, which were always unmyelinated, could not be observed at all the boutons. Some boutons *de passage* were encountered. Granular endoplasmic reticulum was present below the cell membrane at some of the C boutons, but its distribution, apart from this, seemed to be rather random, as was the location of the dendritic spines. Rather many boutons also established synaptic contacts with adjacent dendrites. These boutons with "double synapses" were clustered, since several neighbouring boutons often made synaptic contact with the same adjacent dendrite.

The size and shape of the synaptic complexes and desmosome-like figures are shown for some of the boutons in Fig. 19. Most of the boutons seem to make several desmosome-like contacts with the dendrite. Between every bouton and the dendrite there is often only one synaptic complex, which, in a few cases, nearly reaches the lateral border of the bouton. The synaptic cleft seems to occupy a rather large part of the zone of apposition as compared to the situation in the adult.

When the results of the three-dimensional reconstruction are compared to those obtained in the adult, the following differencies are distinguished: In

the adult, the boutons were bigger and more densely packed. No similar large areas devoid of synaptic contacts, as in the newborn stage, existed, instead the dendrite in the adult stage was covered by myelin at several places. In the adult, some preterminal axons were myelinated, and very few boutons made synaptic contacts with additional adjacent dendrites. Usually several synaptic complexes were found between each bouton and the dendrite in the adult, in contrast to the situation in the newborn.

In the reconstructions analyzed in this study, the number of boutons inside the largest possible square on the dendritic surface were counted. In region A, 106 boutons were present inside 119 μ^2, which corresponds to 89 boutons/100 μ^2. In region B, 100 boutons were situated inside 130 μ^2 or 72 boutons/100 μ^2. Corresponding values for the adult were 17.5/100μ^2 (A) and 22/100 μ^2 (B). Roughly, the extent of regions A and B in the adult and newborn is comparable.

Postnatal changes of the relationships of the motoneuron cell membrane

First three weeks

During the neonatal period, some differences between the animals in the general ultrastructural features of the ventral horn are already apparent. The gestation period for the cat is 65 ± 4 days (Scott *et al.* 1957), which means that kittens are born at different stages of maturation. Individual differences are also met with in the subsequent development; therefore the present description represents a rather rough estimation.

There is a gradual increase in the size of the motoneuron cell bodies, which seems to continue at least during the first two postnatal months (cf. Mellström and Skoglund 1969). The granular endoplasmic reticulum reaches the cell membrane of the motoneuron cell bodies at most places rather soon after birth, and the glycogen-like particles inside the motoneuron cell bodies then disappear. At three weeks the proximal parts of the motoneuron dendrites still look rather thin, as compared to those in the adult, and they still contain relatively little endoplasmic reticulum. In the three-week old kitten, it is moreover often difficult to demarcate the transition between motoneuron cell bodies and proximal parts of the dendrites (Fig. 20). The zone of transition is rather long. The number of spinous projections from the motoneuron cell membrane seems to diminish gradually already from birth on. From studies on developing Purkinje cells there is evidence that the spines disappear in a

centrifugal course from the neuron cell bodies (Larramendi and Victor 1966). Whether this also holds true in the present material was not investigated.

Parallel with the increase in size of the motoneuron cell bodies, the cell/gray coefficient decreases (Mellström and Skoglund 1969). Concomitantly, the number of desmosome-like junctions between adjacent motoneurons decreases rapidly already during the first postnatal week. There is also a rather rapid decrease in the number of boutons making synaptic contact with more than one postsynaptic extension. The diameter of the profiles in the neuropile increases gradually. During the first three postnatal weeks, few axons in the neuropile are myelinated.

As mentioned, glial cells were rather seldom observed to be apposed at the motoneuron cell bodies in the newborn. During the first postnatal weeks both oligodendrocytes and astrocytes were found in such a position to a seemingly increasing extent. Immediate apposition could then also be found between the cell membranes of the oligodendrocytes and the motoneurons. This contrasts to the situation found in mature animals, where a thin astrocytic sheath nearly always was interposed.

As regards the astrocytes, both the degree of ramification of each cell and the total number of cells seemed to increase after birth. The cytoplasm of the astrocytes contained abundant granular endoplasmic reticulum and the Golgi-apparatus was often very prominent. At three weeks, large areas of the motoneuron cell bodies could be found to be covered by astrocytic cell bodies (see below). An increasing amount of astrocytic cytoplasm is also found around the initial axon segment during the same period (Conradi and Skoglund 1969 b). In the adult, astrocytic cell bodies are seldom observed immediately outside the motoneuron cell bodies; instead, large areas of the latter are covered by thin astrocytic sheaths (cf. Conradi 1969 a).

The size of the boutons also increases during the first postnatal month. At one month, boutons are rather infrequently observed to contain glycogen-like particles. One or several mitochondria, instead, are usually seen in most bouton profiles in each single section. Boutons, completely fulfilling the criteria for M boutons in the adult, appear from the third postnatal week.

The development after three weeks

The main ultrastructural changes observed in the ventral horn after 3 weeks were somewhat different from those occurring in the preceding period. The size of the cell bodies continued to increase and the motoneurons were still further separated. The diameters of dendrites, axons and boutons showed a

the adult, the boutons were bigger and more densely packed. No similar large areas devoid of synaptic contacts, as in the newborn stage, existed, instead the dendrite in the adult stage was covered by myelin at several places. In the adult, some preterminal axons were myelinated, and very few boutons made synaptic contacts with additional adjacent dendrites. Usually several synaptic complexes were found between each bouton and the dendrite in the adult, in contrast to the situation in the newborn.

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Table 6. Mean number of bouton profiles on the motoneuron cell bodies in the quantitative study on single sections

Age	Cat 1	Cat 2	Mean
Newborn	47	59	53
3 weeks	46	75	60
Adult	34	35	34

probability, still larger. If so, the postnatal increase in diameter is larger for the more distal than for the proximal parts of the dendrites.

The boutons. Since the dimensions of the bouton profiles were not known, the bouton covering could not be calculated. The tight-packing of the boutons had to be expressed as the ratio: $\frac{\text{membrane length}}{\text{number of boutons}}$ which means mean distance along the cell membrane between the centres of adjacent boutons. Despite this limitation, a few comments on the changes in the bouton population from the newborn stage will be attempted.

The mean distance along the cell membrane between the centres of adjacent boutons has increased from the newborn stage on both cell bodies and dendrites. In all instances, the change from newborn to the three week stage is less than the change between the three week and adult stages.

The total number of bouton profiles on the motoneuron cell bodies in the sections has increased slightly by three weeks. However, the values at both the newborn and three week stages are much higher than those found in the adult material (see Table 6). The possible reasons for this decrease are further dealt with in the discussion.

The proportion of bouton profiles of different types as seen in the single sections at three weeks has also changed to some extent from the newborn stage. The comparatively high proportion of profiles of the S+T-type found in the newborn, especially on the motoneuron cell bodies still holds, although it is less pronounced. The values were: SO=40.7% (newborn 41.4%, adult 35.5%), DA=43.2% (newborn 47.1%, adult 39.6%), DB=50.0% (newborn 49.5%, adult 42.0%). In the first animal, the values resemble more those in the adult than those in the newborn, whereas the reverse is true for the second animal.

The M and big S boutons with axo-axonic contacts are present on about the same regions of the motoneuron surface as in the adult. The numerical proportion in the sections of these profiles, which at 3 weeks fulfil the

criteria used in the adult, is comparable to that found in the adult.

In conclusion, it may be stated that the size of the motoneurons and the location of apposing boutons of different types at three weeks, seem to be intermediate between the newborn and the adult stages. The developmental changes in the parameters investigated during the first three postnatal weeks, however, appear to be less prominent than the changes between the three-week and the adult stage.

Neuronal degeneration

From earlier reports (se Levi-Montalcini 1964, Källén 1965, Bodian 1966 a, Wechsler 1966) there is evidence that neuronal degeneration occurs during the development, at least in the early stages of growth. In the present material, large accumulations of contrast-rich material, similar to the structures described by Bodian (1966 a) as fragmented, were observed inside glial cell bodies during the first three weeks (Fig. 21). Profiles of thin dendrites with a dark cytoplasm were also observed at this time (Fig. 22). Similar figures have been observed at retrograde neuronal degeneration in kittens (Grant and Westman 1969), but whether they represent a sign of degeneration in the present material could not be shown. The profiles were often surrounded by stout astrocytic-like processes.

Profiles, similar to degenerated myelinated axons, were rarely noted. However, as reported in the following paper (Conradi and Skoglund 1969 b) projections from the myelin surrounding the motoraxon, or circular myelin bodies with a fragmented interior located outside the motoraxons, were often encountered. At low magnification, they appeared rather similar to degenerated myelinated axons following axon transection in the adult CNS, as described earlier (see Conradi 1969 d).

Special attention was paid to the relationships of the glial cells, which covered large areas of the motoneuron cell bodies and the most proximal parts of the motoneuron dendrites from the first week. Since, in the newborn, the boutons seemed to be rather evenly distributed on the motoneuron cell bodies, the large areas of apposition of astrocytes and oligodendrocytes shortly after birth might indicate that a disappearance of boutons had taken place. Sometimes, profiles of boutons on the motor cell bodies were situated between the motoneuron cell membrane and the cell body of an astrocyte, thus obviously interrupting a long area of apposition of the glial cell to the motoneuron cell body (Figs. 23 and 24). For the most, the interior of these boutons seemed quite normal. At several places, glial processes intervened

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partly between the motoneuron cell membrane and apposing boutons of an apparently normal appearance (Fig. 25). No boutons were, however, observed to be completely surrounded by glial cytoplasm, as revealed by investigation of series of sections. Some apparently fragmented material was sometimes seen in the glial cytoplasm just outside the motoneuron cell bodies (Fig. 26). Nevertheless, no boutons filled with a contrast-rich material (see Wahlberg 1964, Conradi 1969 d) or neurofilaments (Gray and Hamlyn 1962, Conradi 1969 d) were observed.

DISCUSSION

Glutar aldehyde, dissolved in a 300 mOsm buffer and perfused through the vascular system at a discontinuous pressure, was found to give an acceptable and reproducible fixation of the spinal cord in both the kittens and adult cats. The same is true for the spinal roots (Berthold 1968). The adult animals were more sensitive to the perfusion method than were the newborn kittens, in which it was possible to obtain a good fixation even after perfusions at a high and constant pressure. As pointed out earlier (Conradi 1969 a), the difficulty in obtaining a good perfusion of the spinal cord in the adult cat might be connected with the complicated vascular supply of the cord, and there might well be developmental changes in this respect.

The volume changes in the tissue during the fixation and preparation procedure for ultrastructural work are not known exactly (see Conradi 1969 a). Furthermore, it was not known whether these changes are similar in the kittens and in the adult cats, even if the same fixation method is used. Due to the diminishing water content in the CNS during the development (Friis-Hansen 1957), it might well be that the reaction of the tissue to the fixative varies with age. This has to be borne in mind, especially when comparing the dimensions of various profiles in the different developmental stages (see also discussion by Mellström and Skoglund 1969).

During the earlier stages of development, the motoneurons were rather small and tightly packed. From the lightmicroscopic studies of Mellström and Skoglund (1969), there is evidence that the size-range of all the neurons in Rexed's lamina IX in the newborn is rather small, a differential rate of growth giving rise to neurons of different sizes in the adult. Thus, there are some difficulties in distinguishing the motoneurons from other neurons at younger stages, at least with the lightmicroscope. In the present work, the biggest neurons in the newborn stage were considered to be motoneurons.

They showed similar cytological characteristics, and C boutons were regularly observed on these cells, but did not seem to be present on the smaller neurons, like in the adult (cf. Conradi 1969 a). The dense packing of the cells in the newborn stage also made the recognition of the various motoneuron nuclei somewhat difficult; this could have interfered with the sampling for the quantitative studies.

Membrane relationships of the motoneurons

General appearance

Although there is no earlier report on the membrane relationships of the motoneurons in the cat during the postnatal development, some general features of developing central nervous tissue have been described in several ultrastructural reports during the last few years.

The ultrastructural development, also including earlier maturational stages than investigated here, has namely been studied on neurons (see for ex. Schwartz *et al.* 1968, Voeller *et al.* 1963, Wechsler 1966, Mugnaini and Forströnen 1967, Caley and Maxwell 1968, Del Cerro and Snider 1968), glial cells (see for ex. Vaughn 1969, Mugnaini and Forströnen 1967, Fleischhauer 1968, Caley and Maxwell 1968, Vaughn and Peters 1967), boutons (see for ex. Konishi 1966, Larramendi 1967, Wechsler 1966, Larramendi *et al.* 1967, Mugnaini and Forströnen 1967, Kornguth *et al.* 1968, Glees and Sheppard 1964, Meller *et al.* 1968) and axons (Fleischhauer and Wartenberg 1967, Bunge 1968) in various regions of the CNS (for references, see Wechsler and Meller 1967). The results obtained in these works have been used for the interpretation of the present findings.

Qualitatively, the developmental changes in the relationships of the motoneurons seemed rather slight. Some of these changes also appeared difficult to correlate to the available physiological data. However, the disappearance of glycogen-like particles and the increase in the number of mitochondria, especially in the boutons, during the first postnatal weeks, could point to important biochemical changes in the tissue. Earlier histochemical work has shown that there is a large increase in the amount of oxidative enzymes on and around the motoneurons during the postnatal development (Skoglund 1967, see also Labedsky and Lierse 1968). The finding that glial cells seemed to be apposed to the motoneurons after birth could imply not only changes in the architecture of the extensions on the motoneuron surface, but also chemical changes in the environment of the motoneurons. The great number of desmosome-like junctions between adjacent motoneurons, during the first

postnatal days, and the occurrence of the "double synapses", during the first weeks, could be of importance for the functional interrelationships of the motoneurons. The apparently continuous growth of the boutons and the synaptic complexes, the increase in the number of synaptic vesicles, as well as the myelination of small axons in the neuropile from the second postnatal month on, ought to be of importance for the synaptic activation of the motoneurons.

tative study

here were thus some general features in the ultrastructural develop-
outons, the size and distribution of boutons of different types
rons during development were thought to be of importance in
ological correlates to the physiological maturation. The
gnized in the adult, except for the M-type, were already
pre motoneurons at birth, and the location of the boutons of
as similar in the newborn and the adult. Due to this, the
dist e boutons had to be analyzed quantitatively. Again, it has
to be po at that the quantitative study, performed in this paper, consists
of a limite aterial.

Detailed studies on the development of connexions of the motoneurons require extensive serial sectioning. On the other hand, some of the findings seemed constant throughout the study. The following main findings from the quantitative study will be discussed briefly.

a) Growth of the motoneurons.
b) Growth of the boutons. In the newborn, the bouton profiles were much smaller than in the adult, and the differences between the types were rather small.
c) Postnatal changes in the percentage of total bouton covering of the motoneurons. The covering on the dendrites increased from the newborn to the adult stage, whereas on the cell bodies, the covering remained constant or decreased. The number of bouton profiles on the cell bodies as seen in single sections, was reduced during development. The reduction seemed to be most prominent after three weeks.
d) Distribution of boutons of different types on the motoneurons. The percentage membrane covering of the S and T boutons decreased, most prominently on the cell bodies, from birth. The covering of F and C boutons increased, this was particularly true of the F boutons on the dendrites.

Growth of the motoneurons

There is rather good agreement between the present results and the earlier quantitative light microscopic study (Mellström and Skoglund 1969) in regards to the dimensions of the motoneurons at birth and in the adult. Probably light microscopic studies are the most appropriate for investigations on the growth of the motoneuron cell bodies and dendrites. In the present material, the postnatal increase in diameter was larger for the dendritic profiles than for the cell bodies. If it is assumed that the proportion between the dimension of a profile at one stage of development, compared to the adult, reflects the maturity of the profile (cf. Hines and Emerson 1951) then the finding could indicate that the cell bodies are more mature than are the distant dendritic branches at the earlier developmental stages, a fact which could be of physiologic importance.

With the methods used in the present study, it is impossible to reveal the detailed mode of growth, not only of whole neurons, but also of the different parts. When, for example, only the cell bodies are concerned, it is of interest to know whether there are any movements in the cell membrane and possibly also in the apposing structures, relative to the interior of the cells, which must be of importance for studies on the membrane relationships. The possibilities which exist are: that newly-formed membrane is pushed away from the cell bodies towards the dendrites, and, that the cell body partially incorporates the proximal parts of the dendrites during development. At three weeks, the difficulty in sometimes defining the border between the cell body and the dendrites could be a reflexion of the latter.

The boutons

In the following discussions regarding the boutons it was assumed that the bouton types are retained during development. This means that the boutons with, for example, flattened synaptic vesicles in the newborn stage were believed to contain such vesicles throughout the development. As shown in the adult material (Conradi 1969 a, b) each bouton type showed several characteristics, apart from the shape of the vesicles; hence the assumption seems reasonable.

Growth. In the newborn, most bouton profiles were of similar size. Both the difference in size between the types and the size-range in every type were less in the newborn than in the adult. This can also be seen in the reconstruc-

Reduction in the number of bouton profiles on the cell bodies

The growth of the boutons appeared roughly similar on the cell bodies and on the proximal parts of the dendrites. This, plus the fact that the bouton covering on the cell bodies did not increase, in contrast to that on the dendrites, together with the evidence that the length of the bouton profiles increased faster than the cell body outline, could indicate that there is a reduction in the number of boutons on the motoneuron cell bodies during development. The reduction ought then to involve the S+T boutons, since the covering of these boutons on the cell bodies decreased noticeably. The F boutons might, however, also be affected, since the covering of these did not show the big increase found on proximal parts of the dendrites and the numerical proportion of S+T and F boutons on the cell bodies remained unchanged during development. From developmental studies on the cerebellum, it has been reported that there are contacts between certain boutons and neuron cell bodies at early developmental stages, and that these contacts cannot be found in the adult (Mugnaini and Forströnen 1967, Kornguth *et al.* 1968). The apparent decreasing proportion of S+T boutons on proximal parts of the dendrites in the present material can also be the result of a reduction in the number of these boutons. From the values for the absolute number of boutons on the cell bodies in the single sections from the newborn, at three weeks and in the adult, it might be concluded that such a loss could occur both before and after 3 weeks.

Judging from observations on single transverse sections, the possibilities for the causes of a reduction in the number of boutons on the cell bodies are several: First, the sampling method underlying the comparison between the quantitative studies might have been incorrect, so that different populations of neurons were investigated at different stages. The vertical increase in the diameter of the motoneuron cell bodies might be larger than the horizontal one, implying that a calculated increase in surface, as obtained from measurements of the diameter in transverse sections, is not correct. Thus, the boutons will get more scattered on the surface during development, and the number of bouton profiles found in the single sections will be reduced. In earlier works (Lhermitte and Kraus 1925, Weil 1927, Scheibel and Scheibel 1966) it has been pointed out that the longest diameter of the motoneurons of adult mammals is in the cranio-caudal direction, and that this tendency increases during phylogeny. The earlier-mentioned possible movements of the motoneuron cell membrane during development may also be of importance.

Another possibility is that the "double synapses", during development retain the contact with the thin dendrites and leave the cell bodies. Boutons

might, as suggested, be removed in some way by the astrocyte-like glial cells, which are found to be apposed at big areas of the motoneuron cell bodies from the first or second week. In the adult nervous system, boutons have been reported to be removed by means of glial cells which surround the cell bodies of regenerating motoneurons (Blinzinger and Kreutzberg 1968). In this context, it must also be pointed out that during the first postnatal fortnight, there is removal of all the boutons of different types on the initial motoraxon segment found in the newborn. This seems to occur parallel with an increase in the extent of apposing astrocytes (Conradi and Skoglund 1969 b). Finally, it cannot be excluded that a disappearance of boutons occurs during the normal ageing process. The age of the cats used in the adult material was not known.

In summary, it was concluded that studies on the developmental changes in the connexions of the motoneurons require detailed knowledge of the mode of growth of the motoneuron cell bodies and dendrites. The growth of the boutons differed between the types, the S+T boutons seemed to grow less than other types of boutons. This could explain, at least partly, the decrease in covering of these boutons on, for example, the proximal parts of the dendrites during development. On the cell bodies, there is probably a removal of boutons starting at the newborn stage. This removal, which could comprise both S+T and F boutons might be accomplished by the uncoupling of "double synapses" or by means of the apposing astrocytes.

Physiologic considerations on the size and distribution of boutons

The growth alone of the motoneurons must have physiologic implications. The fact that the dendrites are much thinner in the newborn than in the adult indicates that longitudinal resistance in them is higher in the newborn stage. Thus, the boutons on the cell bodies might have a comparatively greater influence on the impulse-activity of the motoneurons in the newborn than in the adult. Furthermore, the bouton covering on the motoneuron cell bodies is relatively higher in the newborn than in the adult. Due to this, attention is drawn to the boutons on the cell bodies.

As mentioned, there is evidence for a relatively greater bouton covering of S+T boutons on the cell bodies in the newborn than in the adult stage. If these boutons are excitatory (cf. Uchizono 1965), the situation might well correspond to the many observations of an increased excitability of the motoneurons in the newborn stage (see Skoglund 1966). Since there is some evidence (Uchizono 1965, see Conradi 1969 a) that F boutons are inhibitory, the relative increase in the F bouton covering and a decrease of the S bouton

covering at some places confirms the increase of inhibition during postnatal development.

The location of the motoneurons studied in this paper corresponds to the tibial and peroneal nuclei (Romanes 1951), which are physiological flexors. There is a dominance of the flexors against the extensors in the newborn stage (Skoglund 1966) and, as shown by Ekholm (1967), the concealed flexor reflexes are actually dominant in this stage. It might be argued that the morphologic findings presented here are only specific for flexor motoneurons. However, the extensor motoneurons exhibit the same characteristics with regard to excitation and inhibition when tested monosynaptically; they must, therefore, have much in common with the flexor motoneurons, at least with regard to the boutons of dorsal root origin. Further studies (in preparation) might reveal whether there are fundamental differences between flexor and extensor motoneurons with regard to the bouton population during development.

Naturally, there are many other morphologic parameters that can be discussed in connexion with the changing physiology, but rather than venture beyond the realm of facts, the results of the experiments now in progress, using the intracellular recording technique, plus further morphologic studies, will be awaited.

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SUMMARY

The postnatal development of the membrane relationships of the spinal motoneurons was studied on 49 normal kittens from the newborn stage to two months of age. At birth, the motoneurons lie close together and the surrounding neuropile contains glial cells and numerous small profiles of dendrites, unmyelinated axons and boutons. At this stage, the motoneuron cell membrane is covered by glial processes and many small boutons, which sometimes contain glycogen-like particles. The different bouton types found in the adult (Conradi 1969 a, b) are already present in the newborn stage,

except for one type (M-type). During the first postnatal weeks, glial cells seem to be apposed at the motoneuron cell bodies. Myelination of the axons in the neuropile is evident to a marked degree during the second postnatal month.

The size and distribution of boutons of different types on the motoneuron cell bodies and dendrites was studied on single transverse sections through the lateral motoneurons in the most cranial millimetre of segment L 7 in the newborn stage and at three weeks. The results were compared to those obtained earlier in the adult (Conradi 1969 a, b). In the newborn, the percentage bouton covering of the proximal parts of the motoneuron dendrites was less than in the adult, whereas on the cell bodies, the values were equal or possibly higher in the newborn stage. As in the adult, the boutons with flattened synaptic vesicles (F-type) and boutons with a subsynaptic cistern (C-type) were more frequently found on the motoneuron cell bodies than on the dendrites in the newborn. The reverse was true for most of the boutons with spherical synaptic vesicles (S- and T-types), as in the adult. During development, there was a reduction in the percentage membrane covering of the S and T boutons, especially on the cell bodies.

The membrane relationships of the proximal part of a motoneuron dendrite in the newborn stage were analyzed through serial sections. The boutons were distributed roughly as in the adult, but there was a three to four times greater number of boutons inside 100 μ^2 dendritic surface than in the adult.

The findings are briefly discussed against the background of available physiologic data.

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Fig 23 Long zone of apposition between an astrocytic like glial cell (AS) and a motoneuron cell body (M) Two boutons (B) are situated between the glial cell and the motoneuron. 3 weeks old kitten. ×8,800

Fig 24 Similar as in Fig. 23 the same indications. The glial cell contains dense bodies, many vacuoles and also abundant fibrils (arrow). 3 weeks old kitten ×10,300

Fig 25 Bouton on a motoneuron cell body (M) at three weeks. A glial process (G) with a clear cytoplasm intervenes between part of the bouton and the motoneuron cell membrane ×35,500

Fig 26 A vacuolized inclusion (arrow) inside a glial process (G) just adjacent to a motoneuron cell body (M). One week old kitten. ×24,200




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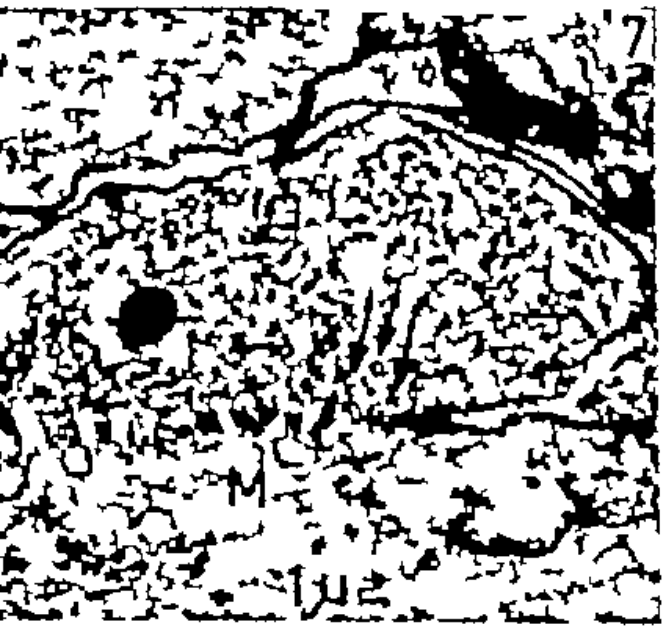


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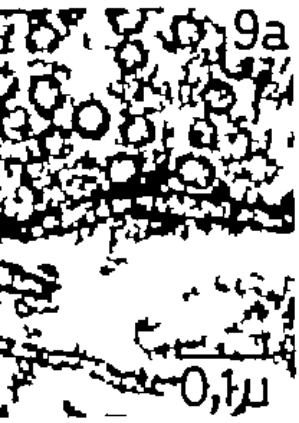


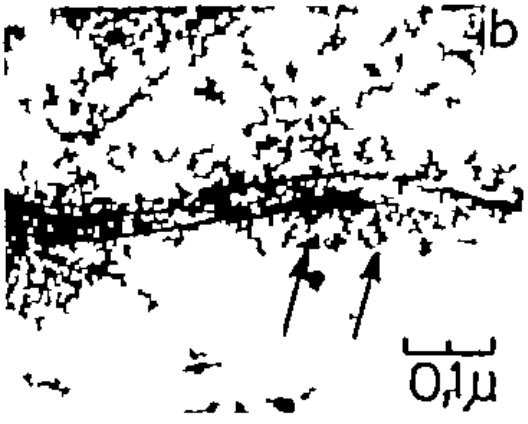
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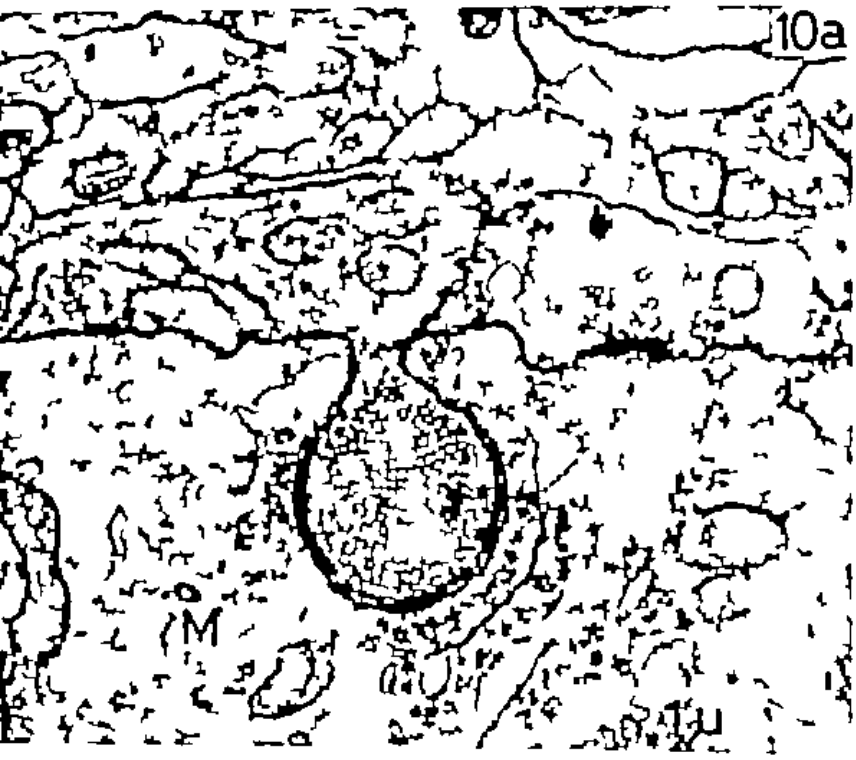

7
M


8
M

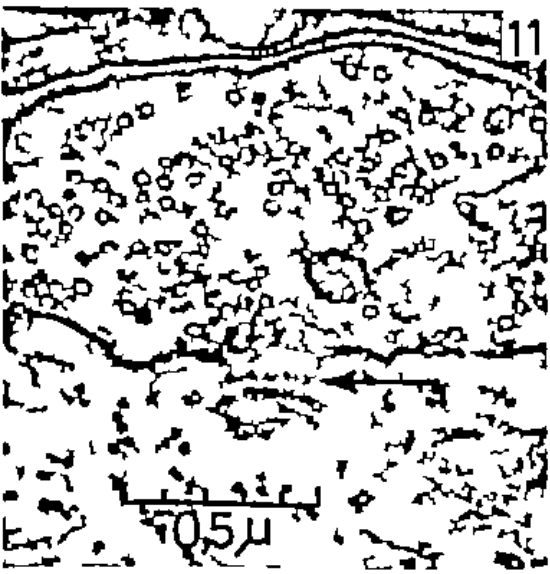

9a
0,1μ

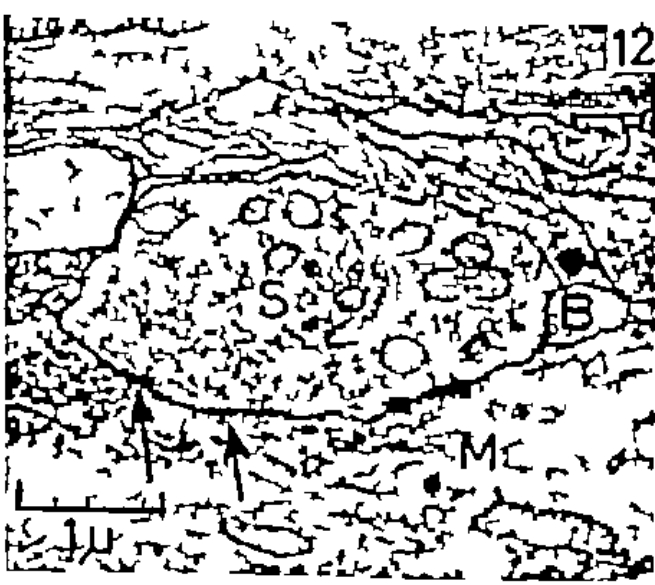

b
0,1μ


c
0,1μ

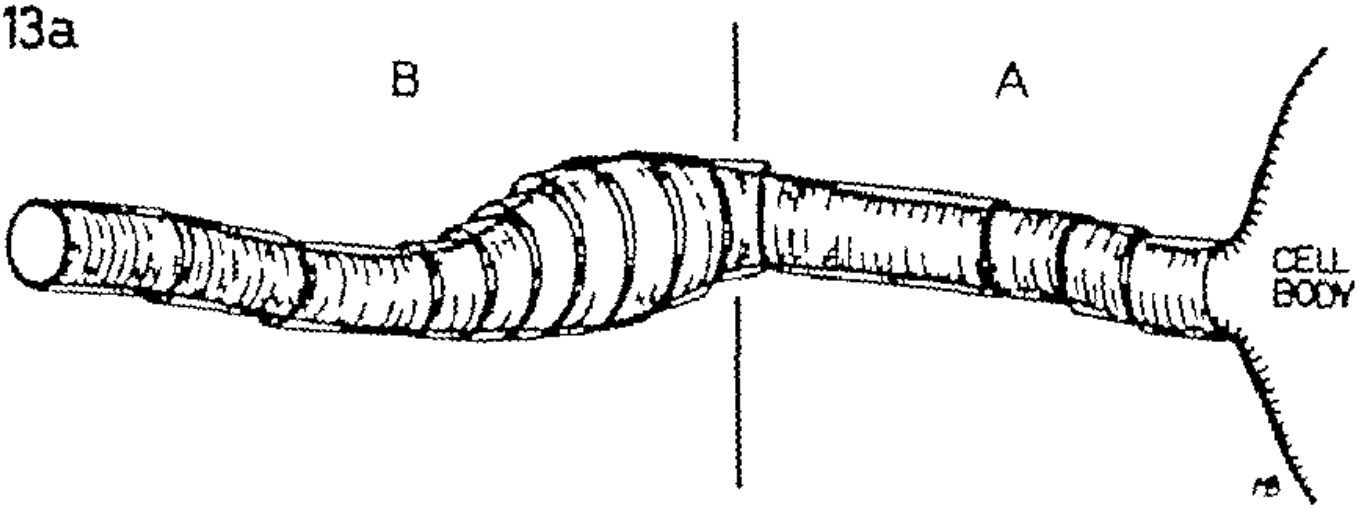

10a
M
1μ


b
0,1μ

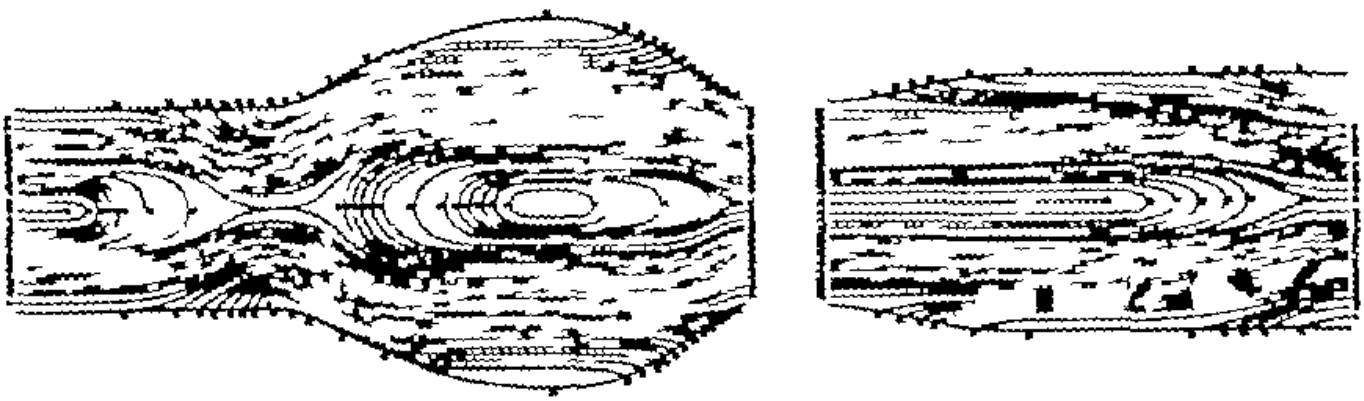

11
0,5μ


12
S
B
M
1μ

13a



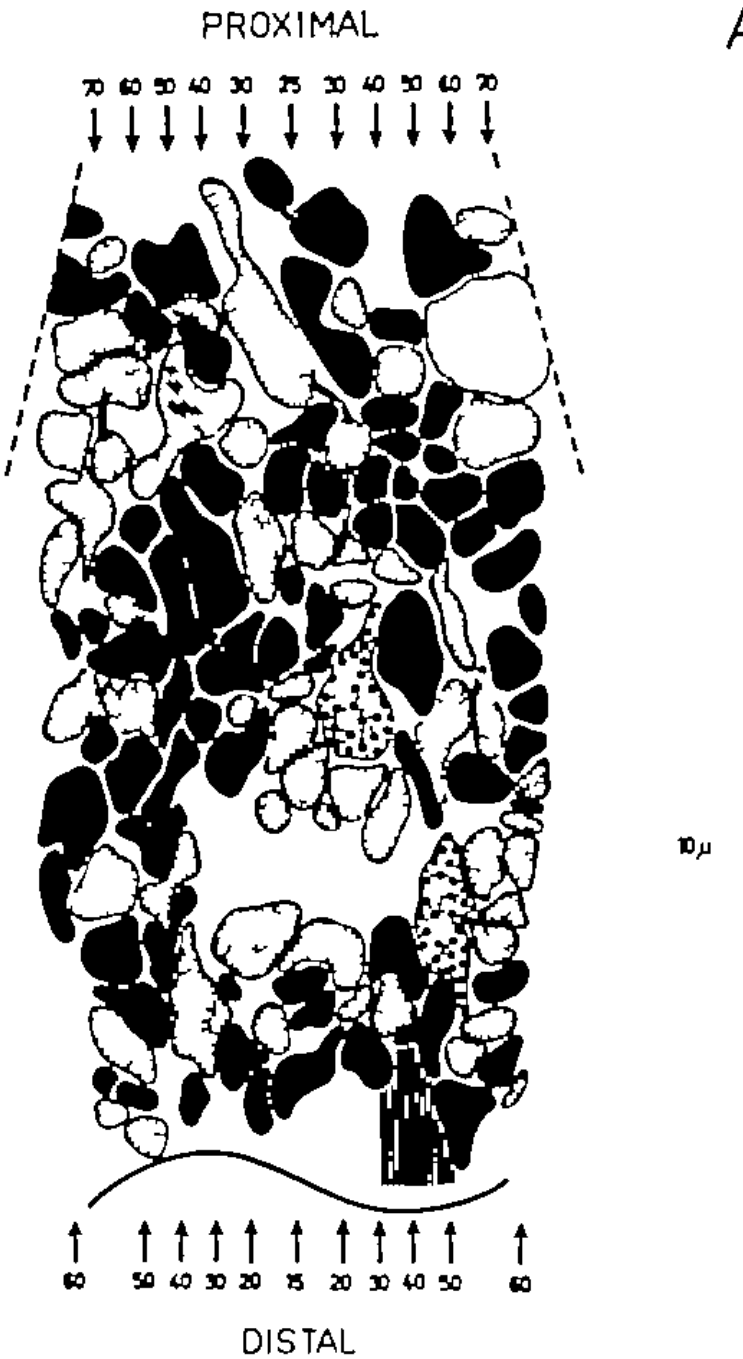
b

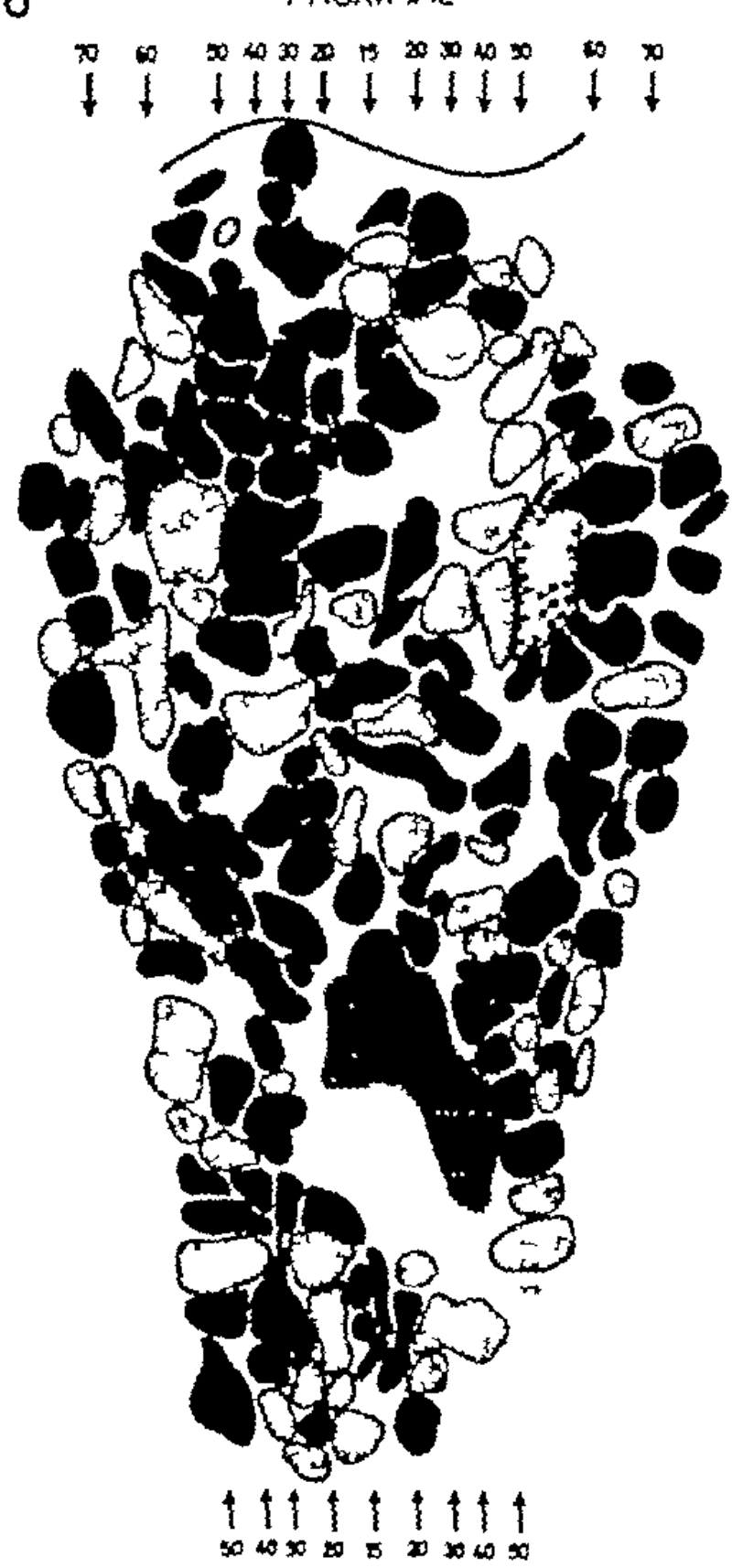


14



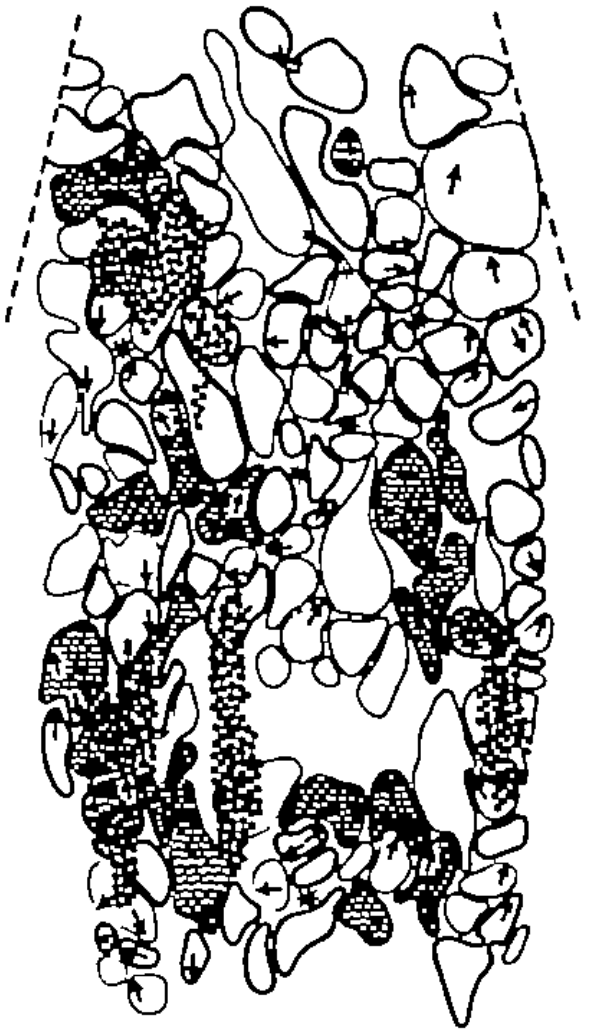
15


PROXIMAL
A
70 60 50 40 30 25 30 40 50 60 70
10μ
60 50 40 30 20 15 20 30 40 50 60
DISTAL
BOUTONS
F (and P)
S
= S with AXO-AXONIC CONTACT
C
DENDRITE ASTROCYTIC LAMELLAE INTERPOSED
MYELIN ASTROCYTIC LAMELLAE INTERPOSED


PROXIMAL
B
70 60 50 40 30 20 15 20 30 40 50 60 70
50 40 30 20 15 20 30 40 50
DISTAL

PROXIMAL

A



DISTAL

BOUTONS

S C

= F (and P)

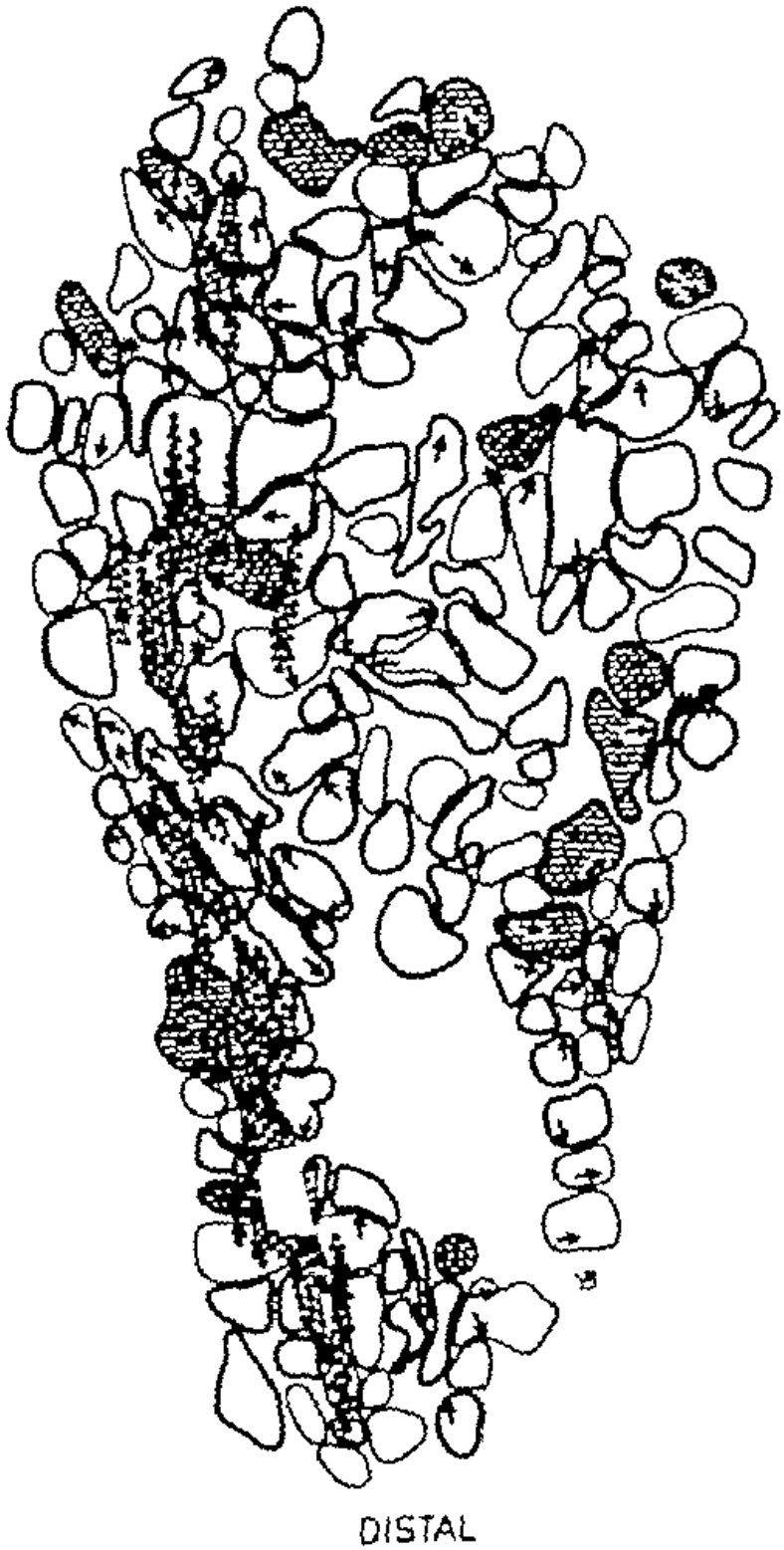
= "DOUBLE SYNAPSE

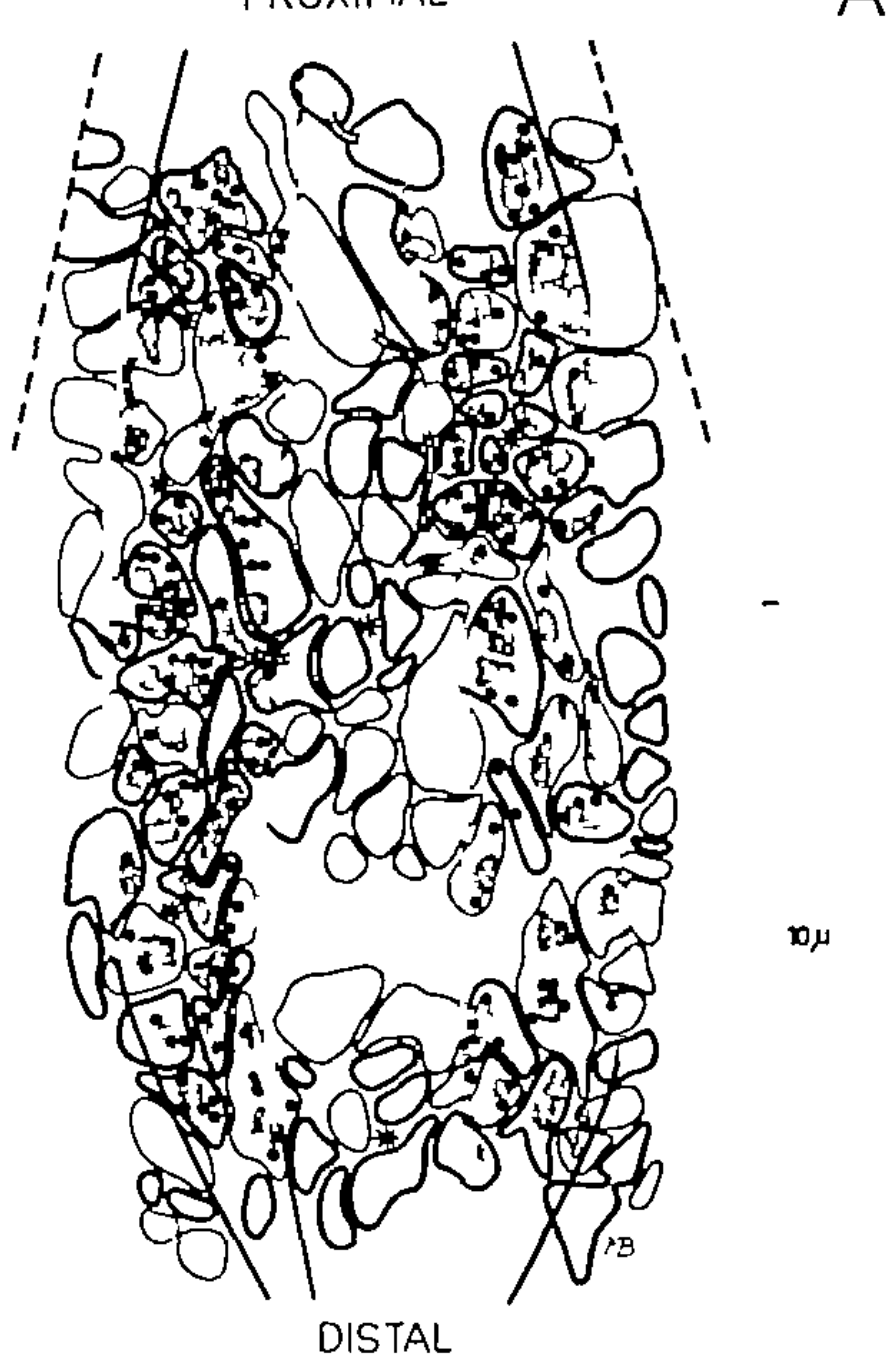
→ = PRETERMINAL AXON UNMYELINATED

= SUBMEMBRANEOUS, GRANULAR ENDOPLASMIC RETICULUM

✱ = DENDRITIC SPINE

18



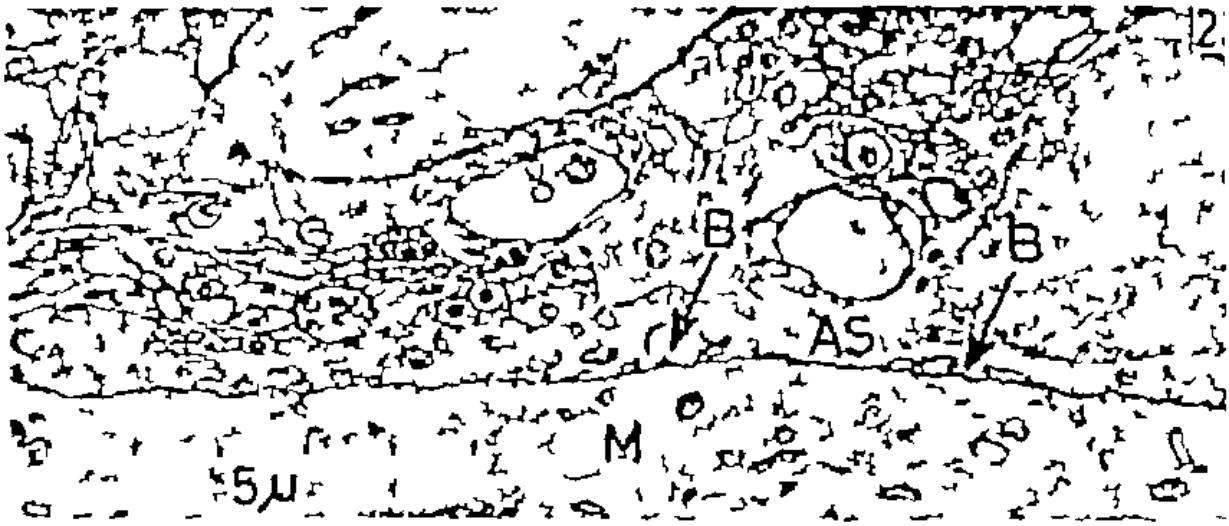

PROXIMAL
A
10μ
DISTAL
= S C
= F (and P)
= SYNAPTIC COMPLEX
= DESMOSOME LIKE JUNCTION
= SUBSYNAPTIC CISTERNA
= DENDRITIC SPINE

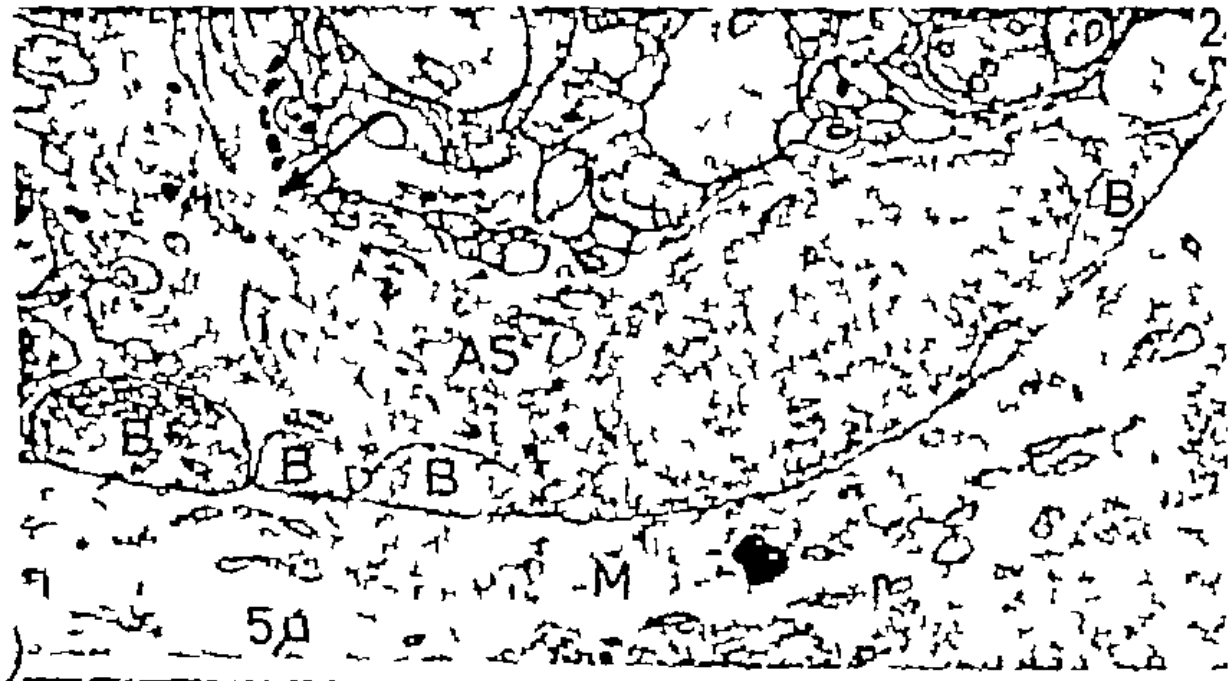
x

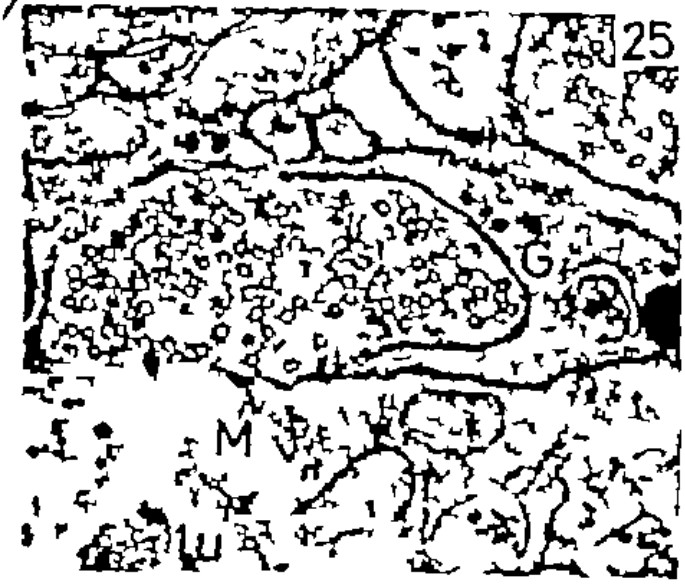

20


21
M
3 µ


22
B
G
G
B
0,5 µ


B
B
AS
M
5μ


AS
B
B
B
B
M
5μ


25
G
M
μ


G
M
μ

Observations on the ultrastructure of the initial motoraxon segment and dorsal root boutons on the motoneurons in the lumbosacral spinal cord of the cat during postnatal development

by

SEBASTIAN CONRADI and STEN SKOGLUND

INTRODUCTION

The present paper describes the ultrastructure of the initial motoraxon segment and the dorsal root boutons on the motoneurons in the kitten. First, the initial axon segment and axon hillock in the newborn stage are described. Some observations on central motoraxon nodes are noted and the findings are compared with those in the adult (Conradi 1969 c). Notes on the development subsequent to the newborn stage are included and finally the ultrastructure of the dorsal root boutons, both in the normal state and following dorsal root section, is elaborated upon.

The initial motoraxon in the newborn is shown to be covered by boutons, which disappear during the first postnatal fortnight. The dorsal root boutons, defined by the same criteria as used in the adult (Conradi 1969 d) are not present until about the third postnatal week. Before this time a few boutons, obviously showing axo-axonic contacts, can be observed and some evidence is presented to show that such boutons degenerate after dorsal root section. The findings are briefly discussed in the light of available physiological data.

MATERIAL AND METHODS

The material of normal kittens, described in the preceding paper (Conradi and Skoglund 1969 a), was used for the study of the initial axon segment and the normal anatomy of dorsal root boutons. Profiles from about 20 initial axon segments, completely or partly cut through, were studied, the majority from the neonatal period.

For the study of the ventral horn after dorsal root section, an additional 28 kittens were used (Table 1). The operation method was roughly the same as the one used for adult cats (Conradi 1969 d), and will be briefly described.

The kittens were anaesthetized intraperitoneally with pentobarbital, 20—40 mg/kg body-weight. Under aseptic precautions and following a wide laminectomy the dura was opened with a mid-line incision. The left dorsal roots L 6, L 7 and S 1 were cut proximal to the ganglion and as distal from the cord as possible. After the operation the spinal muscles and skin were sutured and usually Polymyxin® (Pfizer) unguent was left in the wound. The whole operation lasted for 20—40 min. Postoperatively the kittens were nursed artificially with milk, diluted with water. Treatment with antibiotics was omitted during the postoperative period. Usually the youngest kittens seemed to decrease in weight during the postoperative period and in several cases ascites was noted.

The operated animals were subjected to the same fixation as the majority of the animals in the normal material (Conradi and Skoglund 1969 a). Thus they were perfused through the thoracic aorta with prerinse and fixative at 37° C, and icecold fixative from 1 fusion sets at a discontinuous pressure. The fixative was 5% purified glutaraldehyde, dissolved in 300 mOsm Millonig buffer containing 2.7% Rheomacrodex® (Pharmacia).

Thin, transverse slices of the 7th lumbar segment were made, postfixed in 2% OsO_4 in veronal acetate buffer or 300 OmOsm Millonig buffer and embedded in Vestopal W. In some of the operated animals a slight herniation of the spinal cord occurred. Both in the operated and the normal material, the lateral part of the ventral horn was trimmed out and cut into ultrathin transverse sections, which were collected on one-hole grids. Single sections or short series, comprising 10—50 consecutive ultrathin sections, were studied.

Most of the initial segments studied were continuous with the big neurons in the ventral horn, which were those assumed to be motoneurons (Conradi and Skoglund 1969 a). Some observations were also made on isolated profiles, showing identical

Table 1 Survival time of the kittens

Age at sacrifice	Survival time, days	1	2	3	4	5	6	>6
0—7		1	1	2	3	3		
8—14				2	2	1		2
15—29				2		1		
30—60				1	1	1		
60—					3			

dimensions and characteristics. The description of the myelinated part of the motor axon is based on profiles continuous with the initial segments or isolated profiles in the gray substance, which had the same characteristics and are assumed to be motoraxons (cf. Windle 1930).

The initial motoraxon segment was also studied in longer series of transverse sections through the ventral horn in L 7 from normal newborn animals. The series comprised 50—300 sections, a few random sections were lost. Two initial segments from one of these series were also reconstructed with wax-plates using approximately the same method as was used in the adult animals (Conradi 1969 c). In every second section, the profiles of the initial segments were photographed at a primary magnification of 3 700×. The mean section-thickness in this series was about 670 Å, as calculated from the number of sections required to pass through the longitudinally-sectioned initial segment, which was assumed t be cylindrical. From paper copies with a final magnification of 7,500× the outline of the initial segment and apposing boutons was transferred to 1 mm wax-plates. These were glued together and painted.

One bouton in the newborn stage, which seemed to exhibit an axo-axonic synaptic complex, was also reconstructed with wax-plates. Each section was photographed at a primary magnification of 6 400 and enlarged 3×. The mean section-thickness was here about 600 Å. The bouton was situated on the proximal part of a motoneuron dendrite, analyzed through serial sections (Conradi and Skoglund 1969 a).

RESULTS

The axon hillock and initial axon segment

NEWBORN ANIMALS

Although profiles of many motoneurons are visible in large single transverse sections of the ventral horn in the newborn kitten, it is difficult to recognize the axon hillock and initial axon segment of the motoneurons. The initial axon segment is very thin and does not take a straight course, this explains why complete profiles of longitudinally cut initial segments are almost never observed.

Axon hillock

(Fig. 1) As was the arrangement in the adult, most motoraxons seemed to leave the motoneuron in a horizontal plane. The zone of transition between the parent motoneuron and the axon proper called the axon hillock, usually projected from the motoneuron cell bodies, but in a few cases it was also observed to issue from proximal parts of the motoneuron dendrites (cf.

Strong 1906) In contrast to the situation in the adult the diameter of the hillock was usually not larger than that of the initial segment, therefore the transition between the axon hillock and the cell body was rather abrupt. The cytoplasm in the axon hillock contained scattered ribosomes, longitudinally arranged neurotubuli and neurofilaments some mitochondria and dense bodies The cell membrane of the axon hillock showed the same relationships as the cell membrane of the motoneuron cell body which means that it is covered by astrocytic extensions and boutons. The S- F and C-types of boutons found on the motoneuron cell bodies (Conradi and Skoglund 1969 a) were also present on the axon hillock.

The initial axon segment

The initial motoraxon segment extends from the distal part of the axon hillock to the beginning of the myelin sheath of the motoraxon (Figs 1 and 2) Because the axon hillock is of the same diameter as the initial axon segment, the transition between these two regions was somewhat difficult to distinguish. The cytoplasm of the initial segment contains longitudinally-arranged neurofilaments and neurotubuli the latter often seem to be paired in longitudinal sections, as in the adult (Figs. 3 and 4) Kohno 1964 Peters *et al* 1968 Palay *et al.* 1968 Conradi 1969 c) Mitochondria and dense bodies were observed in some sections and glycogen-like particles may also be found The cell membrane of the initial axon segment showed roughly the same specialization as in the adult (see Conradi 1969 c) At the inner side of the membrane, a 100—300 A thick layer of contrast-rich material is attached Immediately outside the cell membrane there is a rim of granular extracellular material of rather constant thickness, 100—200 A or possibly somewhat less than in the adult (Fig. 3)

The beginning of the myelin sheath

In all instances, the motoraxons were myelinated distal to the initial segment, although the thickness of the myelin sheath varied somewhat (Figs. 1 and 2) As in the adult, the diameter of the axon increased slightly distal to the beginning of the myelin sheath Inside the myelin, the axoplasm contains abundant, mostly longitudinally oriented neurofilaments and numerous mitochondria. The terminal cytoplasmic pockets of the myelin are relatively much larger than in the adult (Fig. 2) as previously found in the ventral root of the newborn kitten (cf Berthold and Skoglund 1968) and they contain a contrast rich cytoplasm They cover about 3 μ of the axon the same as in the adult.

Every pocket reaches the axon membrane which usually is not specialized and ordinarily there is immediate apposition of the cell membranes. Desmosome-like junctions between the cytoplasmic pockets were not found in a number comparable to that observed in the ventral roots (Berthold and Skoglund 1968).

Structural peculiarities of the initial motoraxon segment in the newborn stage as compared to adult

1) As already pointed out, the initial motoraxon segment in the newborn kitten is most often curved. The direction of the deviation occurred in both the vertical and horizontal planes and did not seem to be the same for the different neurons (Fig. 1). (This can also be seen in Fig. 6). In a few cases, profiles quite similar to the granular endoplasmic reticulum of the motoneuron cell bodies were observed in the distal part of the initial segment.

2) There are rather many spinous projections from the initial segment in the newborn. Most often these are found near the beginning of the myelin sheath, either just proximal to this, or at the region where the terminal cytoplasmic pockets (Conradi 1969 c) of the myelin cover the axon (Fig. 2). In a few cases a slender collateral was also observed to arise from the initial segment, often just proximal to the beginning of the myelin sheath. As a rule, these thin extensions from the initial segment could not be followed for any longer distance (Fig. 1 this can also be seen in Fig. 6).

3) Outside the extracellular material surrounding the axon are many thin glial extensions, obviously of astrocytic origin, just as in the adult (Fig. 3). In contrast to the situation in the adult, however no glial cells with a dark cytoplasm, similar to the oligodendrocytes or microglial cells, are observed adjacent to the initial segment in the newborn. (Figs. 1 and 3). Sometimes the initial segment is surrounded by big astrocytic-like glial cells at birth, sometimes this occurs later (Fig. 4 see below).

4) In contrast to the arrangement in the adult, boutons are located on the initial motoraxon segment in the newborn (Figs. 2, 3, 6 and 7). The boutons are rather sparse, but obviously distributed over the entire length of the naked axon. On single sections boutons are not observed on all profiles of initial segments. At the area of apposition of the boutons, the cell membrane specialization of the axon is absent (Fig. 3). the same has been observed to

Table 2 Dimensions of the axon hillock and initial segment

		Axon Hillock		Initial Segment		Length AII+IS
		Length µ	Diam. base µ	Length µ	Diam. µ	µ
Newborn.						
Model	1	8	4	27	1.8	35
	2	8	4	22	1.5	30
Single section profile no.						
	1	9	4	14	1.6	23
	2	9	5	22	1.8	31
	3	8	4	19	1.3	27
	4	13	4	0	1.7	33
	5	9	3	20	1.5	29
3 weeks						
Single section profile no.						
	1	8	6	21	2.3	29
	2	17	9	31	2.4	48
	3	12	9	19	2.8	31

be true in several regions in the adult (cf Palay *et al* 1968 Peters *et al.* 1968) The boutons have the same ultrastructural characteristics and are of the S- F and C-types, the same as on the motoneuron cell bodies (Conradi and Skoglund 1969 a) (Figs 6 and 7) The boutons were often found at the spinous projections on the initial segment. Sometimes the boutons establish a synaptic complex also with a thin dendrite as did the boutons on the motoneuron cell bodies *In rare instances, the boutons on the initial axon segment* and adjacent boutons on neighbouring dendrites were found to arise from the same preterminal axon. In none of the observed cases were there any axo-axonic contacts on the boutons on the initial axon segment.

Dimensions of the axon hillock and initial motoraxon segment

The dimensions of the axon hillock and initial segment were measured in ten cases. The wax-models (see below) were used in two cases for the rest, the measurements were made on profiles in single sections. Seven of the measurements refer to the newborn stage and three measurements to the three week stage (See Table 2)

Table 3 Number and proportion of boutons of different types on the axon hillock and the initial axon segment in the newborn stage and in the adult (Conradi 1969 c) Two models from each stage.

Bouton type	Newborn animals								Adult animals							
	1				2				1				2			
	AH		IS		AH		IS		AH		IS		AH		IS	
	no.	%	no.	%	no.	%	no.	%	no.	%	no.	%	no.	%	no.	%
S+T	15	36	7	37	8	21	5	33	11	34	—	—	19	40	—	—
F	26	62	8	42	28	74	8	53	21	66	—	—	27	59	—	—
C	1	2	4	21	2	5	2	14	—	—	—	—	—	—	—	—
Sum	42		19	38			15		32				46			

The mean total length of the axon hillock and initial segment in the new born, according to this material, is about 75% of the adult value (Conradi 1969 c) The mean diameter of the initial segment is about half of that found in the adult There seems to be a rather rapid increase in the diameter of the base of the axon hillock after birth

Three-dimensional ultrastructure of the axon hillock and initial motoraxon segment

In order to gain knowledge of the three-dimensional morphology of the axon hillock and initial axon segment, and to get more exact information on the distribution of boutons on these regions, wax-reconstructions were made to illustrate two cases (Figs. 6 and 7) From both figures the following observations were made. the axon hillock and initial segment were of about the same diameter the initial segment was somewhat curved and the motoraxon was myelinated One of the initial segments gave off a thin branch just proximal to the beginning of the myelin (Fig. 6) and boutons of the S- F and C-types were distributed on both the axon hillock and initial axon segment.

The boutons were rather densely packed on the axon hillock, but on the initial axon segment they were scattered In both cases, there seemed to be a random distribution of the different types, except for some possible clustering of F boutons on the axon hillock in both the cases.

The number and proportion of the boutons of the different types on the models are shown in Table 3 The values from the two reconstructions in the adult are included for comparison.

It is understood that the two reconstructed cases from each stage may not be representative however in the newborn stage, boutons containing flattened

synaptic vesicles (F-type) seem to occur more frequently than most of the boutons with spherical synaptic vesicles (S+T type) both on the axon hillock and initial segment. The proportion of the F boutons is similar to or somewhat larger than that on the motoneuron cell bodies (Conradi and Skoglund 1969 a) as in the adult (Conradi 1969 c) The total number of boutons on the axon hillock does not seem to change markedly from newborn to adult. The proportion of F boutons on the axon hillock seems similar between newborn and adult. On the initial segment, several boutons are present in the newborn but could not be observed by the adult stage

The myelinated part of the motoraxon

On the myelinated part of the motoraxons (Figs 5 and 8) the incisures of Schmidt Lantermann are abundant, as in the ventral root in newborn animals (Berthold and Skoglund 1968) The incisures are very prominent, and the glial cytoplasm inside them is much like that in the terminal cytoplasmic pockets (Fig. 8) Usually glial cytoplasm is also found inside of the myelin sheath nearest the axon (Fig. 5) In the newborn this seems to be a typical feature of developing myelin (for references, see Bunge 1968) A layer of contrast-rich glial cytoplasm, obviously of oligodendrocytic origin (Mugnaini and Walberg 1964 Bunge 1968 Vaughn 1969) was also seen just outside the myelin sheath. Often the myelin sheath showed slender evaginations, usually filled with glial cytoplasm, but more frequently rounded myelin bodies not continuous with the myelin sheath (Fig. 5) were found in the oligodendrocytic cytoplasm outside the myelin sheath. The interior of these bodies was often fragmented, giving them an appearance similar to the profiles of degenerating myelin bodies, observed in the Schwann cells in the developing ventral root (Berthold and Skoglund 1968) The profiles could easily be mistaken for profiles of degenerating myelinated axons as earlier described in the adult CNS (for references, see Conradi 1969 d)

The central nodes of Ranvier

At the central motoraxon nodes in the newborn (Fig. 9) the cell membrane of the axon is specialized in a manner similar to that found at the initial segment. There is thus a thin layer of contrast rich material on the cytoplasmic side of the membrane Just outside the nodal axon membrane there is only a narrow rim of extracellular material, in contrast to the situation in the adult (Fig. 9) The length of the node-gap is 0.5—1 μ The big cytoplasmic pockets of the myelin, quite like those found distal to the initial segment, are situated

at both sides of the node (Fig. 9) where they cover about 3 μ of the axon, or about the same distance as in the adult Every cytoplasmic pocket reaches the axon membrane (Fig. 9) as in the ventral root of the newborn kitten (Berthold 1968) The node gap is filled with astrocytic extensions Boutons were sometimes found just outside the nodal axon membrane but none was observed to make synaptic contact with the axon.

In one instance, the profile of a big myelinated axon in the gray substance was observed to send out an apparently short branch from a central node of Ranvier About 5 μ from the parent axon, the branch was somewhat distended, and in the distention were several mitochondria and membrane bound dense bodies (Fig. 10) There was also a desmosome-like junction between the distention and an adjacent dendrite. At the zone of emergence of the branch from the axon there was a well-delimited region containing a granular material (Fig. 10) The granular material also reached a short distance into the branch. Similar regions of granular material could be observed inside some profiles of motoraxons these axons otherwise were completely covered by myelin. At these or other places, the contour of both the axon and the myelin could show evaginations (See Fig. 12)

In the newborn, all the small axons in the neuropile were unmyelinated at birth, which made them difficult to distinguish from other types of extensions, e.g., glial processes and small dendrites Thus, whether there are any boutons and synaptic complexes apposed on the small axons, could not be determined with certainty When unmyelinated axons could be identified, they were never observed to be postsynaptic in a synaptic complex. At three weeks, some of the axons in the neuropile had begun to be myelinated. Rather often, profiles of axons, the length of which was only partly covered by myelin, could then be observed. In such instances, boutons generally did not establish synaptic contact with the naked parts of the axons.

POSTNATAL DEVELOPMENT OF THE INITIAL AXON SEGMENT

Already at birth, or shortly thereafter large areas of the initial axon segment could be surrounded by glial cell bodies and thick glial extensions containing a clear ground substance, in which the granular endoplasmic reticulum and Golgi apparatus were prominent. This is seen in Fig. 4 These cells seemed to be of astrocytic origin (Vaughn and Peters 1967 Vaughn 1969 see also Conradi and Skoglund 1969 a) Glial cells with a dark cytoplasm, similar to oligodendrocytes or microgliacytes in the adult (Vaughn 1969 see also Conradi 1969 c) were not found adjacent to the initial segment until later Even at three weeks, such cells were not regularly observed. From the third postnatal

week boutons were no longer found on the initial axon segment, according to observations on single sections (Fig. 11) It was not possible to obtain any information on the process by which the boutons disappear from the initial segment As pointed out earlier the membrane specialization of the initial segment is absent below the boutons Already in the newborn stage areas of the initial segment can be found which lack the membrane specialization, but which are covered by astrocytic cytoplasm Such areas, which can be seen in Fig. 3 were not observed at three weeks.

As was the case on the motoneuron cell bodies (Conradi and Skoglund 1969 a) some boutons on the initial segment in the neonate period appeared to be surrounded by astrocytic cytoplasm some of which was interposed between part of the bouton and the motoneuron cell membrane However no boutons showing any degenerative changes according to conventional criteria (see Conradi 1969 d) were observed

By three weeks the diameter of the initial axon segment had increased and the diameter of the axon hillock exceeded that of the initial segment (Fig. 11) The course of the initial segment had, however not yet straightened completely The terminal cytoplasmic pockets of the myelin both distal to the initial segment and at the nodes, had grown considerably (Figs 12 and 13) but seemed to cover about the same distance as in the newborn or 3—4 μ Every cytoplasmic pocket reached the axon membrane although the thickness of the myelin sheath had increased considerably as well as the number of cytoplasmic pockets

At three weeks there were still many evaginations of the myelin sheath of the motoraxons and many compact myelin bodies were found just outside the myelin (Fig. 12) as in the newborn and adult stages At the central nodes, the motoraxons still seemed to be covered by only a thin rim of extracellular material and outside this, big astrocytic like extensions were found (Fig. 13) The ultrastructure of the central nodes of the motoneurons during development was not studied in further detail

Dorsal root boutons on the motoneurons

NORMAL MATERIAL

Newborn animals

In a preceding communication (Conradi 1969 d) evidence was presented to show that two specific types of big boutons, both containing spherical synaptic vesicles are of dorsal root origin in the adult. One of the types, de

noted M is characterized by a big size, 4—6 μ and a large synaptic complex, which is irregular in shape and contains a large number of postsynaptic dense bodies. The second type, which was somewhat difficult to distinguish on single sections is of roughly the same appearance as other boutons with spherical synaptic vesicles, but bigger On the convex side of both these types of boutons, small boutons (P-type) containing small irregularly shaped, synaptic vesicles, were always found to be apposed. The P boutons seemed to be presynaptic in a synaptic complex with the bigger boutons.

In the newborn animals no clearcut M boutons could be observed However on the proximal parts of the motoneuron dendrites at this stage, there were profiles of comparatively big boutons, containing spherical synaptic vesicles and having another bouton apposed at the convex side (Figs. 14 and 15) The bigger boutons established one or more synaptic complexes with the motoneuron. The layer of postsynaptic material in these cases was often rather thick, 200—300 A, and irregular in width. In some instances, profiles of small postsynaptic dense bodies could be observed at these boutons (Fig. 15) the same was seen at the boutons, judged to be of the T-type in the newborn (Conradi and Skoglund 1969 a) The boutons apposed at the convex side of these bigger boutons contained synaptic vesicles of a somewhat irregular shape, 250—400 A in diameter (Figs 14 and 15) some of which were apposed at the membrane facing the bigger bouton.

In sections cutting the zone of apposition between the boutons perpendicularly a thin and very small extracellular space could be observed between the membranes at the region of the apposed vesicles. The layers of pre and postsynaptic material were very thin. On the proximal part of the motoneuron dendrite analyzed in the preceding paper there were a few boutons of the types just described Only one apposing bouton was observed in these cases, but it covered a large area of the bouton with spherical synaptic vesicles. The structure similar to a synaptic complex between the boutons seemed to be the only one established by the smaller bouton. A wax reconstruction of one of these cases is shown in Fig. 14 a, together with two of the sections through the bouton.

Postnatal development

From about the third postnatal week, M boutons, quite similar to those in the adult, although somewhat smaller 2—5 μ could be observed on the proximal parts of the motoneuron dendrites (Fig. 16) At that time some big S boutons with axo-axonic contacts also seemed to be present The distribution on the

Table 4 Number of boutons under the heading M and big S in the quantitative studies at different stages.

Age	SO No.	DA No.	DB No.	d No.
Newborn	3	16	5	1
3 weeks	2	19	1	1
Adult	1	16	4	

motoneurons of these two bouton types at the three weeks stage in the adult, seemed quite similar

Whether there are any changes in the number and distribution of these bouton types during the postnatal development is difficult to ascertain In the quantitative estimation on the newborn stage in the preceding paper (Conradi and Skoglund 1969 a) boutons containing spherical synaptic vesicles and showing one large or several smaller profiles of a synaptic complex with postsynaptic dense bodies, were included under the heading, M and big S Under this heading were also included boutons containing spherical synaptic vesicles, with another bouton apposed at the convex side

If the boutons included under the heading M and big S in the newborn are to be regarded as future M and big S boutons with axo-axonic contacts, then the location of these boutons, mainly at proximal parts of the motoneuron dendrites, remains constant throughout development. The number of bouton profiles in this column is rather constant during the development (see Table 4)

It must be pointed out that in the adult, and to some extent also at 3 weeks, the bouton profiles under this heading are considerably bigger than other bouton profiles, thus increasing the chance of these boutons to be included in single sections

OBSERVATIONS AFTER DORSAL ROOT SECTION

In the newborn, as in the adult, the postoperative ultrastructural changes on the operated side were very slight. Since there were almost no myelinated axons in the neuropile during the first postnatal fortnight, neither were there any profiles of degenerated myelinated fibres (cf. Conradi 1969 d) on the operated side This increased the difficulty in distinguishing any signs of neuronal degeneration. As in the adult, just outside the motoneuron cell

membrane, there were rounded, membrane-bound dense bodies inside astrocytic extensions in a few cases and this arrangement was similar to structures earlier described as phagocyted degenerated boutons (Fig. 17) (Colonnier 1964 see Conradi 1969 d) Structures, similar to a postsynaptic membrane specialization were however not observed at the motoneuron cell membrane beneath such profiles; this differed from the situation in the adult. No boutons filled with neurofilaments were observed on the operated side in the newborn

In some newborn animals, boutons with the dense appearance found upon degeneration in various systems in adult animals (see Walberg 1964 Mc Mahan 1967) were found from the 3rd to 5th postoperative day (Figs 18—20) This was not found after dorsal root section in the adult animals.

A rather small number of dense boutons was observed on the operated side and the occurrence of them varied greatly between the sections. A few dense boutons were also observed on the normal side in some of the operated animals this gives grounds for believing that at least some of these boutons on the operated side might be the result of accidental degeneration, due to damage to the cord during the operation. Despite this, the boutons will be described in somewhat further detail since for reasons, given below some of the dense boutons might represent degenerating dorsal root boutons

The interior of the dense boutons was granular and profiles of synaptic vesicles and distended mitochondria could sometimes be observed The boutons were often localized on the proximal parts of the motoneuron dendrites or at medium-sized dendritic profiles in the neuropile, but not on the thinnest dendritic profiles Usually the boutons were surrounded by astrocytic extensions and appeared shrunken, compared to adjacent, normal boutons (Fig. 18) The synaptic complex was often visible and was apparently intact. The layer of postsynaptic dense material was often comparatively thick although no clearcut postsynaptic dense bodies were observed. The thickness and appearance of the postsynaptic dense material were quite similar to that observed at normal boutons with postsynaptic dense bodies, in the newborn (Conradi and Skoglund 1969 a)

In a few instances another bouton, apparently normal, was apposed on the convex side of the dense boutons (Fig. 20) There could be apposition of the synaptic vesicles of the normal bouton facing the region of contact with the dense bouton, thus showing some similarities to a synaptic complex Whether a synaptic cleft existed between the normal and dense bouton was rather difficult to ascertain, since, as pointed out earlier there is often a thin layer of contrast-rich material around dense degenerating boutons, at least in the adult (Holländer *et al.* 1969)

The dense boutons sometimes seemed to be partly invaginated into the postsynaptic profile often at the proximal parts of motoneuron dendrites. Dense membrane-bound profiles quite like the dense boutons were also seen to be completely surrounded by motoneuron cytoplasm judging from observations on single sections (Fig. 19)

There were no clearcut observations of dense boutons on the initial motor axon segment.

After dorsal root section at three weeks, fragments of degenerated myelinated axons were regularly seen. The ultrastructure of these profiles was quite similar to that found in the adult, although the myelin sheath is thinner at three weeks At the stage of development when the M boutons appear about the same pattern of bouton degeneration as observed in the adult (Conradi 1969 d) can be seen, and there are no longer any dense boutons on the operated side

DISCUSSION

As in the preceding paper the largest neurons in the lateral part of the ventral horn were studied. It is likely that these are motoneurons, since the largest neurons in the newborn grow fastest, and the largest neurons in the adult are motoneurons (see Mellström and Skoglund 1969)

During the postnatal development the dimensions of the entire gray substance change (see Mellström and Skoglund 1969) This means that the location of the motoneurons, relative to the zone of emergence of the motoraxons from the gray substance ought also to change If such a change takes place then it might influence the direction of the first part of the motoraxon when it leaves the cell body The observation that the initial motoraxon segment usually is straight in the adult but more often curved in most cases in the newborn, could be a reflexion of these growth changes.

The axon hillock and initial motoraxon segment

The initial motoraxon segment in the newborn exhibits the same membrane specialization as in the adult. From physiologic experiments with the intracellular recording technique on spinal motoneurons in fetal and newborn kittens (Naka 1964) there is evidence that the active impulse of the motoneurons is initiated in the initial axon segment as in the adult animals. The

diameter of the axon hillock in the newborn was of nearly the same size as at the initial axon segment, in the present study In the adult, the axon hillock is much wider at least at the base. The longitudinal resistance of the axon hillock in the newborn would thus be relatively high which could be associated with the difficulty in activating the motoneurons antidromically in the early developmental stages. as noted by Naka (1964)

The boutons on the axon hillock and initial axon segment

The population of boutons of different types on the axon hillock was estimated on only the two models, the same as was done in the adult Therefore, conclusions must be drawn with care At both stages, however the findings were rather similar in the two cases, and since there are no earlier reports on three-dimensional reconstructions of the axon hillock of any types of neurons, at least some remarks might be due Both the total number of boutons and the proportion of different types of boutons on the axon hillock were rather similar between newborn and adult. This might indicate that rather few boutons get apposed at the axon hillock after birth Unfortunately as pointed out in the preceding paper (Conradi and Skoglund 1969 a) the movements of the motoneuron cell membrane during development are not yet known.

Boutons of different types were also consistently found on the initial motoraxon segment in the newborn animals Since there are no boutons at this region in the adult animals—and the same also seemed to be the case at three weeks postnatally—the boutons must somehow leave the initial segment, probably shortly after birth Boutons have also been observed on the initial axon segment of some neurons in the cerebellum during the early developmental stages by Kornguth *et al* (1968) but no information was given in that report on the situation in the adult animals. The findings, as such, that the boutons on the initial segment disappear after birth, lend support to the disappearance of boutons from the motoneuron cell bodies suggested to occur in the preceding paper (Conradi and Skoglund 1969 a) In the absence of direct evidence for the actual mode of disappearance, a further discussion than the one in that paper must pe postponed.

The location of boutons on the initial motoraxon segment ought to have considerable physiologic significance If as suggested, boutons containing flattened synaptic vesicles are inhibitory (Uchizono 1965) and most of the boutons with spherical vesicles are excitatory then the initial axon segment in newborn animals must be subjected to both excitatory and inhibitory influences Assuming that some of the boutons are of dorsal root origin, it

would then be possible to explain the great excitability in the monosynaptic pathway as well as the absence of a subliminal fringe in newborn animals (see Skoglund 1966) However neither any boutons with axo-axonic contacts in the normal material, nor any dense boutons in the operated animals were observed on the initial motoraxon segment in this study Still the possibility exists, that investigations on larger materials than used here might reveal the presence of such boutons and of another yet unidentified bouton type of dorsal root origin. The dorsal root boutons might, namely have another appearance in the newborn stage than in the adult

The motoraxon and the myelin

In the earliest stages, thin and obviously short extensions seemed to project from the motoraxon, both at the initial segment and at the nodes in the lateral part of the gray substance. In later stages and in the adult, no collaterals from the initial segment were observed. From Golgi studies collaterals from the myelinated part of the motoraxons have been reported to leave the latter in the ventral part of the gray substance (see, for example, Scheibel and Scheibel 1966 Szentágothai 1967) It may be that there are transient, short collaterals from the motoraxons during the earlier developmental stages In a Golgi study of the motoraxons of newborn kittens, several collaterals, some apparently short, were noted to leave the motoraxons for the entire course through the gray substance (Prestige 1966)

In a recent study on the postnatal development of the motoraxons in the ventral root of the cat (Berthold and Skoglund 1968) it was concluded that there is a reduction in the number of internodes after birth Bearing in mind that the entire length of the axons is myelinated except at the nodes, this must mean that there is a movement of the myelin along the axon during development. Furthermore there must be a discrepancy between the growth of the myelin and the axon (Berthold and Skoglund 1968) If the latter two mechanisms are also valid for the central part of the motoraxons, there could be an overgrowth of myelin on regions, where collaterals were found during the early developmental stages The bulges of the myelin along with the compact or partly fragmented myelin bodies just outside the myelin could represent morphologic signs of an imbalance between the growth of the motoraxon and the myelin sheath also in the CNS. Contrary to the situation peripherally compact myelin bodies are also found along the motoraxons in the adult (Conradi 1969 c) The myelin bodies observed in this report may be the counterpart of the granules observed light microscopically along the central part of the motoraxon in newborn kittens (Grant 1968)

Normal material

Some evidence was presented, suggesting that axo-axonic contacts were found on some boutons already in the newborn stage The presynaptic boutons showed about the same characteristics as in the adult material but at least in some cases in the newborn animals, the presynaptic bouton was rather big, compared to the postsynaptic bouton. Physiologically there is a strong late inhibition of the monosynaptic reflex in the newborn (see Skoglund 1966) which has been considered to be presynaptic (Eccles *et al* 1963)

Degenerative bouton changes

Due to the small number of them degenerating dorsal root boutons are difficult to identify even in the adult stage (Conradi 1969 d) Two types of boutons disappeared after the operation in the adult. In the newborn kitten, on the other hand, at least the M bouton type could not be clearly identified, thus multiplying the difficulties for investigating the bouton degeneration after dorsal root section. In addition to this difference between newborn and adult, another type of degenerative bouton change seemed to be present, namely the dense type, which occurs commonly in the systems hitherto studied in the adult (see Walberg 1964 Mc Mahan 1966) However a direct conclusion that this type of degeneration is typical of the newborn stage in the system studied is somewhat questionable, since in some cases, similar changes were observed on the unoperated side

Some of the dense boutons found in this study were, in fact, partly invaginated into the postsynaptic extension a process which seemed also to occur in the operated adult animals. Moreover normal boutons apposed at the convex side of dense boutons, could be observed, and this might indicate that at least some of the dense boutons were of dorsal root origin, judging from the observations in the adult animals. Finally the location of the dense boutons might also point to a dorsal root origin agreeing with the findings in the adult.

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SUMMARY

The ultrastructure of the axon hillock and initial axon segment of spinal motoneurons in the cat during postnatal development is studied on the normal kittens used in a preceding paper (Conradi and Skoglund 1969 a). At birth, the initial axon segment is covered by boutons of different types, which, however have disappeared by three weeks, but both the internal structure and the membrane specialization of the initial motoraxon segment are similar to those found in the adult. In newborn kittens, the initial segment is often curved and lacks apposition of dark glial cells. The distribution of boutons of different types on the axon hillock and initial segment in the newborn is studied on two wax models made from serial sections. Some observations on the appearance at birth and the postnatal development of the motoraxons in the gray substance are also included.

The postnatal development of the boutons, described earlier to be of dorsal root origin in the adult (Conradi 1969 d) is investigated also on the material of normal kittens. The changes after dorsal root section are studied on an additional 28 kittens, which had been subjected to dorsal root section 1—8 days before fixation. At birth, some boutons with spherical synaptic vesicles and obviously showing axo-axonic contacts are present on the proximal parts of the motoneuron dendrites. Some evidence is presented to suggest that such boutons show the dense type of degenerative change after ipsilateral dorsal root section. From about the third postnatal week, both the normal ultrastructure of the dorsal root boutons and the changes after dorsal root section are similar to those found in the adult.

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Fig 18 Dense bouton with a somewhat fragmented interior on the proximal part of a motoneuron dendrite (M). One-week old kitten, three days postoperatively The bouton is covered by a big glial process (G) and occupies a much less area on the surface of the motoneuron than do the two adjacent, normal boutons. ×39,800

Fig 19 Profile of a dense bouton, partially invaginated into the cytoplasm of a neuron cell body (M) First postnatal week, four days postoperatively ×35 000

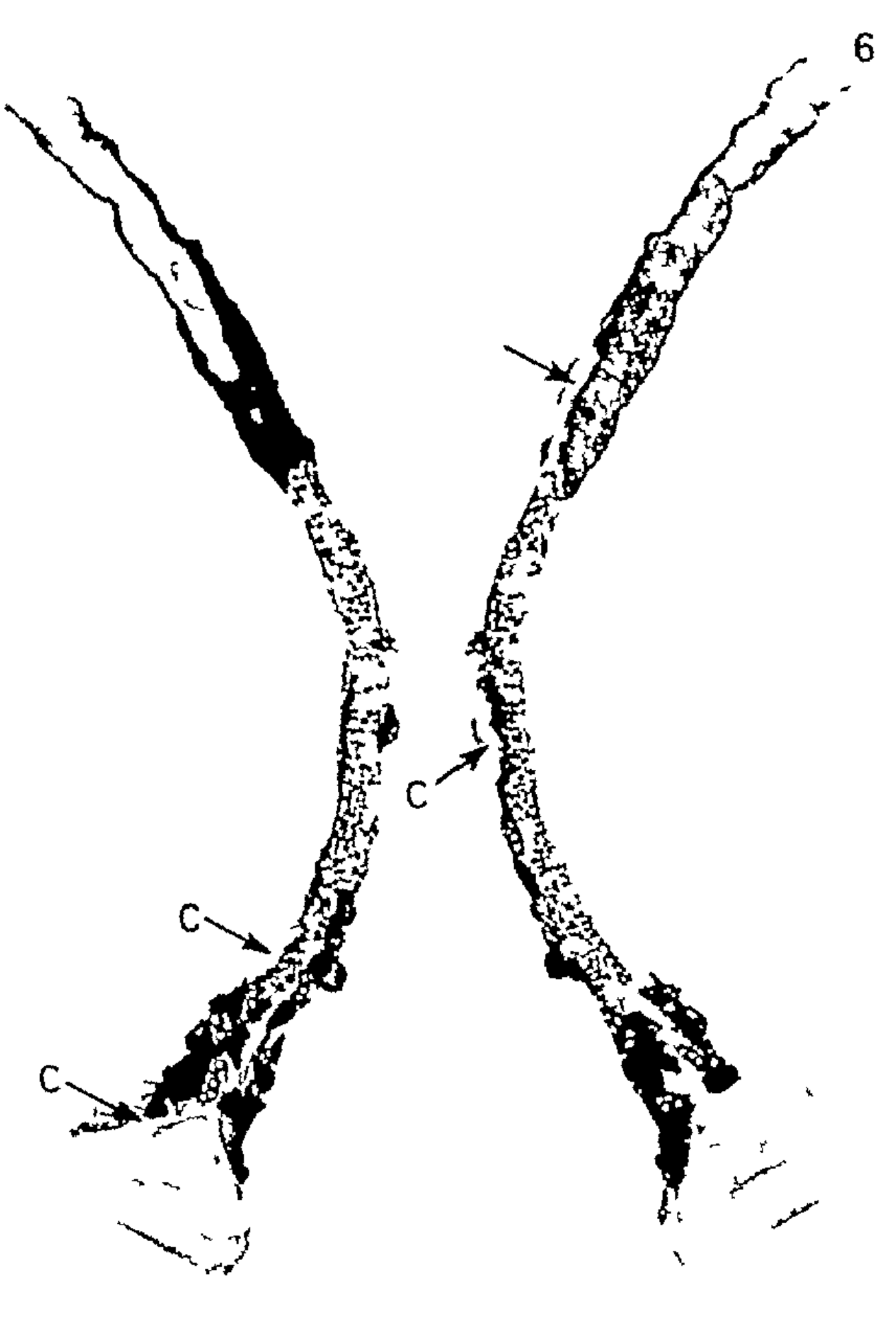
Fig 20 A dense (M) bouton on a big dendritic profile in a kitten, 1 week old, four days postoperatively The synaptic vesicles of an adjacent bouton (B) are apposed towards the membrane facing the dense bouton. ×67,300

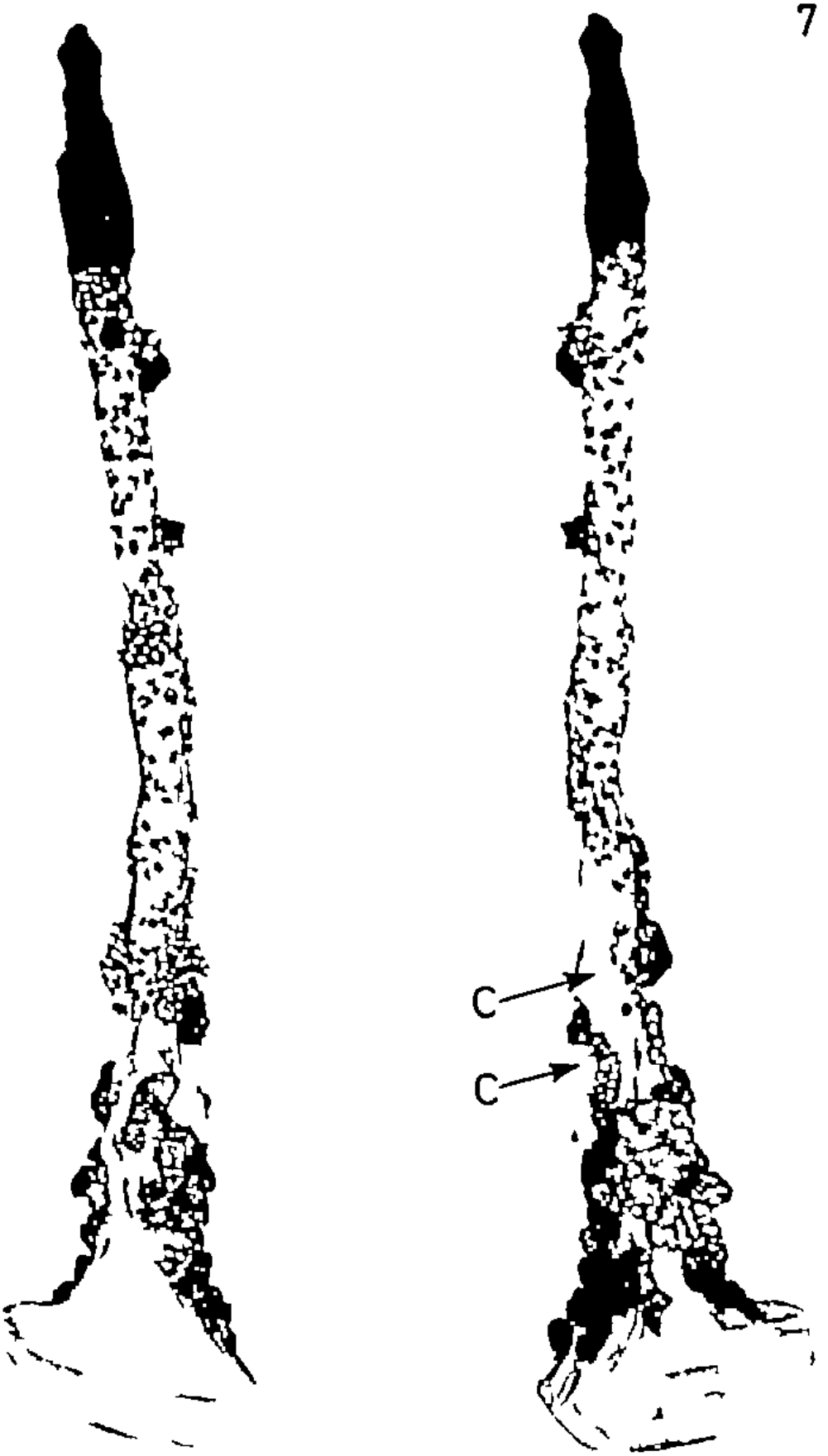

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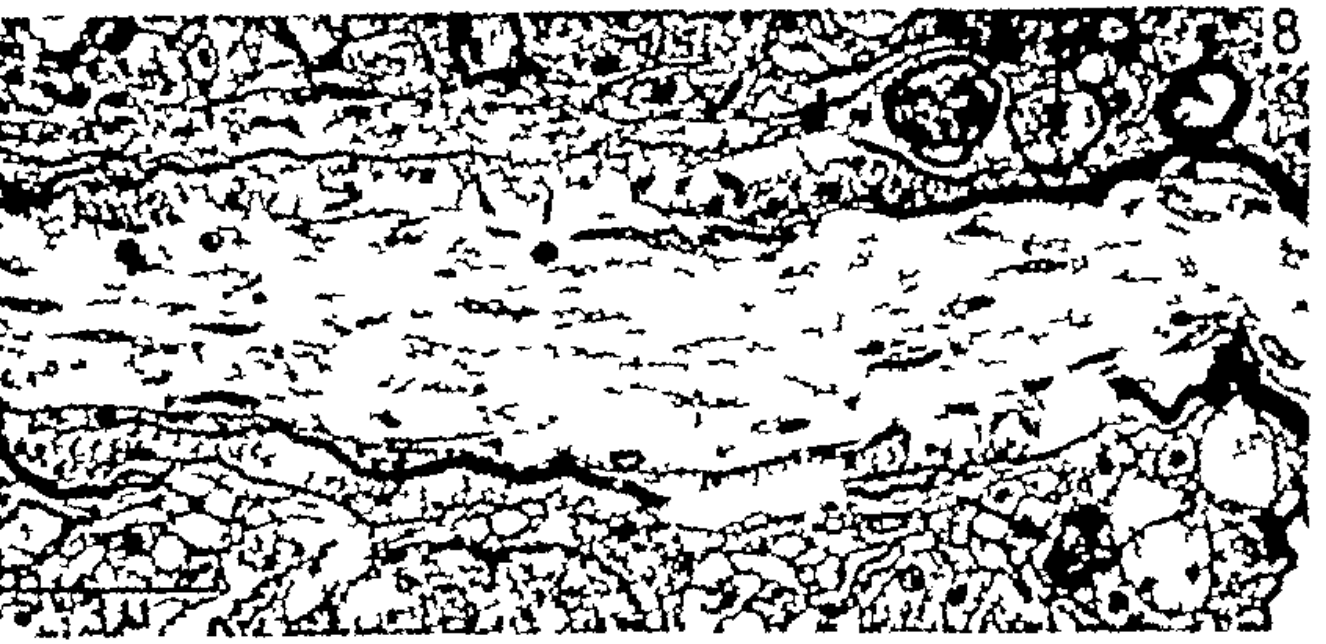

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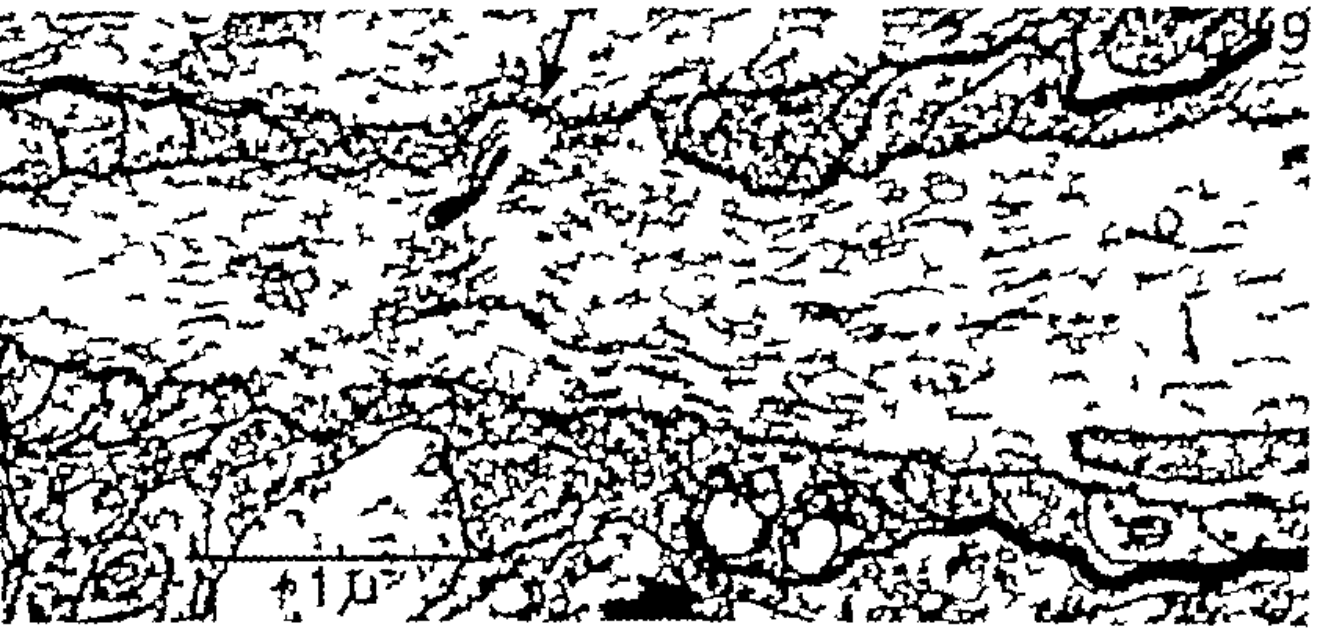

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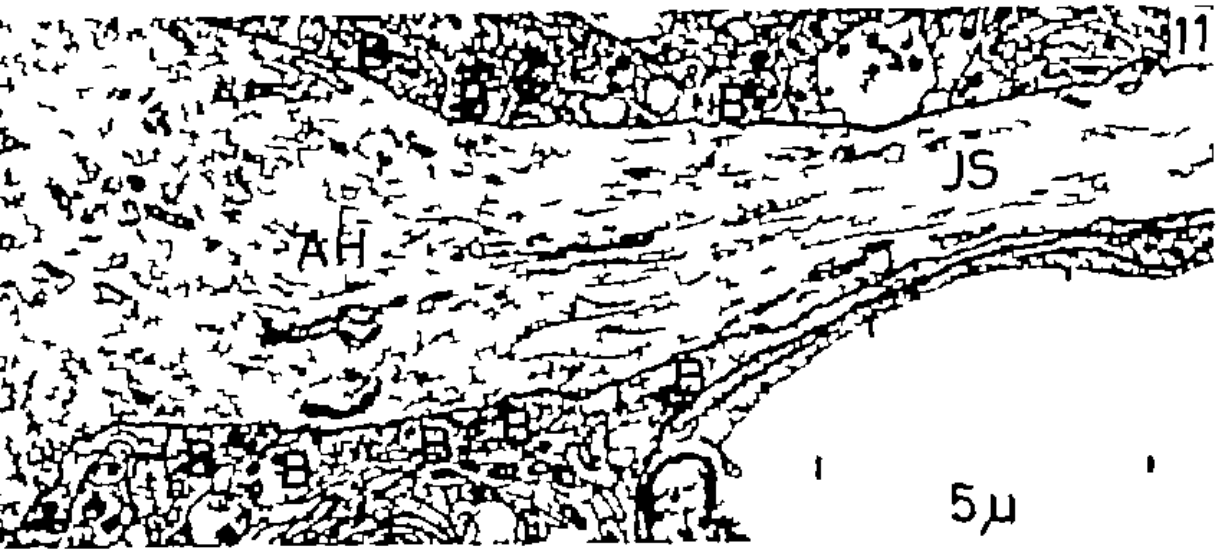

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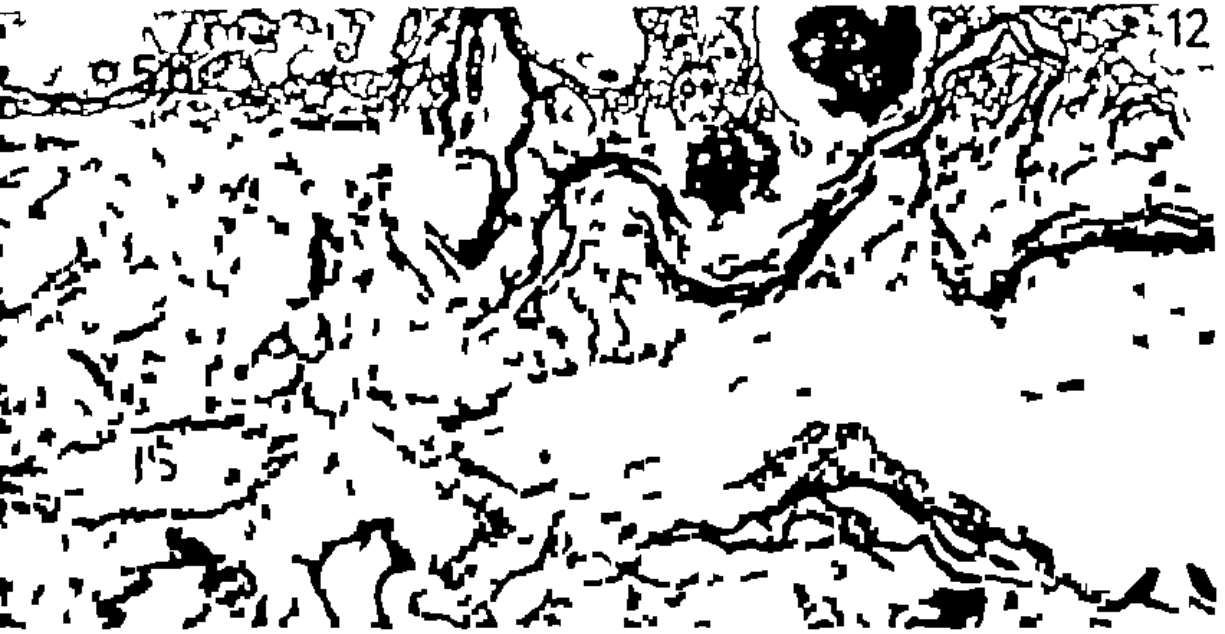

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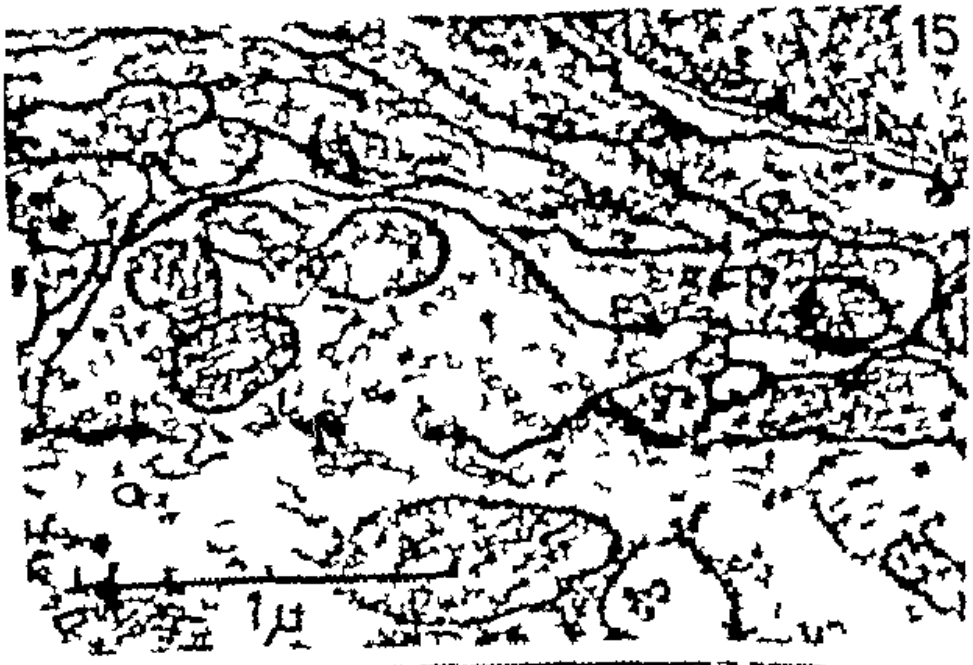

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ACTA PHYSIOLOGICA SCANDINAVICA
SUPPLEMENTUM 334

EFFECT OF OXYGEN SUPPLY ON WOUND HEALING AND FORMATION OF EXPERIMENTAL GRANULATION TISSUE

BY

JUHA NIINIKOSKI

TURKU 1969

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FROM THE DEPARTMENT OF MEDICAL CHEMISTRY UNIVERSITY OF TURKU
TURKU 3, FINLAND

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To the Memory of
My Father

Turku Kirjapaino Polytypos 1969

CONTENTS

ABBREVIATIONS 4
INTRODUCTION 5
REVIEW OF THE LITERATURE 6
Clinical sequences of wound healing 6
Development of granulation tissue 6
Cellular aspects 6
Ground substance 8
Collagen 8
Production of experimental granulation tissue 9
"Supernormal" wound healing 10
Role of oxygen in wound healing and collagen synthesis 10
Physiology of oxygen transport 12
Oxygen toxicity 13
OBJECTS OF THE PRESENT STUDY 15
MATERIAL AND METHODS 16
Experimental animals 16
Induction of granuloma formation 16
Variation of the supply of oxygen 17
Tensile strength measurements 19
Incubation of granuloma slices 20
Incorporation of ^{3}H thymidine and ^{32}P phosphate *in vivo* 21
Analysis of granulomas 22
Extraction and fractionation of collagen from granulomas 22
Processing of incubated granuloma slices after the incorporation of ^{3}H proline 23
Extraction of nucleic acids from granulomas 23
Extraction of acid soluble nucleic acids from incubated granuloma slices 23
Extraction of extranuclear lipids from granulomas 24
Analytical methods 25
Statistical calculations 27
RESULTS 28
I. Effect of oxygen supply on the tensile strengths of a healing skin wound and experimental granulation tissue 28
Effect of various oxygen-nitrogen mixtures at 1 ATA 28
Treatment started one day after implantation 28
Treatment started five days after implantation 30
Effect of hyperbaric oxygenation 32
Effect of reduced barometric pressure 33

II. Effect of oxygen supply on the chemical composition of experimental granulation tissue 34
Effect of various oxygen-nitrogen mixtures at 1 ATA 34
Treatment started one day after implantation 34
Treatment started five days after implantation 45
Effect of hyperbaric oxygenation 40
Effect of reduced barometric pressure 46
III. *In vivo* and *in vitro* studies on the mechanism of the action of oxygen in the synthesis of various granulation tissue components 47
Studies on energy metabolism 47
Utilization of glucose *in vitro* 47
Formation of lactic acid *in vitro* 48
Lactate dehydrogenase *in vitro* 49
Incorporation of ^{3}H proline *in vitro* 50
Incorporation of ^{3}H thymidine *in vivo* 51
Incorporation of ^{3}H-cytidine *in vitro* 51
Incorporation of ^{32}P phosphate *in vitro* 52
DISCUSSION 54
Comments on material and methods 54
Discussion of results 55
Effect of oxygen supply on the tensile strengths of a healing incised wound and experimental granulation tissue 55
Effect of oxygen supply on the chemical composition of experimental granulation tissue 56
The mechanism of the action of oxygen in wound healing 57
SUMMARY 60
ACKNOWLEDGEMENTS 63
REFERENCES 64

ABBREVIATIONS

ATA	atmosphere absolute pressure
Hypro	hydroxyproline
OHP	oxygen under hyperbaric pressure
psi	pounds per square inch

INTRODUCTION

Knowledge of the process of wound healing and the relevant factors is of great significance to the surgeon. Despite extensive studies in the field of connective tissue regeneration, healing is still an obscure process in many respects. We know general and topical agents that retard wound repair. We also have information about the beneficial effects of various systemic and local applications on healing. In fact, most of these do not speed up the healing but merely restore the rate to normal. Attempts have been made to solve the problem of "supernormal" healing, but no practical way has yet been devised to accelerate normal wound repair.

The present work was prompted by the finding that a hyperoxic atmosphere speeds up the synthesis of collagen *in vitro* (Kao *et al.* 1963) and by the knowledge that wound healing is accelerated in divers living under a high pressure below sea level. The author wished to extend the study to animals living in atmospheres of varying oxygen tension. Preliminary reports on the results of the present study have appeared (Niinikoski *et al.* 1966, Niinikoski *et al.* 1967, Kulonen *et al.* 1967, Kulonen and Niinikoski 1968).

In 1967 J. Englebert Dunphy wrote: "It seems a long way from the operating room to oxygen tension in wound fluid, and even longer to the hydroxylation of proline in an homogenate of chick embryo. But this is the very bridge between surgery and the basic sciences which must be strengthened."

REVIEW OF THE LITERATURE

According t definition, "a wound is a break or solution in continuity of tissue resulting from external force, whether mechanical, physical, chemical or bacterial in origin (Haley and Williamson 1962). Most of the following discussion of wound healing will be in terms of injury by mechanical force and will deal with the healing of skin and connective tissue. Some selected features of current knowledge on wound healing are discussed without any complete review of the available literature. Arey (1936) has published a thorough review of the early literature on wound healing. Edwards and Dunphy (1958a, b). Dunphy (1960). Haley and Williamson (1962). Viljanto (1964). Schilling (1968) and Dunphy and Van Winkle (1969) have reviewed the recent knowledge on wound healing and granulation tissue development.

CLINICAL SEQUENCES OF WOUND HEALING

The clinical division of the healing process into various phases has been based on the rate of gain in wound tensile strength (Fig. 1) or on the rat f contraction of the wound edges (Carrel 1910).

Figure 1 shows that at the beginning there is a quiescent *latent* period of 4—7 days, during which the wound strength does not increase appreciably. The latent period is characterized by the in asion f immature fibroblasts int the wound area. *Proliferation f f broblasts* takes place during the first two weeks. Cellular proliferation is partly accompanied and f llowed by high rates f *synthesis f coll gen* and noncollagenous proteins. During this period the tensile strength rises rapidly. After the third week the rat of collagen synthesis decreases and the tensile strength curve levels ff and there f llows a *period f volution* or maturation that lasts until healing is complete.

DEVELOPMENT OF GRANULATION TISSUE

Cellular aspects

The proliferation f fibroblasts begins from the periphery of the wound and proceeds toward the central regions f the regenerating area. During this proliferation, which is the first stage of fibroblast activity, the young cells vary in size and shape. Th second functional stage is dominated by collagen formation and accompanied by waning growth potential (Schilling *et al.* 1953). In th third, final stage fibroblasts lose their blast potential nd mature into fibrocytes. As maturation progresses, the fibroblasts lengt and orient themselves in parallel patterns between collagen fibers.

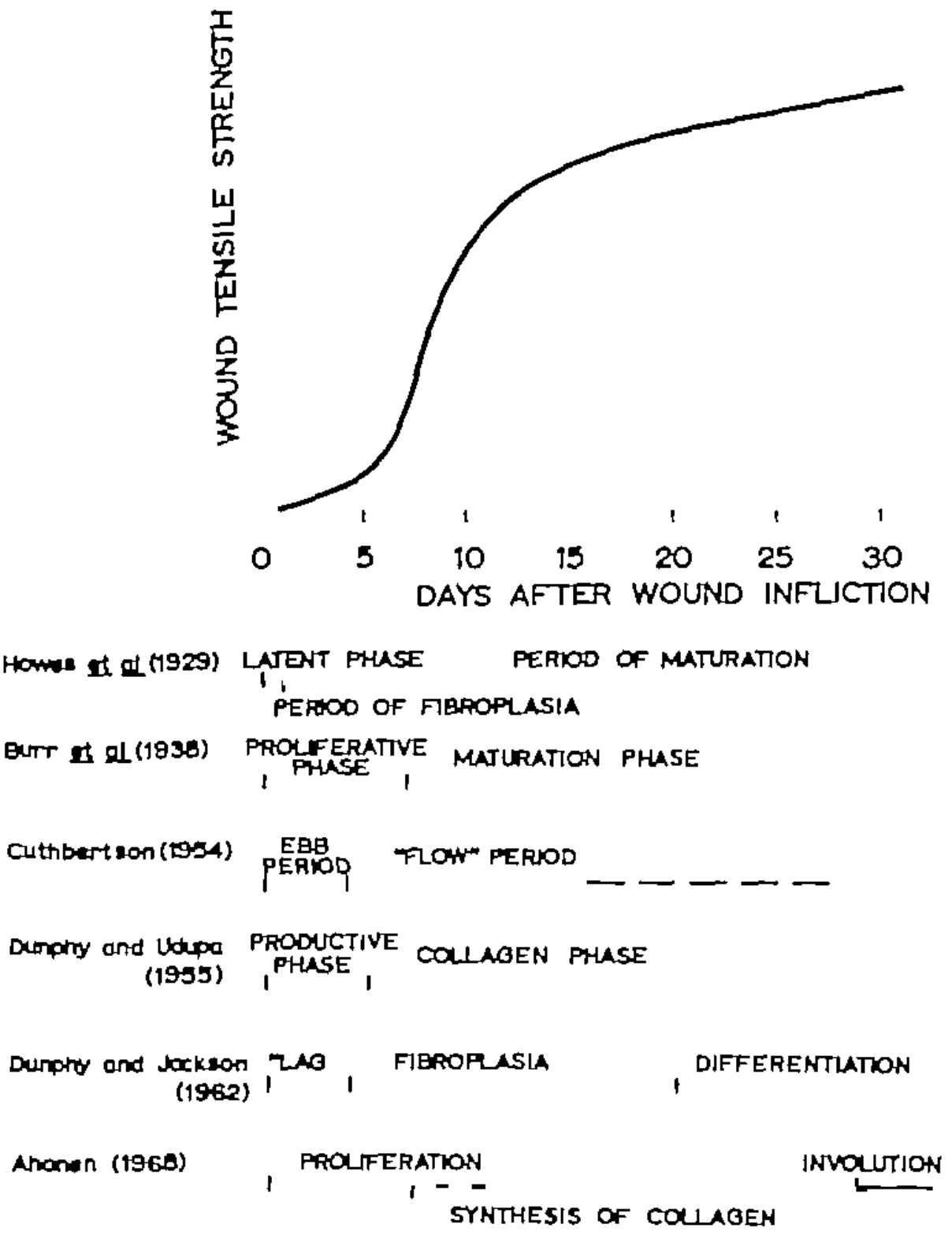


Fig 1 Phases of wound healing as revealed by the increase in tensil strength.

The content of nucleic acids in granulation tissue reflects the number and activity of cells. The contents of DNA and RNA in healing wounds are highest on the 8th day (Hosoda 1960, Williamson and Goschlbauer 1961) In Ivalon and laroue cellulose sponge granulomas, the maximum level of DNA is reached on the 20th day whereafter

the concentration decreases slightly (Woessner and Boucek 1961, Viljanto and Kulonen 196.., Lampiaho and Kulonen 1967) The peak DNA content was attained on the *0th day also in carrageenin granulomas (Boucek and N ble 1961) Chvapil and Cmuchalová (1961) observed the highest level of DNA in carrageenin granulomas already on the 6th— th day probably because of partial resorption of the implants. The peak of fibroplasia was reached in turpentine granulomas on the 5th day as revealed by DNA determinations (*Trnavský et al.* 1961)

The maximum contents of RNA in experimental granulation tissue are attained during the synthesis of collagen (Williamson and Guschlbauer 1961 Viljanto and Kulonen 1962) In granulomas 60—90 days ld the contents of RNA were nearly constant although the synthesis of collagen had almost ceased on the 40th day (Ahonen *et al.* 1964, Lampiaho and Kulonen 1967)

Ahonen (1968) studied the incorporation of ^{14}C-glycine into nucleic acids and proteins during the development of viscose cellulose sponge granulomas. Most of the DNA was synthesized during the first two weeks. Cell proliferation was accompanied and followed by high rates of synthesis of RNA, collagen and noncollagenous proteins.

Connective tissue formation requires the activity of many cell types in addition to fibroblasts. These have been highlighted by Edwards and Dunphy (1958a)

Ground substance

The name ground substance has been given to the amorphous interfibrillar matrix of connective tissue. In healing wound this metachromatically staining material contains acid and neutral mucopolysaccharides, glycoproteins and proteins (Shethar *et al.* 1959, White *et al.* 1961) The metachromasia reaches its maximal intensity on the 5th—6th day and then diminishes (Dunphy and Udupa 1955 Kodicek and Loewi 1955) The accumulation of mucopolysaccharides is connected with the prolifera tion of fibroblasts. Mucopolysaccharides have been suggested to participate in the precipitation and orientation of collagen fibers (Gross *et al.* 1952, Kodicek and Loewi 1955, Loewi and Meyer 1958, Wood 1960)

The content of hexosamine has been used a a measure of mucopolysaccharide content (Dunphy and Udupa 1955, Ahmad 1961) However other evidence has cast doubt on the validity of this. Jackson *et al.* (1960) showed that most of the bound hexosamine in sponge biopsy material is associated with saline-soluble proteins. The ratio of hexosamine to nitrogen in th unextracted sponges indicated that the hexosamine was derived largely from serum glycoproteins rather than from mucopolysaccharides.

Collagen

Structure f collagen. Collagen has a unique amino acid composition with high proportions of glycine, proline and hydroxyproline and low proportions of aromatic amino acids such as tyrosine. The basic structural unit of collagen is called tropo-collagen. Th tropocollagen macromolecule is in the native form an longated, stiff rod 2800 nm long and 14 nm in diameter and its molecular weight is approximately 300,000.

Fibrinogenesis and formation of collagen Tropocollagen molecules are synthesized by fibroblasts and polymerize t fibrils adjacent to these cells. In experimental granula tion tissue the synthesis of collagen begins on the 4th—5th day as judged from the incorporation of proline and synthesis of hydroxyproline by the tissue *i vit o*. The rate f collagen synthesis rises to a peak on the 10th— 1st day and the declines. On the 40th day the incorporation of proline is almost negligible (Lampiaho and Kulonen 1967) In both healing wounds and experimental granulomas the increase of collagen content is paralleled by a gain in tensile strength (*e.g* Dunphy and Udupa 1955, Viljanto and Kulonen 1962)

Hydroxylation of proline in th biosynthesis of collagen. According to current knowledge collagen hydroxyproline and hydroxylysine are formed by a unique process, in which the hydroxylation of proline and lysine takes place after they have been incorporated into a polypeptide precursor *of collagen (Peterkofsky and Udenfriend* 1963, Prockop and Juva 1965, Lukens 1965, Juva and Prockop 1966a, Gottlieb *et al.* 1966, Prockop *et al.* 1966) The proline- and lysine-rich substrate for these hydroxyla tions has been called protocollagen (Juva and Prockop 1966a)

Molecular oxygen is required for hydroxylation (Fujimoto and Tamiya 1962, 1963, Lamport 1963, Prockop *et al.* 1963, Hutton *et al.* 1967) In a nitrogen atmosphere hydroxylation is inhibited (Prockop and Juva 1965) protocollage accumulates inside the fibroblasts (Juva *et al.* 1966) and no collagen is synthesized.

Hutton *et al.* (1967) tested the dependence of the rate f proline hydroxylation *i vitro* on oxygen concentration over the range f 0.5—14.9 volumes per cent. When a partially purified proline hydroxylase was used, a high correlation was found. The Michaelis constant for oxygen was found to be 2.6 volumes per cent.

The hydroxylating activity of a supernatant from an experimental granulation tissue reaches its peak value in 20-day-old granulomas and then declines (Juva 1965) Juva concluded that the hydroxylating activity of granulation tissue reflected the intensity of collagen synthesis and that the hydroxylation reaction might prove to be a regulator of collagen biosynthesis.

PRODUCTION OF EXPERIMENTAL GRANULATION TISSUE

Experimental granulomas induced by various subcutaneous implants offer system for the study of developing granulation tissue. Boucek and Noble (1955) employed nonresorbable polyvinyl sponges implanted under the dorsal skins of rats. The amount of granulation tissue growing into the implant was directly proportional to the size of the sponge. Polyvinyl sponge was employed also by L. C. Edwards *et* L (1957) Schilling *et al.* (1959) R. H. Edwards *et* L (1960) Jackson *et* L (1960) Hruza and Hlavičková (1963) and Sandberg (1964) Viljanto and Kulonen (1962) introduced viscose cellulose sponge as an inductive matrix, and Viljanto (1964) employed it in his comprehensive study on wound repair Experimental granulomas have been induced also by carrageenin (Black 1957 Robertson *et al.* 1959, Chvapil and Cimchalová 1961) stainless steel wire mesh cylinders (Schilling *et al.* 1959) tantalum wire mesh cylinders (Schilling *et al.* 1963) polyurethane (Salvatore *et al* 1961) Dacron-*collagen mixture* (Wesolowski *et* L 1961) silicate powders (W lman and Wolman 1956, Daniel-Mousard and Quesson 1961) agar gel (Kuano and Tetsuka 1964) paraffin (Löhlich

and Arambasic 1961) oil of turpentine (Berenson and Dalferes 1960 Trnavsky *et al.* 1961) alginic acid (Kimoto *et al.* 1960 Mikuochi and Kurume 1960) and cotton pellets (Saikku 1956, Ngy *et al.* 1960)

In experiments where comparisons have been made between different types of experimental granulomas, sponge implants have been found the most useful as they are homogeneous and induce more reproducible granulation tissue formation than, for example carrageenin. In contrast to carrageenin (Viljanto *et al.* 1962) polyurethane (Salvatore *et al.* 1961) and a Dacron-collagen mixture (Wesolowski *et al* 1961) viscose cellulose sponge is not resorbed (Viljanto 1964) The yield of granulation tissue is smaller in wire mesh cylinders than in sponge implants (Schilling *et al.* 1959) Granulation tissue that develops in a viscose cellulose ponge is similar both chemically and histologically to the tissue formed in a healing wound (Viljanto 1964)

"SUPERNORMAL WOUND HEALING

Beneficial effects of various topical or systemic agents on the healing of wounds have been reported, but in many cases continued studies have revealed that the favorable effect has been due to elimination of factors that retard healing

Factors that enhance wound healing are summarized in Table I. It should be noted, however that no factor has attained general clinical significance.

ROLE OF OXYGEN IN WOUND HEALING AND COLLAGEN SYNTHESIS

Wound healing Oxygen tension is much lower in the centers of small muscular wounds than in wound edges nd the surrounding tissue as long as vascularization of the wound is incomplete (Hemensnyder and Majno 1968) Hunt *et al.* (1967) registered respiratory gas tensions and hydrogen ion concentrations in fluid aspirated from developing wire mesh cylinder granulomas. The oxygen tension was 1.. mmHg on the 5th day and rose as healing progressed to 18 mmHg on the 25th day Ten days after metal cylinder implantation, the carbon dioxide tensi n was elevated, probably because of increased production of carbon dioxide by the healing tissue. The low pH values were mostly due to the high carbon dioxide tensions of the wound fluid.

Lundgren and Sandberg (1965) concluded from tensile strength measurements that wound healing was retarded in rats kept intermittently in pure oxygen. However after an cut hemorrhage, oxygen under hyperbaric pressure (OHP) appeared to have stimulating effect on wound healing (Lundgren and Sandberg 1966) Beckham and Hitchcock (1965) obtained quit opposite results. Intermittent exposures to pure oxygen at 2 ATA levated the tensile strengths of wounds in dogs. Repeated skin grafting and OHP together speeded up the closure of open wounds in rats (Shulman and Krohn 1967) A beneficial influence of OHP on ischemi ulcers was observed by Illingworth *et l.* (1961) Boffes *et al.* (1966) Hall *et al.* (1966) and Black *et al.* (1966) on tra matic ischemia by Smith *et al.* (1961) and Black *et al.*

Table I

Factors promoting wound healing

Factor	Effect	References
"Secondary suture"	Increased tensile strength of disrupted and resutured wounds	*e.g.* Calnan *et al.* (1964)
	Enhanced healing from an initially more vascularized tissue	Weiber (1961)
Cartilage powder:		
Locally applied heterologous cartilage powder	Increased tensile strength of wounds	Prudden *et al.* (1957)
	Increased tensile strength of wounds (clinical study)	Prudden and Allen (1963)
Parenterally injected saline extract of cartilage powder	Accelerated closure of open granulating wounds	Sabo *et al.* (1965)
Histamine:		
Intraperitoneally injected histamin and histamine liberator (compound 48/80)	Increased tensile strength of wounds and histologically confirmed acceleration of healing	Boyd and Smith (1939)
Intraperitoneally injected histamine liberators (*compound* 48/80 and polymyxin B)	Increased tensile strength f wounds and enhanced synthesis of collagen	Sandberg (1964)
Elevated temperature	Increased rate of cicatrization	Ebeling (1922)
The first trimester of pregnancy	Increased tensile strength of wounds and enhanced synthesis of collagen	Sandberg and Sandberg (1967)
Locally injected species-specific soluble collagen	Increased tensile strength of wounds and histologically confirmed acceleration of healing	Hernández Richter *et al.* (1965)
Parenterally applied skin extracts	Shortening f the healing period and quicker epithelization of wounds	Teir *et al.* (1951)
Tanning with formaldehyde	Increased tensile strength of wounds	Peacock (1966)
Locally applied hyaluronic acid	Increased tensile strength of experimental granulation tissue	Vilkkinen and Viljanto (1965)
Application of collagen films containing tissue culture cells	Acceleration of skin defect closure	Pappas *et al.* (1961)
Reinforcement of incision by Dacron mesh	Increased tensile strength of wounds	Adler *et al.* (1962)
Orally administered zinc sulphate	Accelerated healing of granulating wounds (clinical study)	Pories *et al.* (1967)
Parenterally injected protamine-zinc insulin	Increased tensile strength of wounds	Rosenthal (1963)
Application of amino-oxidase inhibitors	Stimulated fibroplasia	Scherbel (1961)
Orally administered oxyphenbutazone	Increased tensile strength of wounds	Brunius and Zeder feldt (1965)

(1966) on experimental burns by Marchal *et al.* (1960) and Nelson *et al.* (1966) and on ischemic skin flaps by Perrins (1966) and Niinikoski (1969). Local application of pure oxygen at 1 ATA promoted the healing of burns (Lucedoni *et al.* 1961, 1963) and of torpid ulcers in the lower extremities (Chieregato and Pittoni 1964). Intra-arterial injections of hydrogen peroxide improved the healing of leg ulcers (Balia *et al.* 1964). The closure of excision wounds in dogs was more rapid at sea level than at an altitude of 3000 m (Utkina 1964).

Hyperbaric oxygen affects bacterial growth and may prevent wound infection. Studies conducted with common coliform organisms and staphylococci indicated that *the response of bacteria to OHP both in vivo and in vitro is biphasic as the oxygen* tension increases (Ollodart 1966). Elevating the oxygen tension from normal (pO_2 160 mmHg) to 1 ATA (pO_2 760 mmHg) increases the growth rate of *E. coli*. Pure oxygen at pressures above 2 ATA, however, inhibits bacterial growth.

Collagen synthesis. Kao *et al.* observed in 1963 that *in vitro* synthesis of collagen by sponge biopsy connective tissue was accelerated when the atmospheric oxygen concentration was raised from 21 to 95 volumes per cent. A similar finding was made by Lampinho and Kulonen (1967). Tsurufuji and Ogata (1963) suggested that maturation of collagen and synthesis of tropocollagen depends on aerobic cellular activities. Chvapil *et al.* (1963) found that the synthesis of collagenous and non-collagenous proteins in chick embryo skin slices was stimulated by oxygen at concentrations up to 20 volumes per cent. No effect on protein synthesis was observed at higher tensions. However, as judged from the relative proportion of insoluble collagen hydroxyproline, the maturation of collagen appeared to be a function of pO_2 in the range of 20—95 per cent. Collagen synthesis exceeded its degradation up to an oxygen concentration of 60 per cent but at higher tensions synthesis and degradation of collagen took place at equal rates. The studies of Stern *et al.* (1966) revealed that the rate of collagen synthesis in bone slices increased concurrently with increasing oxygen concentration but that collagen degradation was accelerated even more. Bassett and Herrmann (1961) found that variation of oxygen supply could determine the type of connective tissue that *differentiated in a culture of cells arising from bone; an* oxygen concentration of 35 volumes per cent favored collagen fiber formation. Shaw and Bassett (1967) investigated the effects of various oxygen concentrations on osteogenesis and embryonic cartilage formation *in vitro*. Maximum osteogenesis was achieved at an oxygen concentration of 23 volumes per cent. Osteogenesis and the formation of collagen fibers were retarded by lowering the oxygen concentration to 5 per cent and, to a lesser degree, by elevating it to 95 per cent. High oxygen concentration (95 per cent) caused extensive chondroclasia. Intermediate concentrations of oxygen (40—65 per cent) produced numerous large collagen fibers while some chondrocytes underwent alteration in morphology and came to resemble osteoblasts.

PHYSIOLOGY OF OXYGEN TRANSPORT

After diffusion from the alveoli of the lungs, oxygen is transported to tissues combined with hemoglobin and dissolved in the plasma. At sea level the hemoglobin in arterial blood is about 95 per cent saturated with oxygen, and a hemoglobin value of 15.3 g/100 ml corresponds to 19.5 volumes per cent oxygen in the blood. The oxygen

pressure in arterial blood is normally 90 mmHg The final transport of oxygen to the cell takes place by diffusion and thus depends on the pO_2 in the plasma and the cell. The higher the cellular oxygen consumption, the steeper is the oxygen gradient. The oxygen uptake of the cell is decreased only beyond a critical value of about 1 mmHg (*cf* Tribukait 1963b) The oxygen content in the plasma conforms to Henry's law and depends on the solubility of oxygen in plasma and upon the pO_2 of the gaseous mixture in the alveolar space. Under normal conditions, the dissolved oxygen amounts to 0.26 volumes per cent. In 100 per cent oxygen at sea level, it amounts to 2.2 volumes per cent (Bohr 1903) and in oxygen at 2 ATA to 4.3 volumes per cent. The latter concentration covers sufficiently the oxygen demand f a resting mammal (Haldane 1895) Boerema *et al.* (1960) confirmed this finding by a dramatic demonstration of the possibility of sustaining lif in a bloodless pig perfused with saline solution while the animal breathed oxygen at 3 ATA.

OXYGEN TOXICITY

The pulmonary manifestations of oxygen toxicity involving changes in the lung parenchyma, small arteries, capillaries, alveolar membranes and the pulmonary surface active material are well known (Ohlsson 1947 Bruns and Shields 1954, Pfister *et al.* 1964, Brooksby *et al.* 1966, Cederberg *et al.* 1965, Klatter *et al.* 1966, Spencer *et al.* 1966, Wittner and Rosenbaum 1966 Wood 1969) The changes may be so severe that the pulmonary function fails and the animal dies from paradoxical anoxia and acidosis. Tribukait (1963a) subjected rats to oxygen concentrations f 30—100 per cent for 8—10 weeks. The lungs of the rats were unaffected by oxygen at concentrations up t 80 per cent. According to Hedley Whyte and Winter (1967) however prolonged in halation of 60—70 per cent oxygen can cause lung damage.

The most dramatic and commonly encountered form of oxygen toxicity in short term hyperbaric oxygen therapy at more than 3 ATA is a classic grand mal seizure. This can result in cell destruction in the central nervous system and death.

Increased tolerance to oxygen at 3 ATA (Kaufman *et al.* 1966) and prolonged survival times of animals (Ackerman and Brinkley 1966) were achieved by the use of intermittent hyperbaric oxygenation instead of continuous treatment. Th general safety limit for CHP in human beings is 4 hours' exposure to oxygen t 2.8 ATA (Yarbrough *et al.* 1947) Some investigators, however have stated that probably no absolutely safe threshold can be established for all subjects even in selected group because of the great individual variation in susceptibility (*e.g.* Donald 1947)

Another feature of oxygen toxicity is retrolental fibroplasia in premature infants (*cf* Haugaard 1965)

The mechanism of the toxic actio a high oxygen pressure has been a controversial subject. Complete saturation f hemoglobin in venous blood by oxygen inhibits the transport of carbon dioxide and leads to accumulation of CO_2 in tissues and pH changes. Cellular mechanisms of toxic action of oxygen have been highlighted recently by Haugaard (1968) In 1965 Haugaard summarized the evidence in favor of the view that the toxicity is due to oxidation of sulfhydryl groups in the so-called SH enzymes. On the other hand, Chance *et al.* (1965) observed that oxygen at 11—17 ATA selectively inhibited the energy linked reduction of nicotinamide-adenine dinucleotide

(NAD) by succinate. However Straub (1937) showed that oxygen at 3 ATA had no effect on the energy linked reduction of NAD by succinate in rat liver mitochondria, and so this system does not appear to be the site of the toxic action of oxygen at this pressure.

Low temperature prevents the development of oxygen toxicity whereas an elevated temperature has the opposite effect (Hulpieu and Cole 1944, Smith *et al.* 1960) Increased thyroid activity is an important factor in oxygen toxicity because accelerated metabolism augments the toxic effects of oxygen (Smith *et al.* 1960)

Young individuals are less susceptible to oxygen toxicity especially as manifested by convulsions, than older individuals. This has been ascribed to the immaturity of the adrenals (Bean 1965)

Hulpieu and Cole (1944) observed that the ambient humidity is important in the control of oxygen toxicity At a relative humidity of 30 per cent the toxicity is weaker than at relative humidities below 20 per cent or above 90 per cent.

OBJECTS OF THE PRESENT STUDY

The purpose of the present work was to study the effect of oxygen supply on wound healing and the development of experimental granulation tissue in the rat. The following were the main objects of study:

(I) The influence of various respiratory oxygen tensions on the tensile strengths of healing skin wounds and experimental granulomas.

(II) The relation of various respiratory oxygen tensions to the chemical composition of experimental granulomas.

(III) Mechanism of the action of oxygen in the synthesis of various granulation tissue components.

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Hulpieu and Cole (1944) observed that the ambient humidity is important in the control of oxygen toxicity At a relative humidity of 20 per cent the toxicity is weaker than at relative humidities below 70 per cent or above 90 per cent.

VARIATION OF THE SUPPLY OF OXYGEN

Experiments in various oxygen nitrogen mixtures at 1 atm absolute pressure. The experimental setup is shown schematically in Fig 2. After the first postoperative night, Wistar rats were housed two to a box. The boxes were opened once every day for a period of about 5 min for feeding cleaning and ventilating The oxygen nitrogen mixtures (containing 15 21, 50 and 100 volumes per cent of oxygen) were supplied by AGA Oy Turku, Finland. When the gas flows were adjusted to 6 l/h, the final concentrations of oxygen in the boxes were 12 ± 1, 18 ± 1 35 ± 3 and 70 ± 5 % v/v respectively as measured with Scholander s apparatus (Scholander 1947) The final oxygen tension was reached within 8 hours. The carbon dioxide concentration inside the boxes rose to a level of about 3—5 % during the 24-hour period (Fig 3 next page)

Higher oxygen concentrations were obtained by raising the gas flow This decreased the carbon dioxide concentration When small con

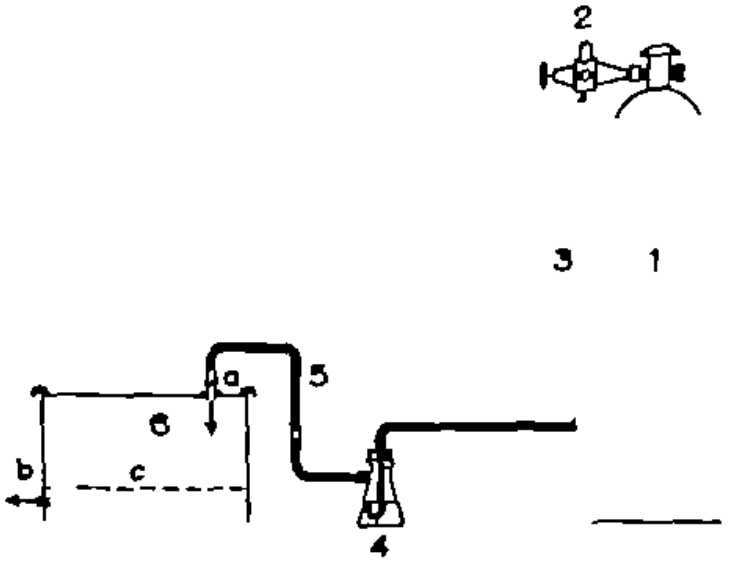


Fig 2 Experimental arrangement. The oxygen-nitrogen mixture flows from a gas cylinder (1) through a reducing valve ( ) and 5 mm tubing (3) to a wash bottle (4) which serves both as crude flow-meter and humidifier and then through further tubing (5) to box (6) where two rats are kept. The air-tight box (volume about 23 l) with transparent cover is made of Perspex and has two small holes of diameters 5 and 3 mm for the entry (a) and exit (b) respectively of the gas. The rats live on a removable mesh floor (c) of stainless steel wire nd the bottom of the box is covered with saw dust which is replaced every third day

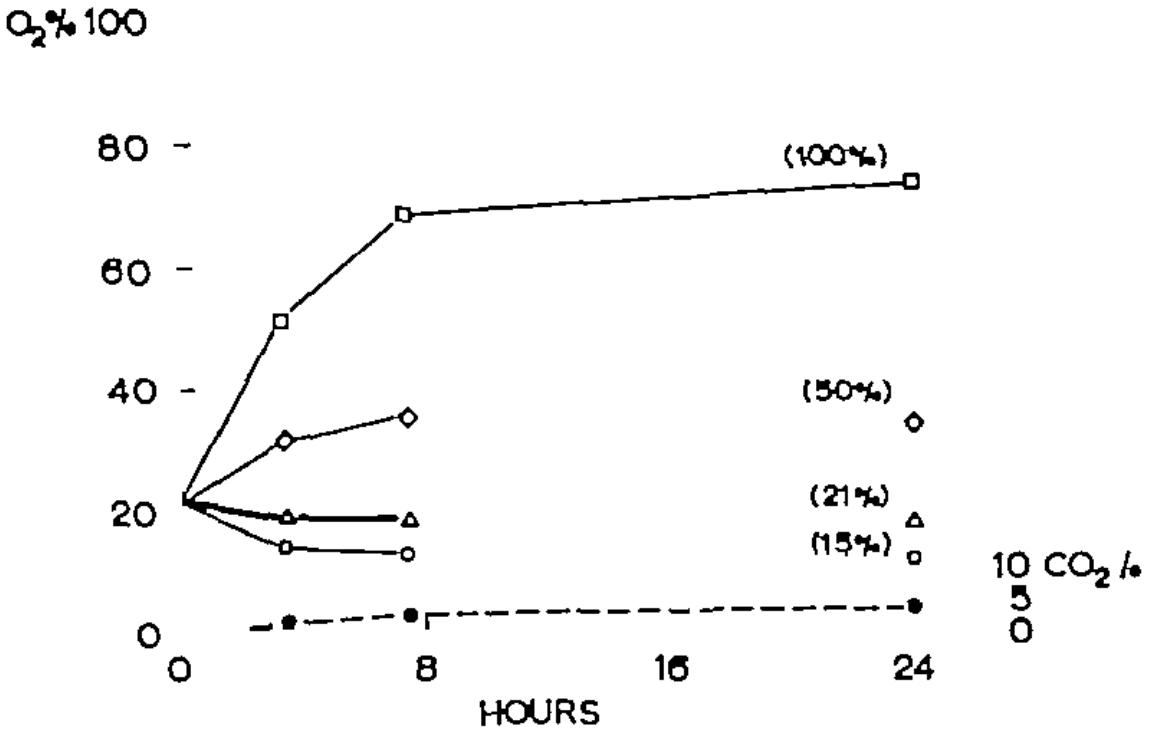


Fig 8 Actual O and CO concentrations inside the boxes during the 24-hour period. The respective O_2 concentrations in the gas cylinders are given in parentheses. Gas flow = 6 l/h.

tainers of soda lime (Sofnol® 5—10 mesh, A G Field & Co Great Britain) were employed as absorbers inside the boxes, the carbon dioxide concentration remained under 1 % Table II reveals the correlations between the flow rates of the oxygen-nitrogen mixtures and the final oxygen and carbon dioxide concentrations inside the boxes. The relative humidity in the boxes was about 90 % and the temperature 2—3 C above room temperature.

Table II

The flow rates of the oxygen-nitrogen mixtures and the O and CO concentrations (volumes per cent) inside the boxes after the 24-hour period

Gas cylinder O_2 %	Gas flow l/h			
	6	8	12	15
15	1[illegible] % O_2/4 % CO_2	14 % O_2/< 1 % CO_2	—	13—14 % O_2/3 % CO_2
21	1[illegible] % O_2/4 % CO_2	19 % O_2/< 1 % CO_2	19 % O_2/[illegible] % CO_2	19—20 % O_2/3 % CO_2
50	3[illegible] % O 4 % CO_2	40 % O_2/< 1 % CO_2	40 % O_2/2 % CO_2	45 % O_2/3 % CO_2
100	1[illegible] % O 4 % CO_2	75 % O_2/< 1 % CO_2	—	90 % O_2/3 % CO_2

CO_2 absorbed by soda lime

The following discussion of the concentrations of oxygen and carbon dioxide will be in terms of volumes per cent reached inside the animal boxes during the 24-hour period if not stated otherwise.

Experiments under hyperbaric oxygen After the first postoperative night, Sprague-Dawley rats were exposed to oxygen at 2 atm absolute pressure for a period of 2 hours twice every day (at an interval of 2 hours) The hyperbaric treatment was extended to the 3rd or 9th day after wounding The rats were killed 4 or 10 days after the implantation. The exposures to compressed oxygen took place in a hyperbaric animal chamber (volume about 33 l) manufactured by Vickers Research Establishments, Sunninghill, Berks., England. Ten rats were housed in the chamber Compression and decompression were carried out at the rate of 3 psi/min i.e in 5 minutes. The flow of oxygen was 1 l/min/rat. The highest carbon dioxide content in the breathing gas during the experiments, as ascertained with a Luftanalysator Type K 1/64 (the Laboratories of Aviation and Naval Medicine, Karolinska Institutet, Stockholm, Sweden) did not exceed 0.1 % The temperature in the chamber increased by 2°C and the relative humidity decreased from about 50 % to 35 % during the 2-hour exposure. The control rats were housed in ordinary cages in the animal room.

Experiments in air at ½ atm absolute pressure These experiments were carried out in an altitude chamber (volume about 10 m) After the first postoperative night, Wistar rats were subjected to ½ atm absolute pressure corresponding to an altitude of 5500 meters for 9 days. Decompression was accomplished in 5 minutes and the reduced pressure was maintained in the range at altitudes of 5300—5700 meters. Recompression also took 5 minutes. For feeding the rats, the pressure was elevated to normal once a day for a period of 20 minutes. Fresh air was pumped into the chamber three times every day Sixteen rats were housed simultaneously in the chamber The relative humidity in the chamber was approximately 50 % and the temperature 1—3 C above room temperature during the experiments. The control rats were housed in ordinary cages in the animal room. All the rats were killed 10 days after the implantation.

TENSILE STRENGTH MEASUREMENTS

Wounds. The rats were anesthesized with ether and killed by a blow on the neck. The tensile strengths of the skin wounds and the granulomas were measured as described by Viljanto (1964) The wound with its environment was cut away and the sutures were removed. One skin strip 1 cm wide was cut at right angles to the wound incision with a razor blade

with the aid of a Perspex sheet containing two parallel slits. The tensile strength was determined by a flowing water method The effect of static stress was avoided by adjusting the height of the water level and the diameter of the rubber tube so that one assay took 20—40 sec. The skin strip was fixed between two clamps with the jaws of the clamps parallel to the wound and suspended at the end of the beam of a triple beam balance. Water was allowed to flow at a uniform rate (250—500 ml/min) into a plastic container on a scale pan When the wound edges were torn apart, the wound strength was calculated from the weight of water that had flowed into the plastic vessel. From the 5th day on the wounds became so strong that 2 thread stitches were made at each end of the skin strip and the wound strength was tested by pouring small lead shot (3 mm in diameter and weighing 50 mg) at an even rate into the plastic container to tear the wound open within a period of 20—40 sec.

Granulomas The granulomas were removed from the subcutaneous space and freed from the connective tissue capsules surrounding them The cotton thread holding the halves of the implant together was cut and removed. The granulomas were fastened to the two serrated clamps of the tensile strength apparatus with the jaws exactly parallel to the section surfaces. The tensile strengths of the granulomas were assayed by the flowing water method in the same way as the tensile strengths of the wounds. After the granulomas had been torn apart, they were cooled in test tubes on crushed ice and stored frozen until analyzed. On the 15th day after implantation, the granulation tissue in the sponges was so strong that the halves could no longer be torn apart and the granulomas broke near the clamps. For this reason reliable tensile strength determinations could be carried out on the granulomas only on the first ten days.

INCUBATION OF GRANULOMA SLICES

For the *in vitro* experiments, the granulomas were removed from the rats under light ether anesthesia, decapsulated and cooled on crushed ice, after which the pieces were cut into thin slices with a Stadie-Riggs microtome in the cold room. If not stated otherwise, the incubations were carried out in air or 40 % oxygen in a thermostated shaker (Compensiat, Gallenkamp & Co. London England) at 37 C The nutrient medium was the Krebs Ringer phosphate solution, pH 7 4 (Cohen 1957) supplemented with glucose (5 4 mM) No antibiotics were added to the medium In the incorporation experiments, 20 ml of the medium and the slices

from 4 granulomas (wet weight about 4 g) were added to a 100-ml flask. After a preincubation period of 30 min a radioactive compound was added to the medium and the incubation was continued with vigorous shaking of the mixture. The atmospheres inside the flasks were renewed every second hour.

Studies on energy metabolism. The experiments on glucose utilization and lactic acid production of the slices of granulation tissue were carried out entirely in air. Slices from half a granuloma (wet weight about 0.5 g) and 3 ml of the medium were added to a 25-ml flask and incubated for 3 hours. No preincubation was used. Immediately after the incubation, the slices were separated from the medium by centrifuging in a refrigerated centrifuge (MSE High Speed 17. Measuring and Scientific Equipment Ltd., London, England) at 10 000 *g* and 0°C for 10 min.

Incorporation of ^{3}H proline. ^{3}H L-Proline (TRA. 82, 100—1000 mCi/mM, The Radiochemical Centre, Amersham Bucks., England) was added in a dose of 80 μCi in 0.22 ml of 0.9 % sodium chloride solution to the incubation mixture. The incorporation was stopped by centrifuging the incubation mixture at 30 000 *g* and 0°C for 10 min and discarding the supernatants.

Incorporation of ^{3}H-cytidine. ^{3}H-Cytidine (TRK. 198. >15 000 mCi/mM, The Radiochemical Centre) was added in an amount of 30 μCi in 0.30 ml of 0.9 % sodium chloride solution to the incubation mixture. The incorporation was stopped by addition of ice-cold perchloric acid to give a final concentration of 0.5 N.

INCORPORATION OF ^{3}H THYMIDINE AND ^{32}P PHOSPHATE IN VIVO

Incorporation of ^{3}H-thymidine. A dose of 100 μCi of ^{3}H-thymidine (TRK. 61. >10 000 mCi/mM, The Radiochemical Centre) in 1.0 ml of 0.9 % sodium chloride solution was injected intraperitoneally into anesthetized sponge-bearing rats on the 8th and 9th postimplantation day and the implants were removed 48 and 24 hours later respectively. The rats were kept in an atmosphere containing 19 or 40 % O_2. On the 10th day the sponge implants were decapsulated and cooled on crushed ice.

Incorporation of ^{32}P phosphate. A dose of 1.0 mCi of ^{32}P phosphate (Sodium Phosphate-^{32}P Injection, PBS. 2P. phosphorus content 1 mg/ml. The Radiochemical Centre) in 1.0 ml of 0.9 % sodium chloride solution was injected intraperitoneally into anesthetized sponge-bearing rats exposed

to 19 or 40 % oxygen On the 10th postimplantation day after incorpora tion periods of 1—19 hours, the implants were removed decapsulated and cooled on crushed ice

ANALYSIS OF GRANULOMAS

Extraction and fractionation of collagen from granulomas

The extraction and fractionation procedures were carried out as described by Viljanto (1964) except that the acid-soluble collagen was extracted with 0.5 M acetic acid solution instead of a 0 1 M citrate buffer pH 3.5 The collagen of the granulation tissue was divided into three fractions according to solubility neutral salt-soluble acid-soluble and insoluble collagen The detailed extraction procedure is presented in Fig 4

Fig 4 Extraction a d fractionati f collagen from granulomas.

The specimen consisting of 4 granulomas (all the implants from one rat) was homogenized in 20 ml of water in revolving blade homogeniser (Omni Mixer®, Type OM, Ivan Sorvall Inc., Norwalk, Conn., U.S.A.) at 20 000 rpm and 0 C for 1 min. The homogeniser cup was rinsed with 5 ml of water The mixture was centrif ged in a refrigerated centrifuge (MSE Super Speed 50S, Measuring and Scientific Equipment Ltd., London, England) t 35 000 *g* and 4 C for 30 min.

Sediment extracted with 10 ml of 1 M sodium hloride solutio by shaking t 4 C for 2 hrs, centrifuged at 35 000 *g* and 4 C fo 30 min extra tion, shaking and centrifugation repeated.

Supernatant:
Hemoglobin
Hexosamine
Hyd oxyproline
Nitrogen

Combined supernatants
Hydroxyprol and n itrogen f vaporized extral salt-sol ble collagen

Sediment extracted by shaking igorously with 10 ml of 0.5 M acetic acid solution at 4 C for 2 hrs mixture centrifuged at 35 000 *g* and 4 C for 30 min extraction, shaking and centrif gation repeated.

Combined supernata t
Hydroxyprol ne and itrogen f vaporized acid-sol ble ollage

Hydrolysis of sediment in 6 N hydrochloric acid at 130 C f 3 hrs.

Hydrolysate:
Hydroxyproline and nitrogen f vaporized insoluble collagen

Processing of incubated granuloma slices after the incorporation of H proline

The method employed has been described by Juva (1968) A detailed scheme of the process is presented in Fig 5 Because proline and hydroxy proline occur in collagen in nearly equal amounts, the radioactivity of non collagenous protein was calculated from the total activity assuming that the radioactivity of collagen was twice that of the hydroxyproline.

Fig 5 Management of incubated granuloma slices after the incorporation of H proline

Four sliced granulomas were washed twice with 20 ml of ice-cold distilled water a d the mixture centrifuged at 30 000 *g* nd 0 C for 10 min.

↓

The sediment was homogenized in the Omni Mixer® homogenizer at 20 000 rpm and 0°C for 1 min in 20 ml f ice-cold distilled water Half a milliliter of toluene and 10 mg of carrier proline were added t the homogenate, which was then dialyzed gainst running tap water for 48 hrs.

↓

The dialysate was hydrolyzed in 6 N hydrochloric acid at 120°C for 12—16 hrs.

↓

The hydrolysate was evaporated to dryness on a boiling water bath and the residue was dissolved in 4 ml f distilled water

↓

0.2 ml was taken for the determination f total radioactivity

3.8 ml was used for the assay f labeled hydroxyproline.

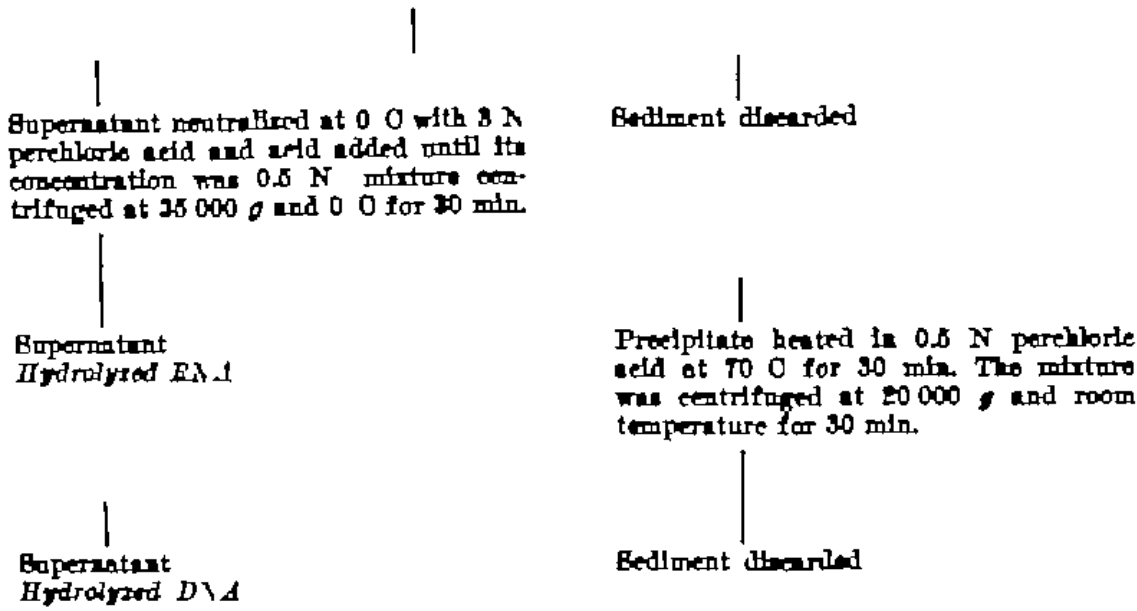
Extraction of nucleic acids from granulomas

The method was a modification of the original Schmidt-Thannhauser procedure (Schmidt and Thannhauser 1945) The detailed extraction scheme is presented in Fig 6 (next page)

Extraction of nucleic acids from incubated granuloma slices

Four sliced granulomas were washed twice with 20 ml of ice-cold 0.5 N perchloric acid and centrifuged in the MSE High Speed 17 cen trifuge at 35 000 *g* and 0 C for 20 min The granulation tissue was homogenized in 20 ml of 0.5 N perchloric acid in the Omni Mixer® homogenizer

Fig 6 Extraction of nucleic acids from granulomas



at 20 000 rpm and 0 C for 1 min. The homogenate was centrifuged at 35 000 *g* and 0°C for 30 min. The sediment was washed twice with ice-cold 0.5 N perchloric acid and centrifuged at 35 000 *g* and 0°C for 30 min. The sediment was hydrolyzed in 10 ml of 0.3 N potassium hydroxide solution at 37 C for 20 hours. After the hydrolysis, the procedure was continued as described in Fig 6

Extraction of extranuclear lipids from granulomas

Two granulomas removed from the same side of a rat were homogenized in 24 ml of chloroform methanol (1 1) in the Omni Mixer® homogenizer at 20 000 rpm and 0 C for 2 min. To remove the nuclei, the homogenate was centrifuged in the MSE High Speed 17 centrifuge at 600 *g* and 4 C for 15 min The sediment was extracted further with 2 × 4 ml of chloroform-methanol (1 1) and centrifuged at 20 000 rpm and 0 C for 2 min. The supernatants were combined and a volume of 8 ml was taken for the isolation and purification of total lipids by the procedure described by Folch *et al* (1957) Other aliquots were taken for the measurement of total and specific radioactivity of lipid-bound phosphorus.

ANALYTICAL METHODS

Hemoglobin was extracted from granulomas as described in Fig 4 The opalescence of the aqueous supernatant was removed by centrifugation at 100 000 *g* and 4 C for 30 min in the MSE Super Speed 50S centrifuge. The hemoglobin was determined as cyanmethemoglobin as recommended by the European Society of Haematology (1965) The coefficient of variation of the method was ± 3.8 %

Water-soluble hexosamine was estimated by Blix's (1948) modification of the original Elson Morgan procedure (Elson and Morgan 1933) The sample was first hydrolyzed in 2 N hydrochloric acid in sealed tubes at 100°C for 17 hours. Hexosamines were separated from interfering substances on a Dowex-50 column by the method of Boas (1953) The coefficient of variation of the method was ± 3 4 %

Hydroxyproline was determined as described by Pikkarainen (1968) The procedure was based on the method of Stegemann (1958) as modified by Woessner (1961) Extraction with benzene was not used. The coefficient of variation of the method was ± 2.5 %

Radioactive hydroxyproline was assayed according to Juva and Prockop (1966b) The coefficient of variation of the method was ± 2.6 %

The amino-acid composition of the granuloma collagen was kindly determined by Dr J Pikkarainen from the gelatin extracts (Pikkarainen 1968)

Nitrogen was estimated as described by Pikkarainen (1968) The method originated from the methods of Stegemann (personal communication to Dr Nänti) and Minari and Zilversmit (1963) The coefficient of variation of the method was ± 2.0 %

DNA was determined by the diphenylamine reaction (Burton 1956) The standard was prepared from DNA ("ex herring sperm L. Light & Co. Ltd., Poyle, England) The coefficient of variation of the assay was ± 1.1 %

RNA was assayed as ribose by the method of Ceriotti (1955) The coefficient of variation of the assay was ± 1.8 %

Lactic acid in the incubation medium was determined as described by Barker and Summerson (1941) The coefficient of variation of the assay was ± 2.8 %

Glucose in the incubation medium was assayed enzymatically with glucose oxidase (Glox® AB Kabi Stockholm, Sweden) The coefficient of variation of the assay was ± 1 5 %

Lactate dehydrogenase (LDH) After decapsulation four 10-day-old granulomas were homogenized in 20 ml of distilled water in the Omni Mixer® homogenizer at 20 000 rpm and 0°C for 1 min. The homogenate was centrifuged at 35 000 *g* and 0°C for 30 min in the MSE High Speed 17 centrifuge. The electrophoresis of the LDH isoenzymes in the supernatant was performed on cellulose acetate according to Barnett (1964) The supernatant was diluted 1 50 with distilled water and the total activity of LDH in the supernatant was measured by the UV method (C P Boehringer & Soehne G.m.b H Mannheim, Germany Biochemical Test Combination, TC-G) using a Beckman DU spectrophotometer

Lipid-bound phosphorus was assayed by the method of Bartlett (1959) with the exception that the digestion reagent employed was that proposed by Svanborg and Svennerholm (1961) The coefficient of variation of the assay was ± 0.8 %

Measurement of radioactivity Radioactivities were assayed usually with a Packard Tri-Carb® liquid scintillation spectrometer Model 3214 (Packard Instrument Company Inc., Downers Grove, Ill., U.S.A.) The solvent system (Prockop and Ebert 1963) consisted of 6 ml of ethylene glycol monomethyl ether (methyl cxitol, Shell) and 10 ml of scintillation liquid containing 15 g of 2.5-diphenyloxazole and 50 mg of 4-methyl 5-phenyloxazolylbenzene (Packard) in 1000 ml of distilled toluene. In the measurement of radioactive hydroxyproline, the solvent system did not contain methyl cxitol. The aliquot for the assay of radioactivity was chosen so that the measured counts were at least ten times the background. H-counting efficiencies were individually estimated with tritiated water (specific activity $^{9}72 \times 10^{6}$ dpm/ml, Packard) as an internal standard. ^{32}P radioactivities were assayed with a Packard Tri-Carb® liquid scintillation spectrometer Model 3320 ^{32}P-counts were corrected for decay by *measuring the radioactivity of a standard solution at the same time as* the radioactivities of the sample solutions.

STATISTICAL CALCULATIONS

The statistical calculations were carried out on an IBM 1130 computer in the Department of Applied Mathematics, University of Turku, by Mr Matti Grönroos, B.Sc. The statistical significance of the results was determined by the analysis of variance or by the *t* test.

RESULTS

I. EFFECT OF OXYGEN SUPPLY ON THE TENSILE STRENGTHS OF A HEALING SKIN WOUND AND EXPERIMENTAL GRANULATION TISSUE

Effect of various oxygen nitrogen mixtures at 1 ATA

Treatment started one day after implantation

Figure 7 reveals the effect of various respiratory oxygen concentrations on the tensile strengths of healing wounds and granulomas during the first 15 postimplantation days. Reliable tensile strength values of the granulation tissue were obtained only up to the 10th day. The favorable effect of a high oxygen concentration was clear from the 5th day on, especially in the range of 18—35 % O_2. The tensile strength rose to a maximum in 70 % O. The healing rate was lower in 12 % O than in 18 % O_2. The increase of the tensile strength was greatest for wounds on the 5th—7th day and for granulomas on the 7th—10th day. On the 10th day the tensile strengths of the wounds in rats exposed to 70 % O were 35 % and the tensile strengths of granulomas 108 % greater than in the rats exposed to 18 % O. In the analysis of variance on the 7- 10- and 15-day values, statistically significant differences were observed between the tensile strengths of both wounds and granulomas in rats exposed to different oxygen concentration, $p < 0.001$. Removal of carbon dioxide did not influence the tensile strengths of the wounds essentially.

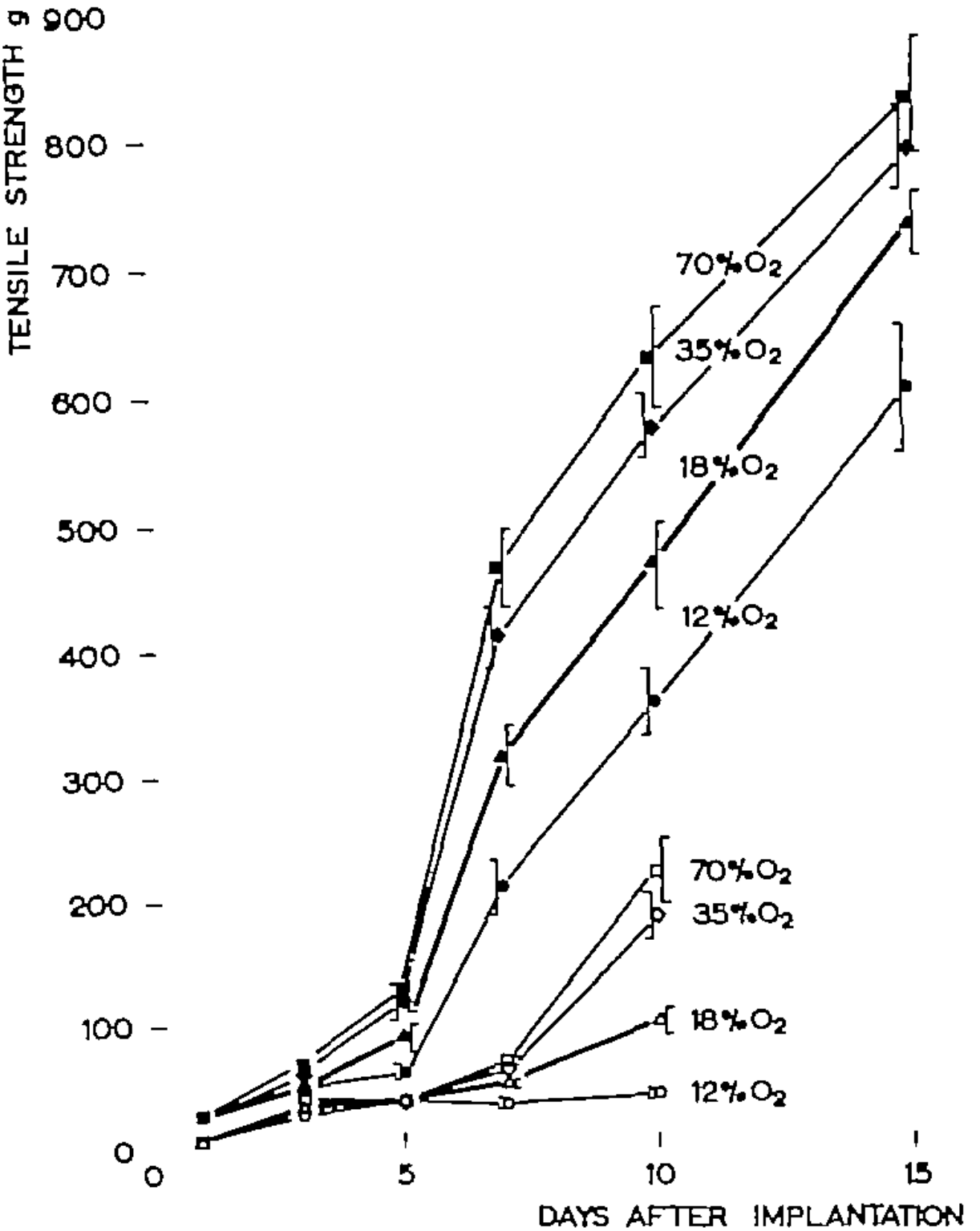


Fig. 7. The tensile strengths of skin wounds and experimental granulomas in rats exposed to different oxygen concentrations during the first 15 postoperative days. The tensile strengths of the skin wounds (dark symbols) are given in g/cm of healing wound width and the tensile strengths of the granulomas (open symbols) in g/cm² of cross-sectional area. All values are means for 8—29 rats or 83—116 granulomas. The length of each vertical bar represents twice the standard error of the mean.

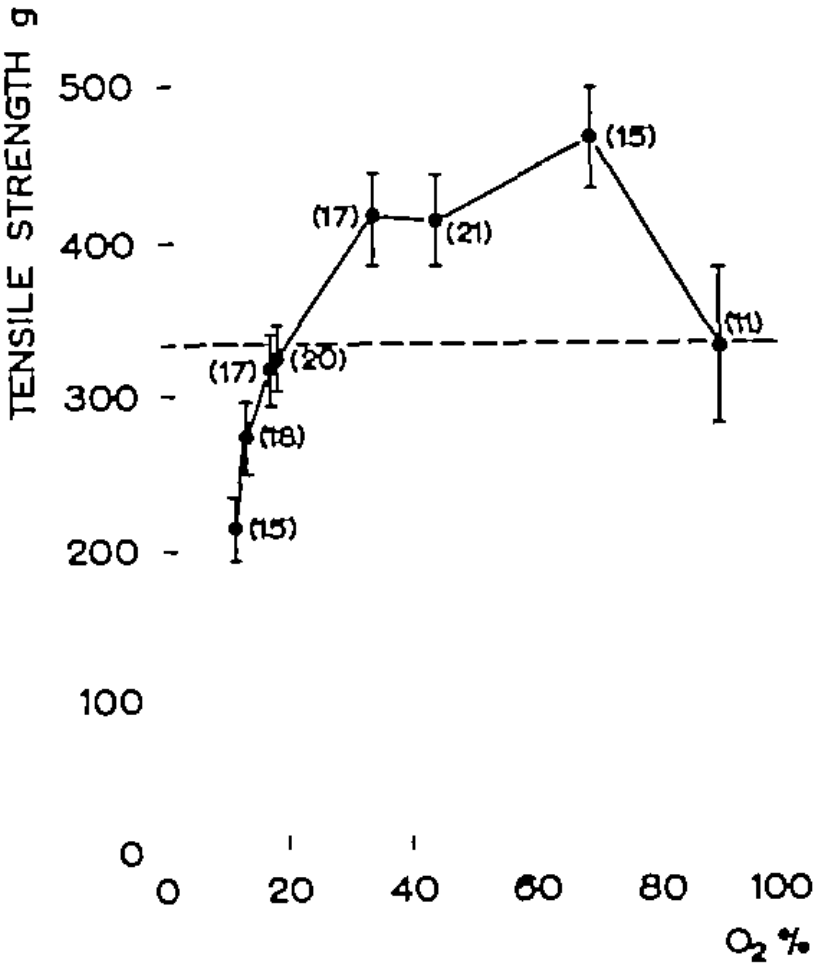


Fig 8 The tensile strengths of 7-day-old skin wounds in rats exposed to different oxygen concentrations. All values are means for 11—31 rats as indicated in the parentheses. The length of each vertical bar represents twice the standard error of the mean.

From Fig. 8 it is evident that the concentration of oxygen in inspired gas for optimal wound healing is 35—70 %. When the oxygen concentration was 90 % the tensile strengths of the 7-day-old wounds were on the same level as in air. In the *t*-test,, the difference between the tensile strengths of the wounds in rats exposed to 70 and 90 % O_2 was statistically significant, $p < 0.05$

Treatment started five days after implantation

To evaluate the significance of the oxygen supply during the latent phase of healing, rats (7—8 in each group) were kept in normal air instead of various oxygen concentrations for the first 5 days after operation

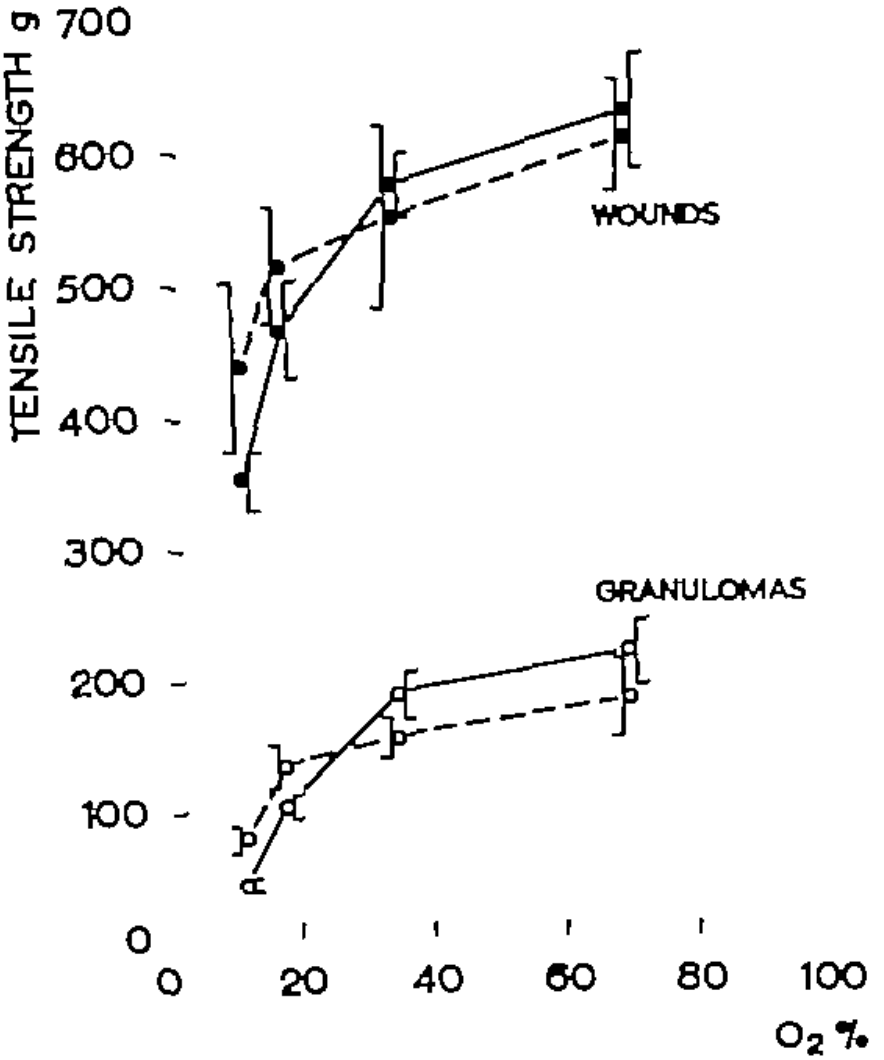


Fig 8 The tensile strengths of 10-day-old skin wounds and experimental granulomas in rats exposed to various oxygen concentrations either from the 2nd (continuous lines) or from the 6th (dashed lines) postoperative day onwards. The length of each vertical bar represents twice the standard error of the mean.

and, then in different oxygen tensions for the following 5 days. Ten days after implantation the rats were killed and the tensile strengths of the wounds and granulomas were measured.

When the inhalation of various oxygen concentrations was started first during the proliferative phase, the relative changes in the tensile strengths of wounds and granulomas of rats exposed to different oxygen tensions were much smaller than in the preceding experiments (Fig 9). Exposure to 12 % O_2 from the 6th day onwards led to lower tensile strengths than exposure to 18 % O_2 (in the t test, $p < 0.05$ for wounds and $p < 0.01$ for granulomas). No statistically significant differences were observed between mean tensile strengths for rats exposed to normal or higher oxygen tensions.

Effect of hyperbaric oxygenation

As seen in Fig 10 the optimal oxygen tension in the breathing atmosphere is passed when the oxygen treatment is extended to hyperbaric conditions. The tensile strengths of wounds and granulomas were clearly lower in rats exposed intermittently to oxygen at 2 ATA than in control rats after both 4 and 10 days. The results were statistically significant (in the analysis of variance, $p < 0.001$ for both wounds and granulomas)

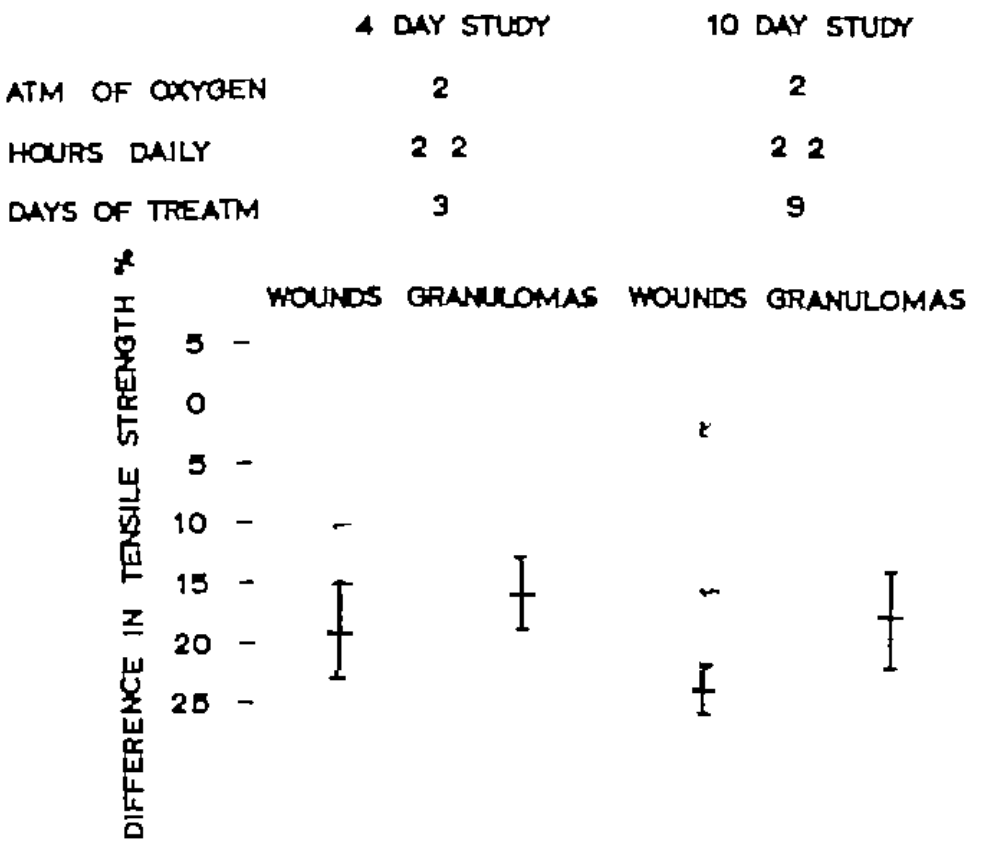


Fig 10 Effect of hyperbaric oxygenation on th tensile strengths of skin wounds and experimental granulomas. The heights of the columns are relative differences (in per cent) between the mean tensile strengths of the experimental and control wounds or granulomas. A decreased tensile strength is indicated by a negative percentage. The length of each thin bar represents twice the relative standard error of the mean (in per cent) for the experimental group. Each group consisted of 16—20 rats.

Effect of reduced barometric pressure

Studies conducted in air at $\frac{1}{3}$ ATA revealed that wound healing was distinctly retarded in unacclimatized animals at a pO of 80 mmHg (Fig

11) Ten days after implantation the mean tensile strength of the skin wounds was 30 % and that of the granulomas 35 % lower than the respective control values (in the t test, $p < 0.01$ for the wounds and $p < 0.05$ for the granulomas)

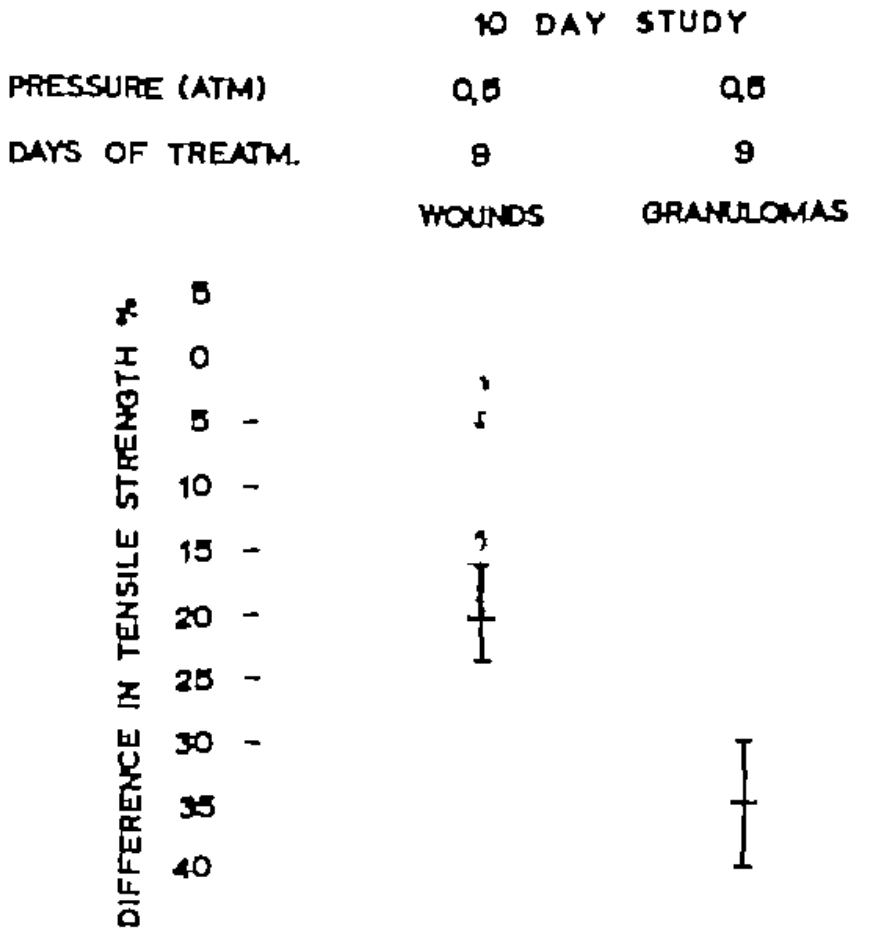


Fig 11. Effect of reduced barometric pressure (½ ATA) on the tensile strengths of skin wounds and experimental granulomas. The heights of the columns are relative differences (in per cent) between the mean tensile strengths of the experimental and control wounds or granulomas. A decreased tensile strength is indicated by negative percentage. The length of each thin bar represents twice the relative standard error of the mean (in per cent) for the experimental group. Both groups consisted of 15 rats.

The reduced pO_2 distinctly accelerated erythropoiesis in the rats. During the 10-day study the mean blood hemoglobin rose from 12.0 g/100 ml to 16.8 g/100 ml ($p < 0.001$) and hematocrit from 45 % to about 65 %.

As a general conclusion, air at a reduced pressure of ½ ATA has an effect similar to that of a hypoxic oxygen nitrogen mixture of 1 ATA (Figs. 7 and 8) and delays healing.

II. EFFECT OF OXYGEN SUPPLY ON THE CHEMICAL COMPOSITION OF EXPERIMENTAL GRANULATION TISSUE

Effect of various oxygen nitrogen mixtures at 1 ATA

Treatment started one day after implantation

Hemoglobin Hemoglobin analyses were carried out to get information on the extent of vascularity in the granulation tissue (Fig 12) A clear increase in the amounts of granuloma hemoglobin occurred in all oxygen tensions from the 1st to the 7th day The highest content was reached in 18 % O on the 7th day Already on the 10th day the hemoglobin contents were clearly lower No marked differences were observed in the amounts of hemoglobin in the 15-day granulomas Throughout the series, the lowest hemoglobin contents were found in granulomas from rats exposed to 12 % O_2.

The contents of hemoglobin determined in the present work are lower

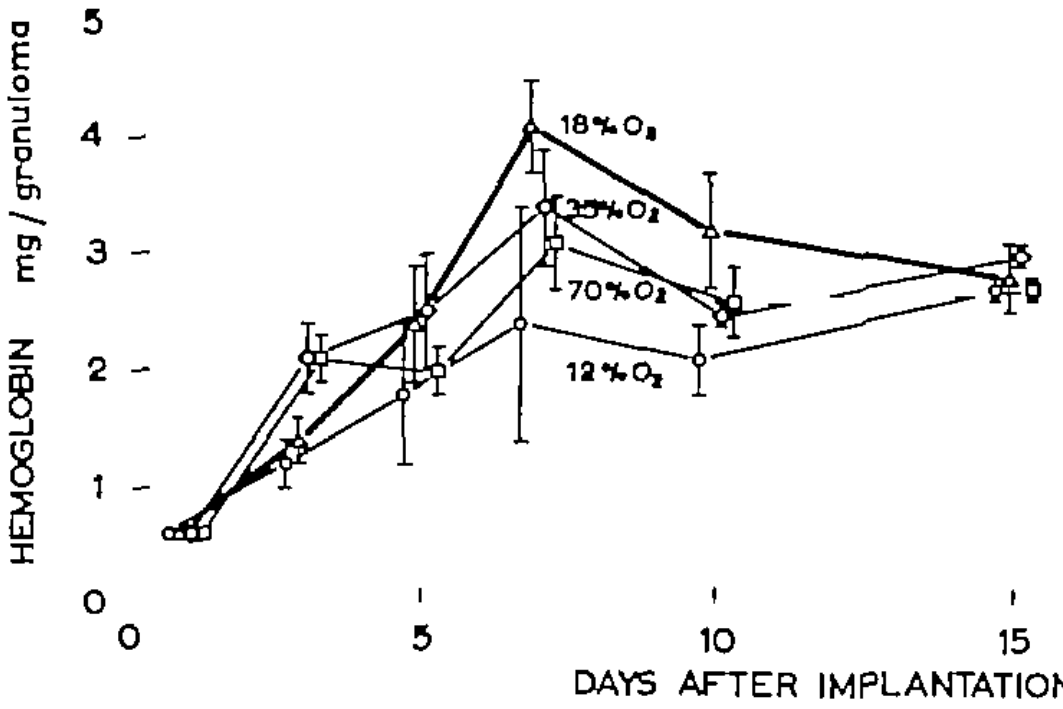


Fig 12. Effect of various respiratory oxygen concentrations on the content of granuloma hemoglobin. All values are means for 4—7 samples (16—23 granulomas) The length of each vertical bar represents twice the standard error of the mean.

than the values reported by Viljanto (1964) This can be due to a different porosity of the viscose cellulose sponge or to loss of granuloma exudate during the decapsulation of the implants.

In addition to vascularity the content of hemoglobin in blood affects the hemoglobin content of the granulomas. On the 15th day blood hemoglobin was 2.8 g/100 ml higher in rats exposed to 12 % O than in rats exposed to 18 % O_2, but no marked changes in blood hemoglobin took place in rats exposed to the other oxygen tensions.

Water soluble hexosamine The formation of mucopolysaccharides in granulomas is partly reflected in the content of hexosamine. As shown in Fig 13, the accumulation of water-soluble hexosamine in the granulomas took place in two phases. In the first phase the hexosamine content rose to

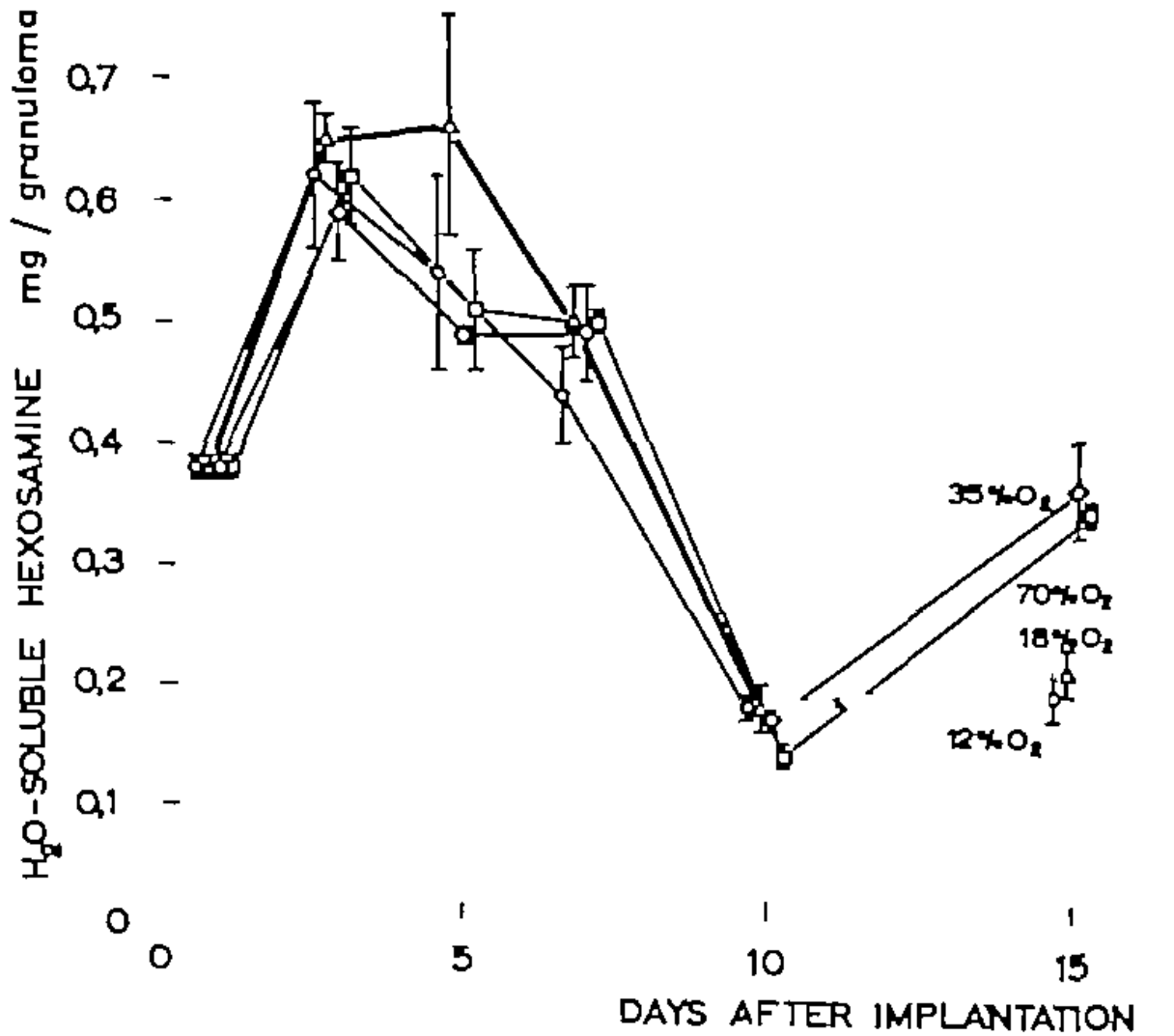


Fig 13 Effect of various respiratory oxygen concentrations on the content of water soluble hexosamine granulation tissue. All values are means for 4—8 samples (16—32 granulomas) The length of each vertical bar represents twice the standard error of the mean.

a maximum already on the third day and then fell steeply to a minimum on the 10th day In the second phase from the 10th to the 15th day the low concentration was maintained in 12 % O and 18 % O_2, whereas a statistically significant increase of the hexosamine content occurred in 35 % O and 70 % O (in the *t*-test, $p < 0.01$)

Nitrogen of unpurified collagen fractions Four fractions were obtained (Fig 4) The total nitrogen contents of the granulomas and of the unpurified collagen fractions are plotted in Figs. 14 and 15

The *water-soluble fraction* originated mainly from the exudate absorbed by the implant on the first few days and from plasma proteins which entered the sponge with circulating blood. The fraction contained also the water soluble nitrogenous compounds synthesized within the implant itself. From the 1st to 7th day the content of nitrogen in the water-soluble fraction was approximately the same in granulomas of rats exposed to different oxygen tensions. From the 10th day onwards, the mean values for rats exposed to 35 % O and 70 % O were higher than those for rats exposed to 12 % or 18 % O (in the analysis of variance $p < 0.001$ on the 15th day)

Nitrogen in the *fraction soluble in 1 M sodium chloride* rose evenly from the 1st to the 15th day From the 10th day onwards, the mean value rose concurrently with increasing oxygen pressure (in the analysis of variance $p < 0.01$ on both the 10th and the 15th day)

The amount of nitrogen in the *acetic acid-soluble fraction* rose from the 1st-day value of 0.25 mg/granuloma to a level of 0.5 mg/granuloma on the 15th day irrespective of the atmospheric oxygen concentration.

The nitrogen in the *insoluble fraction* rose slowly from 1.4 mg/granuloma on the 1st day to 3.5—5.0 mg/granuloma on the 15th day In the 15-day granulomas, the mean value rose with increasing oxygen tension in the hyperoxic environment (in the analysis of variance, $p < 0.01$)

Hydroxyproline in unpurified collagen fractions Chemical analyses on the granulomas confirmed that the tensile strength increased with the content of collagen hydroxyproline (Fig 7 as compared with Figs. 16 and 17) Generally the hydroxyproline contents of the different fractions remained almost unchanged during the first 5 days at various oxygen tensions. After 5 days, however the hydroxyproline contents rose sharply An elevated oxygen concentration accelerated the formation of hydroxyproline whereas a low oxygen concentration retarded collagen synthesis.

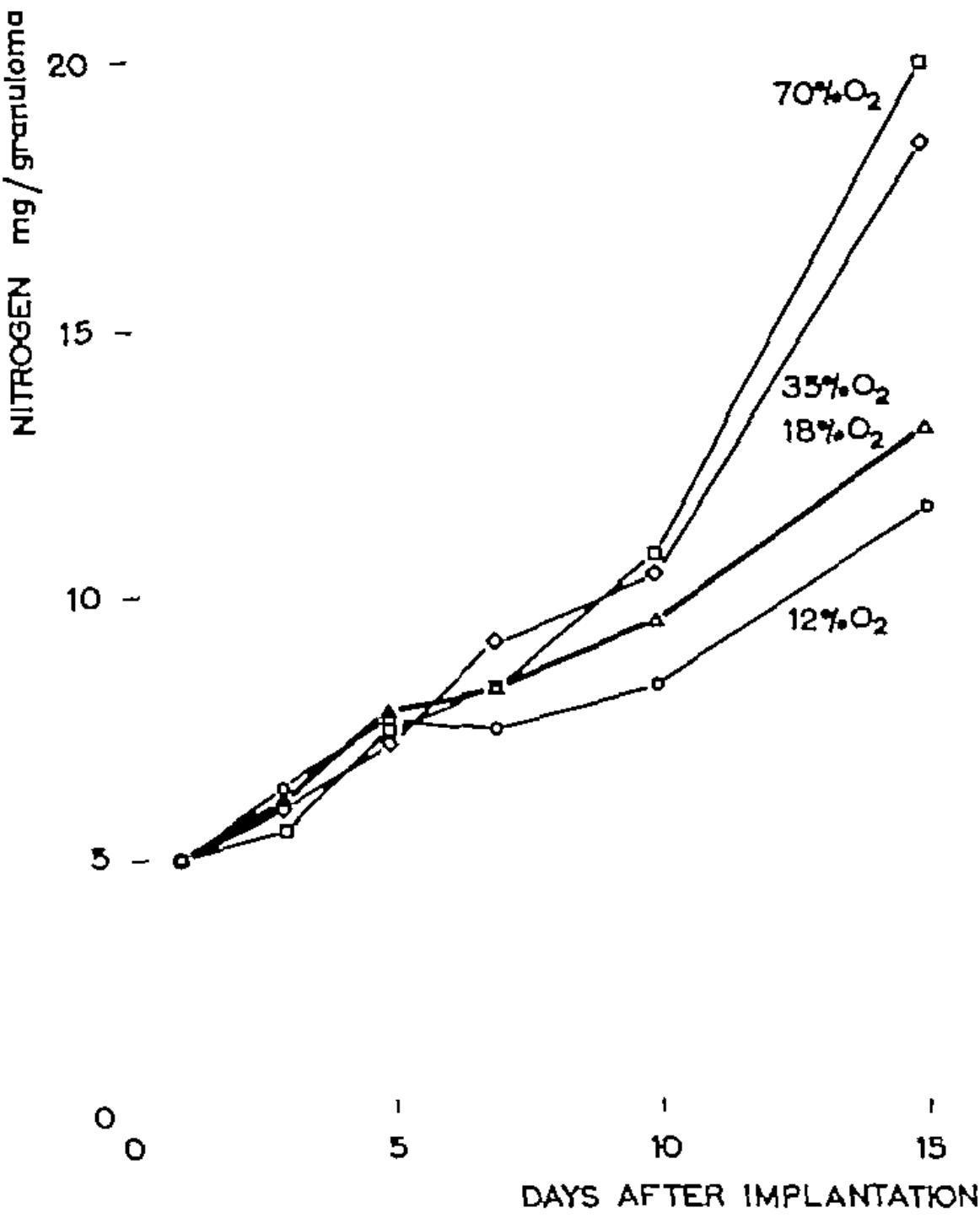


Fig 14 Effect f various respiratory oxygen concentrations on the content f total nitrogen in granulation tissue

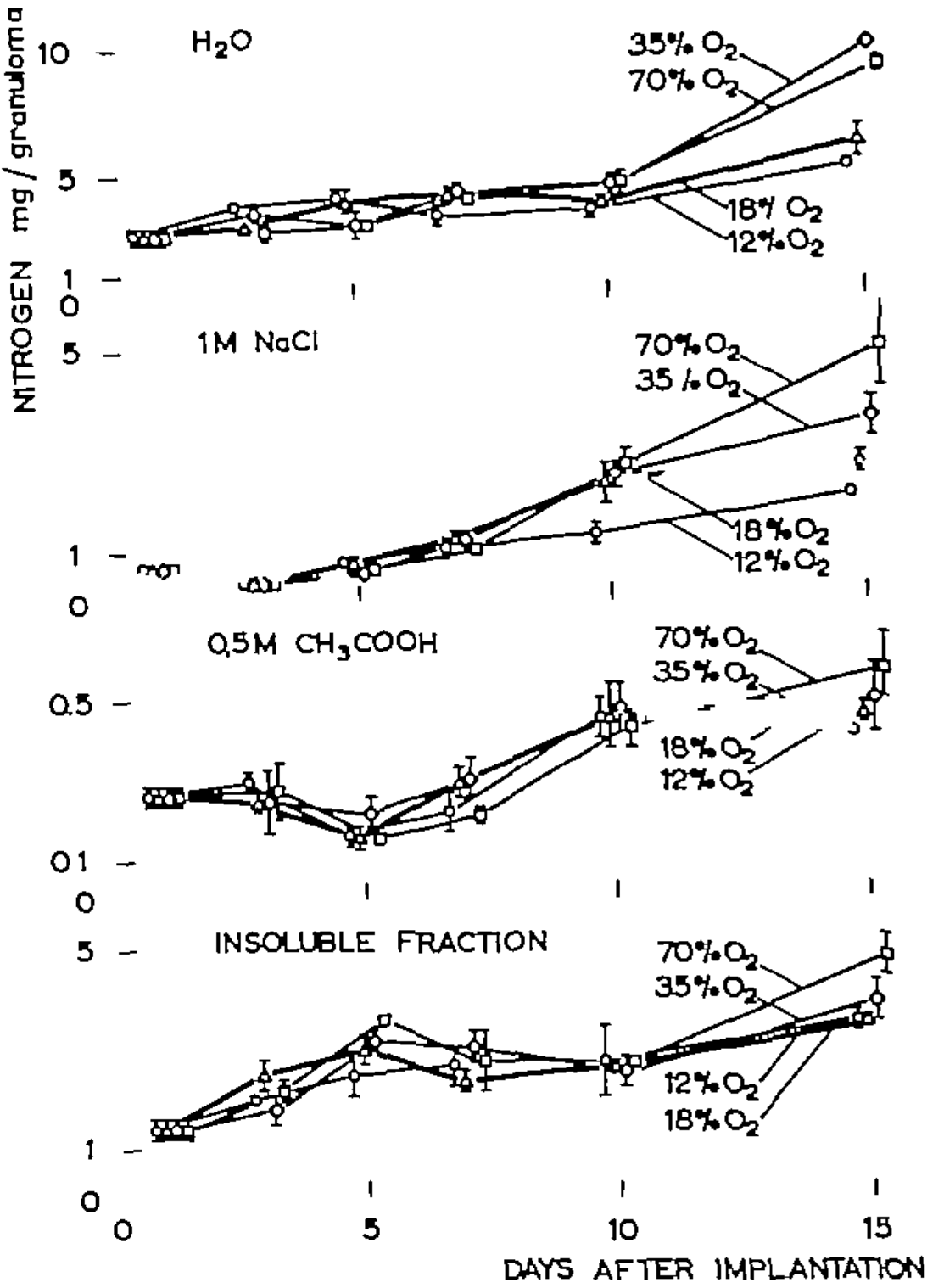


Fig. 15 Eff t various re pirat ry oxygen concentrations on the total nitrogen i unpurified colla fra tions f om gr ulation tissue All alues are means for 4—8 samples (16—1 granulomas) The length of each vertical bar represents twice the standard error the mean. The fractions were obtained as described in Fig 4.

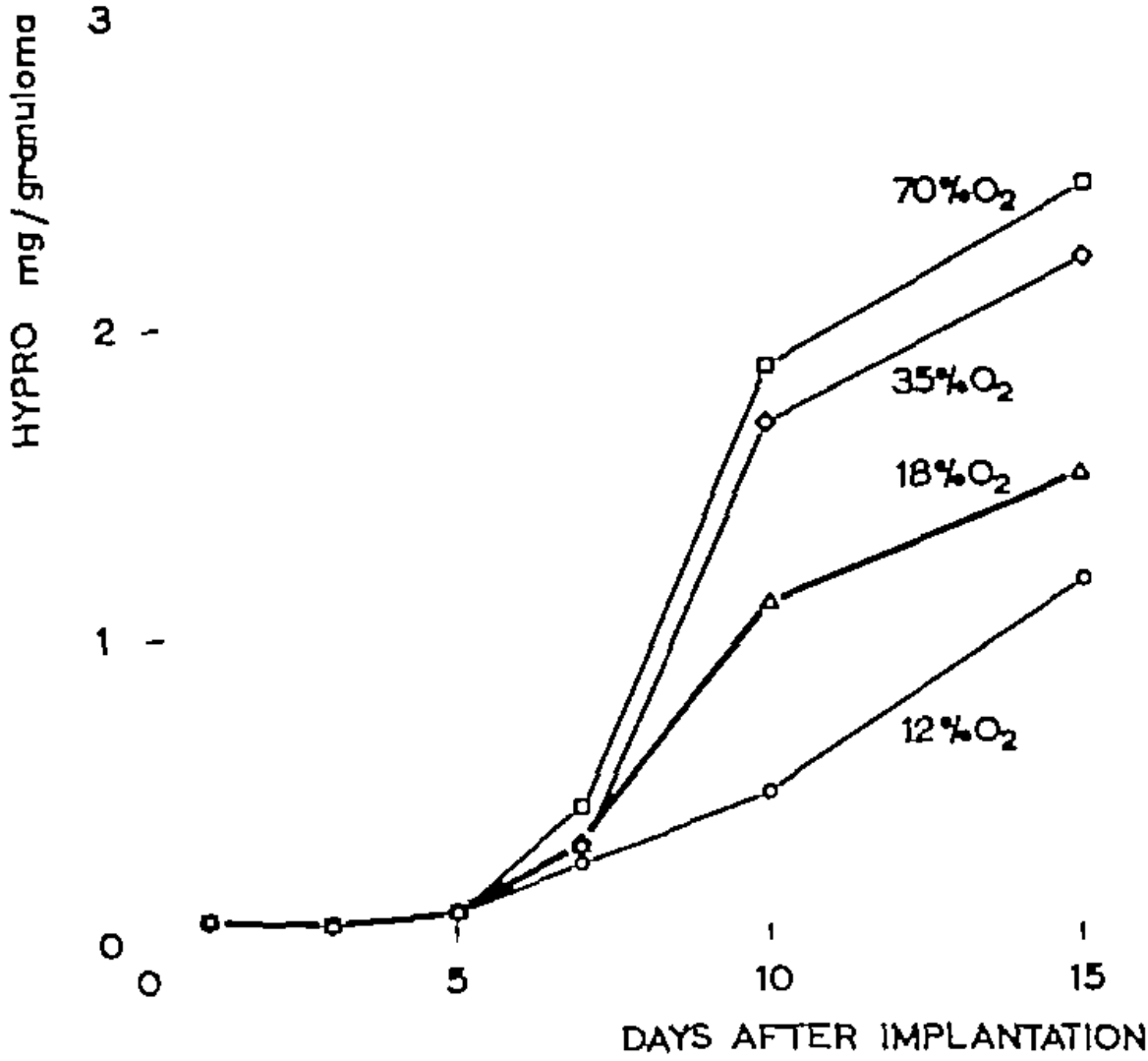


Fig 16. Effect of various respiratory oxygen concentrations on the content f total hydroxyproli in gran lation tissue.

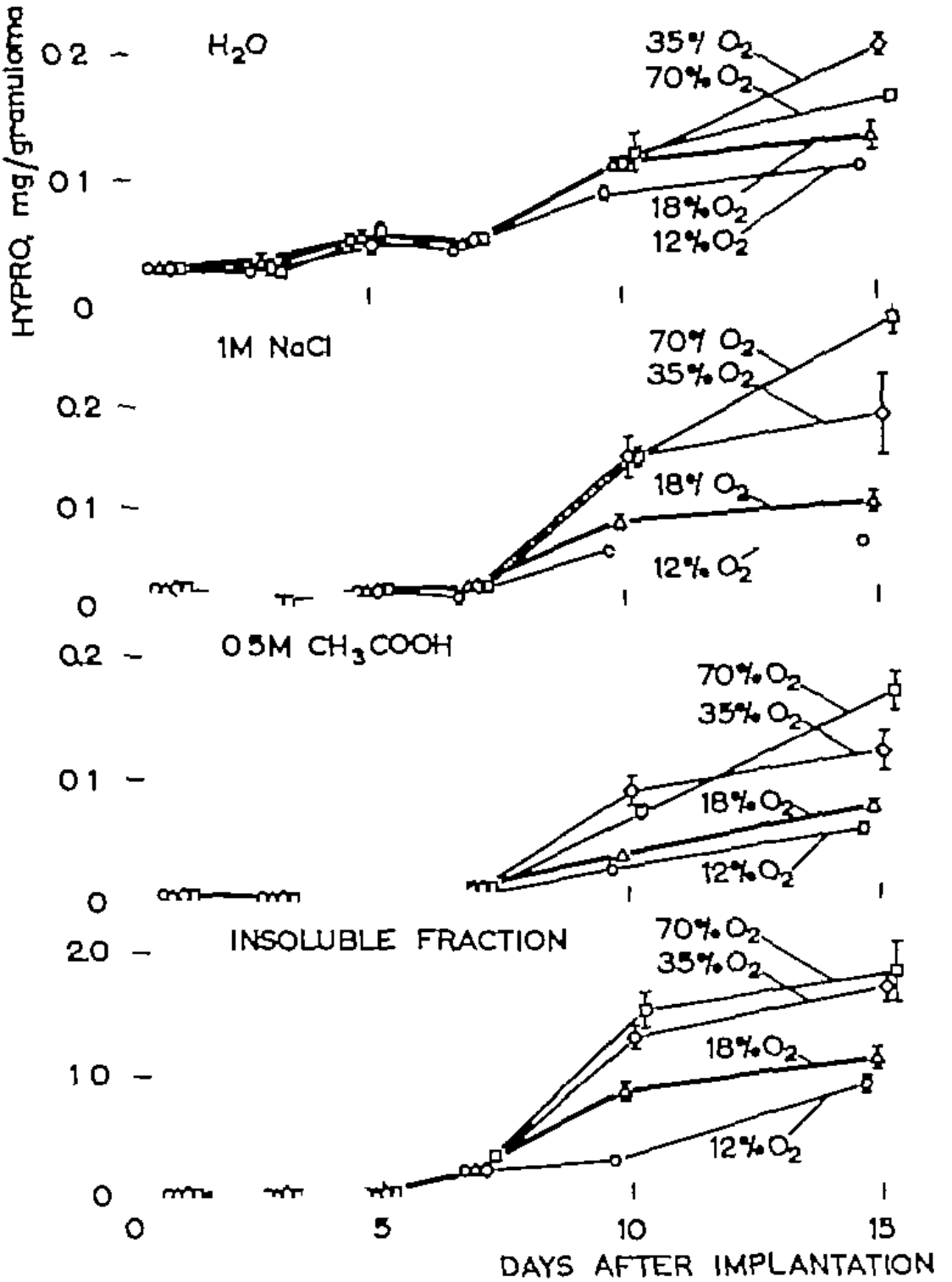


Fig 17 Effect of various ambient oxygen concentrations on the contents of hydroxyproline in unpurified collagen fractions from granulation tissue All values are means for 4—8 samples (16—3 granulomas) The length of each vertical bar represents twice the standard error of the mean. The fractions were obtained as described in Fig 4.

An increase in respiratory oxygen tension elevated significantly the content of *water-soluble hydroxyproline* in the 10- and 15-day granulomas (in the analysis of variance $p < 0.05$ and $p < 0.001$ respectively). According to Viljanto (1964) half of the water-soluble hydroxyproline is free hydroxyproline in normal conditions.

The hydroxyproline of the *neutral salt soluble fraction* rose distinctly faster from the 7th day onwards in 70 % and 35 % O_2 and more slowly in 12 % O than in 18 % O (in the analysis of variance, $p < 0.001$ on both the 10th and the 15th day). Seventy per cent oxygen and 35 % O accelerated and 12 % O retarded the formation of hydroxyproline in the *acid soluble fraction* as compared with the rate of formation in 18 % O (in the analysis of variance, $p < 0.001$ on both the 10th and the 15th day).

The level of hydroxyproline in the *insoluble fraction* began to rise on the 5th day. The greatest increase occurred in 70 % O_2. The formation of hydroxyproline was also accelerated in 35 % O_2, whereas in 12 % O_2 it was retarded as compared with the rate of formation in 18 % O (in the analysis of variance $p < 0.001$ for the 10-day values and $p < 0.01$ for the 15-day values).

Hydroxyproline-proline ratio. For the determination of the hydroxylation ratio of the imino acids, the amino acid composition of gelatin extracted by hot trichloroacetic acid solution and exhaustively dialyzed was assayed by an automated method developed by Spackman *et al.* (1958). The amino acid composition of collagen varied insignificantly in granulomas from rats exposed to different oxygen tensions. The content of glycine was approximately one third of the total amino acid content. Varied oxygenation did not influence the amounts of hydroxy amino acids. The hydroxyproline proline ratio was the same (about 0.7) in the different groups.

Maturation of collagen. The maturation of collagen occurred similarly in the different oxygen concentrations as judged from the ratio of insoluble collagen hydroxyproline to total collagen hydroxyproline. The proportion of insoluble collagen hydroxyproline rose from the 1st-day value of 45 % to 80—90 % on the 7th day and remained unchanged thereafter.

Deoxyribonucleic acid. The DNA contents of the granulomas from rats exposed to various oxygen tensions are shown in Fig. 18. The contents remained nearly constant during the first 5 days. From the 7th to the 10th day the formation of DNA in granulomas of rats exposed to 35 % O was clearly accelerated in comparison with the formation in granulomas of rats exposed to 18 % O (in the *t*-test, $p < 0.05$ on the 10th day). The DNA content in granulomas of the rats exposed to 18 % O rose evenly

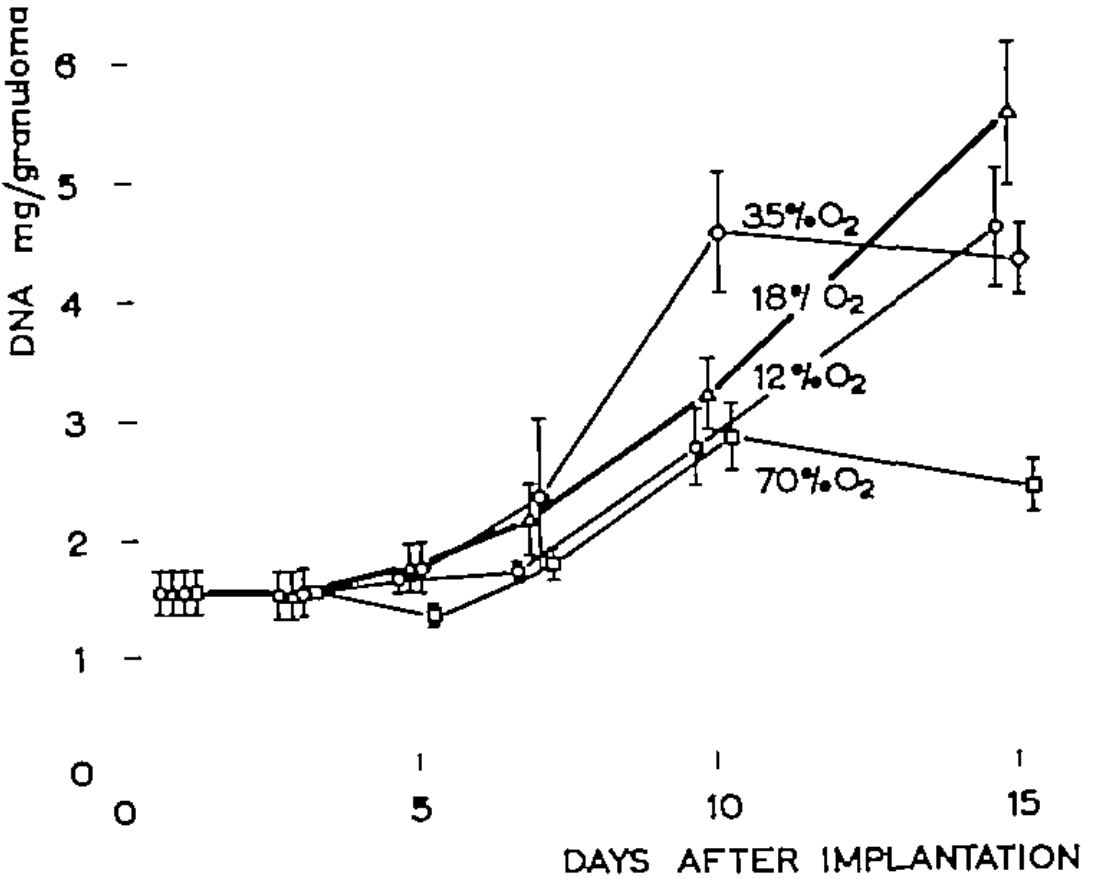


Fig 18 Effect of various respiratory oxygen concentrations on th content f deoxyribon cleic acid in gran latio tissue. All values are means for 4—8 samples (16—83 granulomas) The length of each vertical bar represents twice the standard error of the mean.

from 1 74 mg/granuloma on the 5th day to a maximum of 5 51 mg/granuloma on the 15th day Also in 12 % O_2, the content of DNA increased continuously from the 5th day but to a smaller extent than in 18 % O_2. It is remarkable that the formation of DNA was clearly retarded in 70 % O as compared with the rate of formation in 18 % O (in the *t*-test $p < 0.01$ for the 15-day values) The changes between the 10th and 15th lay were checked by replicate experiments and analytical determinations.

Ribonucleic acid. RNA was assayed as ribose. The changes in RNA ribose content of granulation tissue at various oxygen concentrations are shown in Fig 19 During the first three days the contents were approximately the same in granulomas from rats exposed to different oxygen tensions. This level was maintained in 12 % O until the 7th day The highest RNA ribose contents from the 5th to the 15th day were attained in 3o % O (in the *t*-test as compared with the results for granulomas from rats exposed to 18 % O_2, $p < 0.001$ on the 10th day) The content of RNA

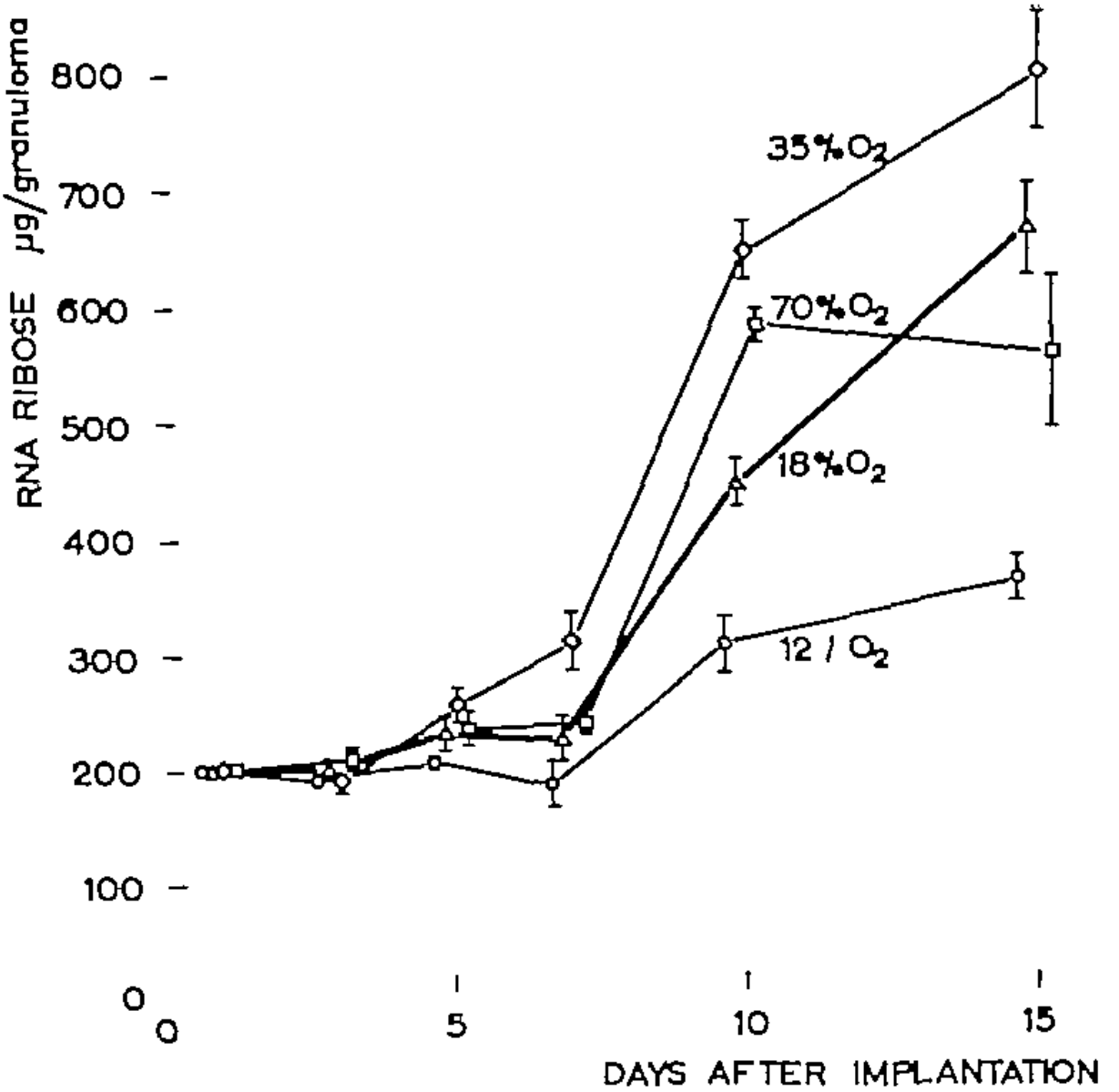


Fig 13 Effect of various respiratory oxygen concentrations on the content of RNA ribose in granulation tissue All values are means for 4—8 samples (16—32 granulomas) The length of each vertical bar represents twice the standard error of the mean.

was greater on the 10th day in rats exposed to 70 % O than in rats exposed to 18 % O (in the *t* test, $p < 0.001$) In 18 % O_2, the RNA ribose rose evenly from the 7th day onwards on the 15th day the mean exceeded the mean in 70 % O Formation of RNA was distinctly retarded at a low oxygen tension (12 % O) as compared with the formation in 18 % O (in the *t*-test, $p < 0.001$ on both the 10th and 15th day) The changes between the 10th and 15th day were checked by replicate experiments and analytical determinations.

RVA ribose.DNA ratio In granulation tissue the ratio of RNA ribose to DNA reflects the potential of fibroblasts to synthesize protein. Furthermore, the ratio can be regarded as an index of cellular differentiation.

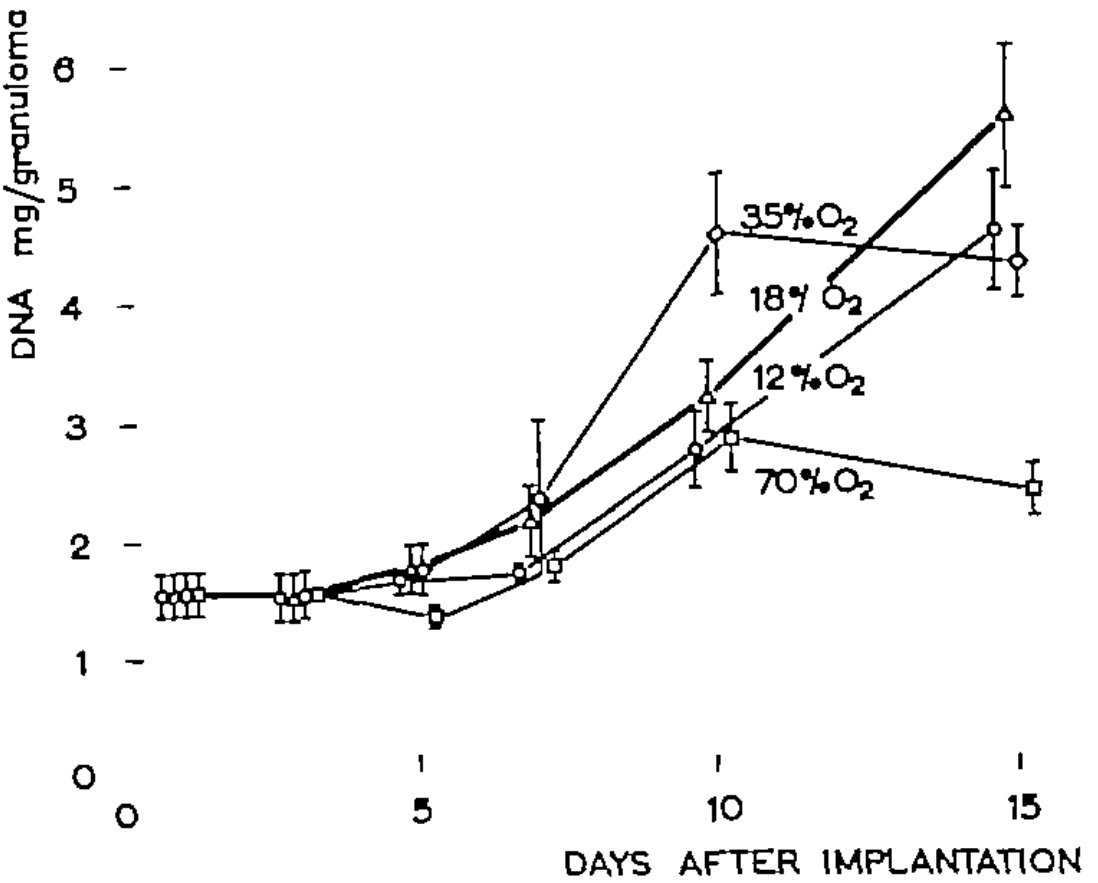


Fig 18 Effect f various respiratory oxygen concentrations on the content of deoxyribonucleic acid granulation tissue All values are means for 4—8 samples (16—33 granulomas) The length of each vertical bar represents twice the standard error of th mean.

from 1 74 mg/granuloma on the 5th day to a maximum of 5.51 mg/granuloma on the 15th day Also in 1° % O_2, the content of DNA increased continuously from the 5th day but to a smaller extent than in 18 % O It is remarkable that the formation of DNA was clearly retarded in 70 % O as compared with the rate of formation in 18 % O (in the *t*-test, $p < 0.01$ for the 15-day values) The changes between the 10th and 15th lay were checked by replicate experiments and analytical determinations.

Ribonucleic acid RNA was assayed as ribose. The changes in RNA ribose content of granulation tissue at various oxygen concentrations are shown in Fig 19 During the first three days the contents were approximately the same in granulomas from rats exposed to different oxygen tensions. This level was maintained in 12 % O until the 7th day The highest RNA ribose contents from the 5th to the 15th day were attained in 3o % O (in the *t* test as compared with the results for granulomas from rats exposed to 18 % O_2, $p < 0.001$ on the 10th day) The content of RNA

Treatment started five days after implantation

Supply of oxygen during the latent phase seems to be important for the subsequent synthesis of collagen and RNA. A low oxygen concentration during the first 5 days inhibited the formation of granuloma hydroxy proline and RNA ribose (Table III) The values for the animals exposed to a normal oxygen tension differ from those for animals exposed to a low oxygen concentration during the latent phase. At high oxygen pressures, differences in the hydroxyproline and DNA contents arose only in granulomas of rats exposed to 35 % O_2. A high oxygen concentration during the latent phase seems to retard the formation of capillaries as judged from the hemoglobin contents. No apparent changes were observed in the nitrogen contents when these were compared with the nitrogen contents after treatment with different oxygen pressures from the second day onwards.

Table III

The chemical compositions of 10-day-old granulomas from rats exposed to various oxygen concentrations from the 6th postimplantation day onwards

The values are means for 4 samples (16 granulomas) The standard errors of the means are also given. The results are compared with those obtained on exposure of rats to various oxygen tensions from the second postimplantation day onwards. The mean relative differences in per cent between the results for the two groups are enclosed in parentheses. A value greater than that obtained after treatment from the second day onwards is indicated by a positive percentage, and *vice versa*. The statistical significance has been tested by the analysis of variance; NS denotes not significant.

Variable (per granuloma)	O_2 % 12	18	35	70	P
Hemoglobin (mg)	2.17±0.60 (+3 %)	2.86±0.04 (+11 %)	3.81±0.7 (+59 %)	4.78±0.6 (+81 %)	<0.05
Nitrogen (mg)	8.0±0.5 (—3 %)	10.1±0.6 (+6 %)	11.1±0.7 (+8 %)	10.4±0.4 (—3 %)	<0.05
Hydroxyproline (µg)	960±268 (+93 %)	1156±50 (+4 %)	1383±77 (—18 %)	1665±156 (—6 %)	<0.05
DNA (mg)	2.47±0.18 (—10 %)	3.33±0.38 (+4 %)	3.63±0.73 (—20 %)	3.96±0.30 (+3 %)	NS
RNA ribose (µg)	464±10 (+52 %)	5 6±30 (+22 %)	635±40 (—3 %)	597±15 (+3 %)	<0.01

Effect of hyperbaric oxygenation

Analysis of granulomas confirmed that the tensile strengths of wounds and granulomas in rats exposed intermittently to hyperbaric oxygen (Fig 10) decreased with the contents of hydroxyproline and nitrogen (Table IV) The reduction of the hemoglobin level was probably due to a diminished vascularity The amount of water-soluble hexosamine was not affected by OHP The slight decreases in the amounts of DNA and RNA ribose were not statistically significant.

Table IV

Effect of hyperbaric oxygenation on the chemical composition of experimental granulomas

The numbers of rats are given in parentheses. The figures express means ± the standard errors of the means. The statistical significance was determined by the *t* test.

Variable (per granuloma)	4-day study		10-day study	
	Control	Experiment	Control	Experiment
Hemoglobin (mg)	2.48±0.1(6)	1.60±0.20(6)	3.04±0.26(5)	1.83±0.38(5)
Water-soluble hexosamine (μg)	340±38(6)	391±20(6)	271±4 (5)	255±15(6)
Nitrogen (mg)	6.3±1.0(6)	5.9±0.7(6)	9.0±0 (6)	9.0±1.3(5)
Hydroxyproline (μg)	*149±11(6)*	*1.4±11(6)*	*1769±163(4)*	*1457±83(6)*
DNA (mg)	1.36±0.03(9)	1.34±0.03(9)	2.83±0.26(8)	2.41±0.18(9)
RNA-ribose (μg)	198±9(9)	184±8(9)	494±18(8)	477±23(9)

Significantly different values, $p < 0.03$.

Effect of reduced barometric pressure

As seen in Table V the formation of all components of granulation tissue was retarded when rats were kept in air at ½ ATA. Low pO retarded the formation of capillaries in the granulation tissue as judged from the content of hemoglobin (by the *t* test, the difference was almost significant, $0.05 < p < 0.1$) A statistically significant decrease was observed in the content of water-soluble hexosamine. The content of collagen hydroxy

proline decreased to approximately half of the control value. The total nitrogen content changed accordingly The decreased contents of DNA and RNA ribose at ½ ATA indicate retarded cellular proliferation

Table 7

Effect of reduced barometric pressure (½ ATA) on the chemical composition of experimental granulomas.

The numbers of rats are given in parentheses. The figures are means ± the standard errors of the means. The statistical significance was determined by the *t*-test; NS denotes not significant.

Variable (per granuloma)	10-day study		p
	Control	Experiment	
Hemoglobin (mg)	3.40±0.31(8)	2.63±0.25(8)	<0.1 NS
Water-soluble hexosamine (μg)	363±41(8)	229±19(8)	<0.03
Nitrogen (mg)	10.1±0.3(8)	8.6±0.3(8)	<0.001
Hydroxyproline (μg)	1418±54(8)	838±59(8)	<0.001
DNA (mg)	2.78±0.34(7)	2.69±0.17(7)	<0.03
RNA ribose (μg)	575±27(7)	408±27(7)	<0.001

III. IN VIVO AND IN VITRO STUDIES ON THE MECHANISM OF THE ACTION OF OXYGEN IN THE SYNTHESIS OF VARIOUS GRANULATION TISSUE COMPONENTS

Studies on energy metabolism

Utilization of glucose in vitro

During the first 10 days, glucose uptake *in vitro* by slices of granulation tissue removed from rats exposed to various oxygen concentrations followed a similar course as the formation of collagen hydroxyproline in granulomas (Fig 71) After a latent period of 7 days, the higher the availability of oxygen *in vivo* the greater was the amount of glucose utilized *in vitro* by the slices of granulation tissue (in the analysis of variance, $p < 0.001$ for the 10-day values) Statistically significant changes were no longer observed on the 15th day The utilization of glucose by slices of granulation tissue was not correlated with the DNA content of the granuloma (Fig 18)

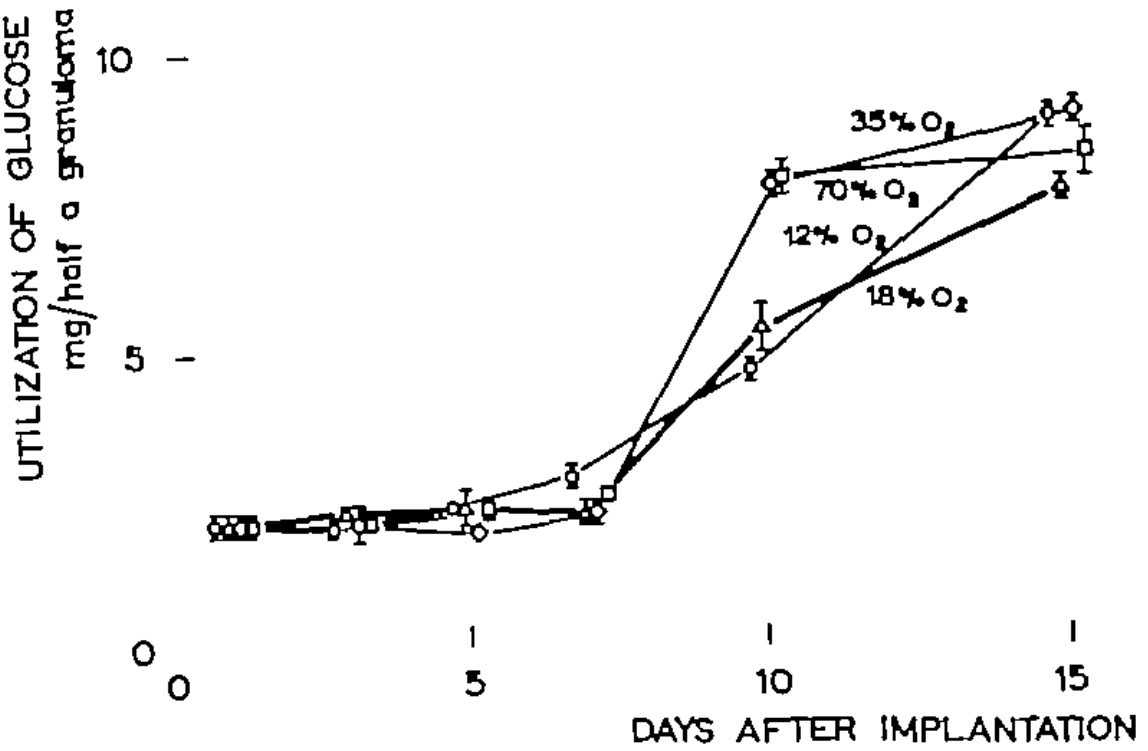


Fig 21. Utilisation of glucose by slices of granulation tissue removed from rats exposed to various oxygen concentrations. Slices of granulation tissue were incubated for 3 hours in a Krebs-Ringer phosphate medium containing glucose. The values are means from 4—12 experiments. The length of each vertical bar represents twice the standard error of the mean (for details, see pp. 20—21)

Formation of lactic acid in vitro

In contrast to the uptake of glucose formation of lactic acid by slices of granulation tissue from rats exposed to various oxygen concentrations had no latent period. As seen in Fig 22, the higher the availability of oxygen *in vivo* the greater was the amount of lactic acid produced by the granuloma slices *in vitro* already from the third day onwards (in the analysis of variance $p < 0.01$ on the third day $p < 0.001$ on the 5th day $p < 0.05$ on the 7th day $p < 0.001$ on the 10th—15th day). However mixtures containing 35 % O_2 and 70 % O_2 had almost the same effect. Again, differences exist between the patterns of granuloma DNA (Fig 18) and the rates of lactic acid formation by the slices.

The utilization of glucose and the formation of lactic acid by slices of granulation tissue reflect the rate of growth of the tissue. The results concur with those of Lampiaho and Kulonen (1967) according to whom glycolysis is a prominent feature in the metabolism of granulation tissue, and especially in the synthesis of collagen.

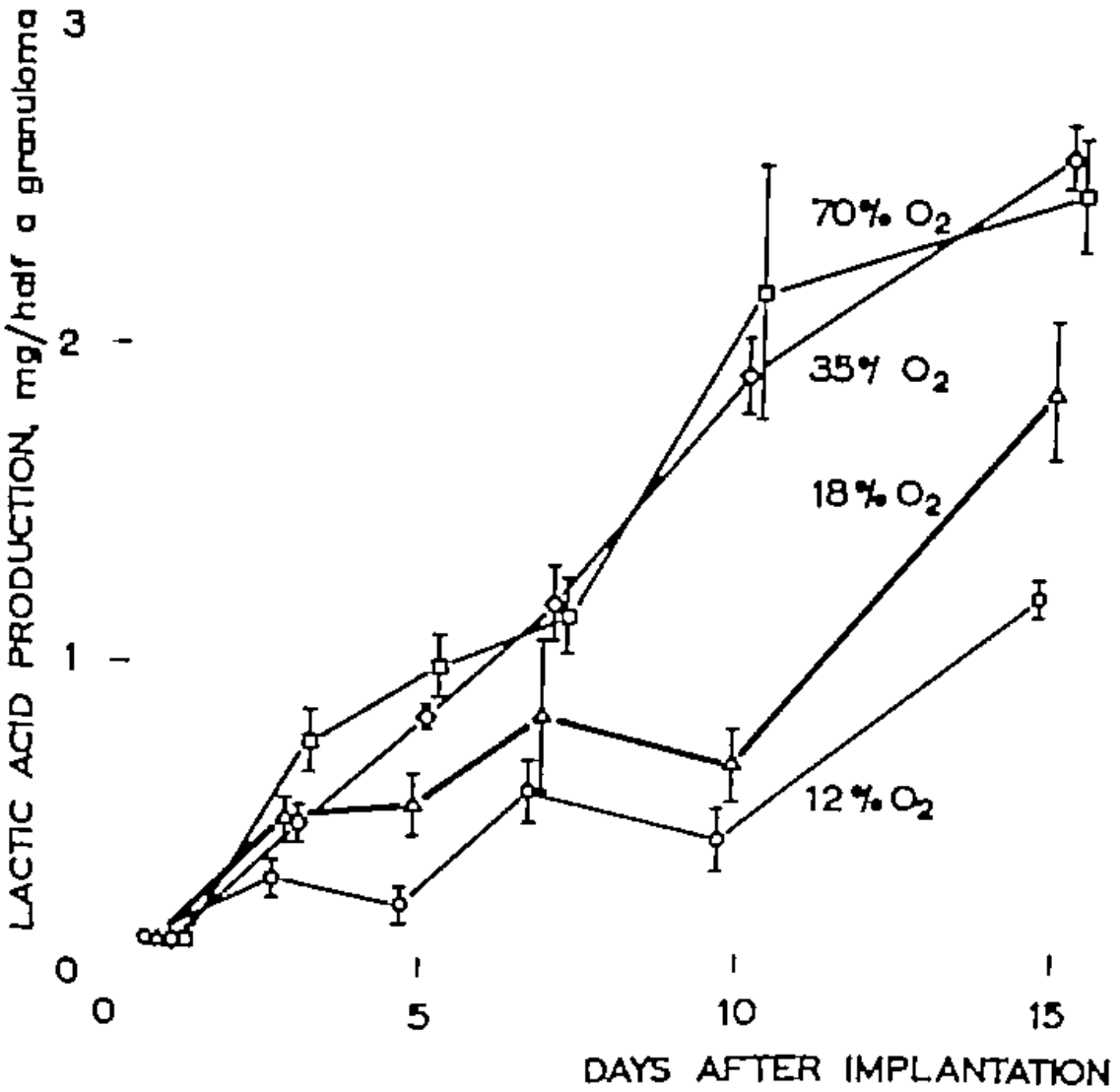


Fig 22. Production of lactic acid by slices of granulation tissue removed from rats exposed to various oxygen concentrations. The experimental conditions were the same as in Fig. 21. All values are means of 4—12 experiments. The length of each vertical bar represents twice the standard error of the mean.

Lactate dehydrogenase in vivo

The lactate dehydrogenase activity of the granuloma supernatant was not dependent on respiratory oxygen tension and varied between 7600 and 11000 mU/granuloma on the 10th postimplantation day. The electrophoretic patterns of the LDH-isoenzymes underwent no changes. Only one anaerobic isoenzyme typical of a tissue in which a glycolytic metabolism prevails was observed.

Incorporation of ^{3}H proline in vitro

Accelerated synthesis of collagen in high oxygen concentrations (Fig 16) was observed also by the incorporation of H proline into collagen of incubated granuloma slices (Table VI) Exposure to 40 % O_2 in vivo or during subsequent incubation had effects which were approximately additive In experiments 1 and 3 40 % O_2 accelerated the synthesis of collagen more than the synthesis of noncollagenous protein as judged from the ratio of H-collagen to noncollagenous H protein. In experiment 2, the ratio was almost the same in the different groups.

Table VI

Effect of oxygen supply on the levels of incorporated H proline and H-hydroxyproline in slices of granulation tissue

The granulation tissues had developed during 10 days in rats breathing the indicated atmospheres. The incubations were carried out in air or 40 % O_2. After preincubation period of 30 min, 80 μCi of ^{3}H proline was added to the medium and the incubation was continued for 4 hours. In the test controls trichloroacetic acid was added to the medium to a final concentration of 5 % to prevent the incorporation (for details, see pp. 20—21 and Fig 5)

Oxygen supply (in vivo/in vitro)	dpm × 10⁻³				
	^{3}H Hypro				
	Total ^{3}H	Spec. ^{3}H/μmol	^{3}H-Collagen	Noncollagenous H protein	^{3}H-Collagen /Noncollagenous ^{3}H protein
Experiment 1					
19 % O_2/air	1	2.4	42	631	0.062
19 % O_2/40 % O_2	3	4	64	640	0.100
40 % O_2/air	33	6.3	106	780	0.136
40 % O_2/40 % O_2	99	7.6	193	712	0.278
Test control	0.01	0.002	0.03	1.1	0.027
Experiment 2					
19 % O_2/air	1	1.0	29	900	0.032
19 % O_2/40 % O_2	18	7	35	1078	0.032
40 % O_2/air	5	1.8	49	1597	0.031
40 % O_2/40 % O_2	1	1.4	42	1579	0.02
Test control	0.02	0.008	0.04	10	0.004
Experiment 3					
19 % O_2/air	29	2	59	1080	0.055
19 % O_2/40 % O_2	53	5	106	133	0.079
40 % O_2/air	67	3.5	113	1688	0.067
40 % O_2/40 % O_2	53	3.1	111	1232	0.090
Test control	0.01	0.004	0.02	3.4	0.006

Incorporation of ^{3}H thymidine in vivo

Preliminary tests showed that H thymidine was not incorporated *in vitro* into the DNA of slices of 10-day-old granulomas that had developed in rats exposed to 19 or 40 % O_2. This indicated that the proliferation of fibroblasts in the slices had ceased or had greatly slowed down

The total amount of H-thymidine incorporated into granuloma DNA on the 10th postimplantation day *in vivo* was essentially the same in 19 and 40 % O (*Table VII*)

Table VII

Effect of oxygen supply on the incorporation of H thymidine into the DNA of granulation tissue in vivo.

The experimental procedure is described on p. 21. DNA was extracted from the granulomas as described in Fig 5. Both experiments were run in duplicate

Sample	Oxygen supply	
	10 % O_2	40 % O_2
Experiment 1		
Total ^{3}H (dpm × 10^{-4})	59	64
Spec. ^{3}H/mg of DNA (dpm × 10^{-4})	8.6	9.5
Experiment 2		
Total H (dpm × 10^{-4})	60	61
Spec. H/mg of DNA (dpm × 10^{-4})	3.1	6.7

Incorporation of ^{3}H-cytidine in vitro

Data on the incorporation of H-cytidine into the RNA of granuloma slices at various oxygen concentrations are presented in Table VIII. The rate of RNA formation was over twice as great in the granulation tissue that developed *in vivo* in 40 % O as in controls. The highest labeling was usually found in granulation tissue exposed to 40 % O both *in vivo* and *in vitro* The lowest incorporations and specific activities were observed in the granulation tissue that had grown in 19 % O *in vivo* and then in air *in vitro*

T ble VIII

Effect of oxygen supply on the incorporation f H-cytidine into the RNA in slices f granulation tissue

Th granulation tissues had formed during 10 days in rats inhaling the indicated gas mixtures. The experimental conditions during the incubations were the same as in Table VI except that in the test controls perchloric acid was added t a final concentration of 0.5 N to prevent the incorporation. After a preincubation period of 30 min, 30 μCi of ³H-cytidine was dded and the incubation continued fo 4 hours *(for details, see pp. 20—31)*

Oxygen supply *in vivo/in v tro*	Total ³H dpm × 10^{-4}	Spec. ³H/μg of RNA ribose dpm × 10^{-4}
Experiment 1		
19 % O_2/air	49	3.1
19 % O_2/40 % O_2	61	3.6
40 % O_2/air	148	7.3
40 % O_2/40 % O_2	106	6.8
Test co trol	8.4	0.7
Experiment 2		
19 % O_2/air	23	1.5
19 % O_2/40 % O	43	2.7
40 % O_2/ai	77	3.2
40 % O_2/40 % O_2	101	3.3
Test control	3.2	0.2
Experiment 3		
19 % O_2/air	27	3.2
19 % O_2/40 % O_2	20	2.4
40 % O_2/air	61	3.8
40 % O_2/40 % O_2	71	4.8
Test control	0.8	0 6

As already shown in Table III adequate oxygenation, especially during the first 5 days, is of great importance for the synthesis of RNA. Ten days after sponge implantation 40 % O stimulated the incorporation of H-cytidine into the RNA of granulation tissue but had no marked effect on the incorporation of H thymidine in.o the granuloma DNA. These data support the finding that the RNA DNA ratio in granulation tissue becomes greater in a hyperoxic environment than in air (Fig 20)

Incorporation of ³²P phosphate in vivo

The experiments with ³²P-phosphate were undertaken to obtain infor mation on the formation of *cytoplasmic membranes of granulation tissue*

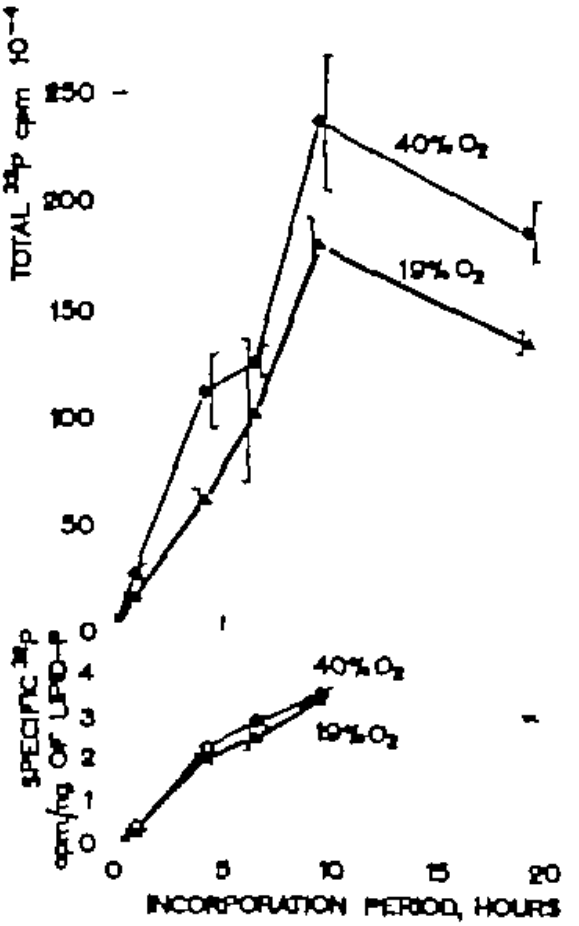


Fig 23 Effect of oxygen supply on the incorporation of ³²P phosphate into extranuclear phospholipids of granulation tissue in vivo. All values are means for 3—8 samples (6—16 granulomas) The length of each vertical bar represents twice the standard error of the mean. The experimental procedure is described on pp. 21—22.

cells in various oxygen tensions. The specific and total radioactivities of the extranuclear lipid bound phosphorus were determined after various incorporation periods. The atmospheres employed contained 19 % O and 40 % O_2.

As shown in Fig 23 the synthesis of extranuclear phospholipids in 10-day-old granulomas was distinctly more rapid in 40 % O than in 19 % O (in the *t* tests, $p < 0.001$ except for the 6.5-hour values which were not significant) It is remarkable that no differences were observed in the specific activities of the extranuclear lipid bound phosphorus at different oxygen tensions. The data indicate that the formation of extranuclear phospholipids took place during the incorporation periods at rates attained already before the injection of ³²P phosphate. The maximum labeling occurred during a period of 9.5 hours.

To conclude, enhanced synthesis of extranuclear phospholipids [illegible] in granulation tissue of rats exposed to 40 % O_2, where also the formation of RNA was augmented (Table VIII)

DISCUSSION

COMMENTS ON MATERIAL AND METHODS

Gas tensions in the breathing mixtures In the experiments at 1 ATA, the oxygen concentrations were lower in the animal boxes than in the gas cylinders (Table II) This was due to entry of air through the exit hole to the oxygen consumption of the animals, and to accumulation of carbon dioxide When the rate of gas flow was 6 l/h the oxygen concentration in the boxes housing the control rats varied between 21 and 18 % O During a 24-hour period, the two rats in each box produced a carbon dioxide concentration of about 4 % (Fig 3 Table II) which caused some hyperventilation However the short term increase of the carbon dioxide concentration had no disturbing effect on the rats as judged from the even rise of the weights of the animals during the experiments and from the fact that the removal of carbon dioxide effected no essential changes in the tensile strength values of the healing wounds. In the experiments in hyperbaric oxygen and at reduced barometric pressure the accumulation of carbon dioxide inside the pressure chambers was almost negligible

Partial pressure of oxygen in the arterial blood and granulation tissue Hunt *et al* (1967) found that the pO in wire mesh cylinder granulomas was 11—17 mmHg but from measurements like these it is not possible to decide if the oxygen tension in granulation tissue cells is below the critical value for respiration. In the present study the pO in the granulomas was not measured. As calculated from the data of Tribukait (1963b) the arterial pO is 175 mmHg in 35 % O_2, 215 mmHg in 40 % O_2, 425 mmHg in 0 % O 570 mmHg in 90 % O and 1500 mmHg in oxygen at 2 ATA In all these conditions, the cellular oxygen pressure should also rise but certainly not proportionately (*cf* Hunt 1965)

Signs of oxygen toxicity Prolonged inhalation of 70 % O_2 caused death of four of about 100 animals on the 10th—15th day At autopsy the lungs of the four animals exhibited diffuse hemorrhagic consolidation and edema. Histologic sections (hematoxylin-eosin or hematoxylin van Gieson stain) revealed patchy consolidation and congestion throughout the lung field,

perivascular edema exudate in the alveolar space and thickened alveolar walls. Histologic sections of lungs from rats exposed to 70 % O for 9—14 days showed perivascular edema around the small vessels. All these findings are signs of oxygen toxicity (Pfister *et al.* 1964 Spencer *et al.* 1966 Wittner and Rosenbaum 1966) No rats exposed to OHP or 35—40 % O showed any signs of oxygen toxicity (such as convulsions or histologic changes in pulmonary tissue)

DISCUSSION OF RESULTS

Effect of oxygen supply on the tensile strengths of a healing skin wound and experimental granulation tissue

The oxygen concentration in breathed gas for optimal wound healing appeared to be 35—70 % O (Figs. 7 and 8) As compared with the controls, an increase of almost 40 % in the tensile strength of a healing wound had taken place in 70 % O on the 7th day after incision. The increase in strength appeared to slow down during the later course of the healing process. The increase in strength on the 10th day was approximately 30 % and on the 15th day no more than about 10 % Prolonged inhalation of almost pure oxygen (90 % O) did not affect the tensile strength of a healing wound (Fig 8) Intermittent short term exposures to hyperbaric oxygen at 2 ATA led to reduced wound strength (Fig 10) as also did prolonged inhalation of mixtures of low oxygen content (Figs. 7 8 and 11) The variation in tensile strength of experimental granulation tissue correlated well with that of the healing wounds.

In the study of Lundgren and Sandberg (1965) hyperbaric oxygenation started on the day of wound infliction retarded wound healing in rats significantly The unfavorable effect of OHP seemed to display a dose response pattern. However when the treatment was started on the second day the tensile strengths of healing skin wounds were the same in the experimental and control groups. This does not agree with the results of present investigation. In 1965 Beckham and Hitchcock established that OHP at 2.5 ATA can influence beneficially the rate of healing of clean surgical wounds in dogs. Two facts cast doubt on the general validity of this finding (1) even the longest observation period of the study was only 4 days, which is too short for reliable tensile strength measurements (2) the dogs were under anesthesia during the whole observation period.

Effect of oxygen supply on the chemical composition of experimental granulation tissue

Hemoglobin The content of granuloma hemoglobin can be regarded as indicative of the volume of circulating blood only after the 5th day when the implant has developed its own vascularity (Viljanto 1964) The peak content of granuloma hemoglobin was attained in 18 % O on the 7th day (Fig 12) Low pO retarded the formation of capillaries. The decrease in hemoglobin content with increasing oxygen tension (Fig 12, Table IV) indicates a decreased vascularity caused by higher oxygenation of tissues, but even vasoconstriction should lead to a lower hemoglobin content Variations in the hemoglobin content of the blood do not seem to be important in this connection

Water-soluble hexosamine The high contents of water-soluble hexosamine during the latent phase originated most likely from plasma glycoproteins that accumulated in the granulomas after sponge implantation (L. C Edwards *et al.* 1957 Schür 1957 Jackson *et al* 1960) The clearly increased hexosamine contents on the 15th day in granulomas of rats breathing 35 and 70 % O probably reflected an accelerated synthesis of mucopolysaccharides (Fig 13)

Collagen Analyses of the granulomas confirmed that the tensile strengths in optimal oxygen tensions were positively correlated with the amounts of collagen synthesized (Figs. 16 and 17 Tables IV and V) Varying the oxygen tension did not affect the amino acid composition of collagen (p 41) The nitrogen contents of the unpurified collagen fractions merely reflected the amount of collagen (Figs 14 and 15)

Maturation of the granuloma collagen was not affected by an elevated or lowered oxygen tension (p 41) The result does not agree with the findings of Chvapil *et al* (1968) and Tsurufuji and Ogata (1965) but concurs with that of Heikkinen (1968) who observed that the formation of insoluble collagen in radioactive skin slices *in vitro* was not affected by anaerobic conditions.

Nucleic acids There is evidence that fibroblasts are more resistant to a subnormal oxygen tension than other cell types. Powell (1959) pointed out that diethyl dithiocarbamate influenced oxidation reduction reactions and thus prevented cell respiration and that it affected sarcoma tumor cells more than fibroblasts. Clark (1964) showed that the growth of adult mouse fibroblasts in tissue culture at oxygen concentrations above 3 % did not differ from the growth in air Kenny and Fink (1966) established that the optimal pO (60 mmHg) for the growth of fibrogenic cells in tissue culture is below the normal oxygen tension of the atmosphere (152

mmHg) Maximum growth of the L strain mouse fibroblasts occurred at oxygen concentrations from 10 to 30 % (Brosemer and Rutter 1961) This finding is in good agreement with the results of present study where the highest levels of DNA *in vivo* were attained at oxygen concentrations from 12 to 35 % (Fig 18) The maximum formation of DNA and RNA took place in 35 % O during the first 10 days (Figs. 18 and 19) In 70 % O the synthesis of DNA was retarded throughout the experiments. However the granulation tissue cells in the rats exposed to 70 % O_2 had rather high contents of RNA (Fig 19) and synthesized protein more efficiently than cells at a lower pO_2 (Fig 20)

Histologic examination of granulomas Microscopic examination of slices of granulomas stained with hematoxylin-eosin revealed changes in the total number of cells, erythrocytes, capillaries and collagen formation at various oxygen pressures which agreed fully with the results of the biochemical studies (the data of the histologic study are not presented)

The mechanism of the action of oxygen in wound healing

Chronic superficial ischemic ulcers in man have been induced to undergo more rapid healing by intermittent exposures to hyperbaric oxygen (Illingworth *et al.* 1961 Baffes *et al.* 1966 Hall *et al* 1966 Slack *et al.* 1966) Skin flaps with impaired circulation have survived with intermittent short-term exposures to hyperbaric oxygen (Perrins 1966 Niinikoski 1969) The favorable effect of adequate circulation has been attributed to an elevation of temperature an increased supply of nutrients and an adequate supply of oxygen (Sandblom 1944)

Wound margins are separated by a minimal fibrin layer at the beginning of the healing process. It is reasonable to assume that increased phagocytosis and fibroblast activity require more oxygen than surrounding normal tissues.

In the present study inhalation of 35—70 % O at 1 ATA had the most favorable effect on wound healing In the presence of excess oxygen, *e.g* at OHP the energy metabolism in the healing wound may be controlled by adenosine diphosphate (ADP) phosphate or substrates, and the kinetics of oxygen utilization will be independent of the oxygen concentration. At decreased oxygen tensions, hemoglobin becomes deoxygenated, large gradients of oxygen tension develop in the tissue and eventually cytochrome oxidase is reduced. The electron flow diminishes. Adenosine triphosphate (ATP) production decreases and the energy transfer is then controlled by the diffusion of oxygen to the respiratory enzymes in the mitochondria.

The increase in ADP and the decrease in the energy reserves may evoke glycolytic activity (Chance 1965)

The oxygen supply is important for healing since collagen cannot be synthesized in the absence of molecular oxygen (Fujimoto and Tamiya 1962, 1963 Prockop *et al.* 1967 Lamport 1963 Hutton *et al* 1967) An increased supply of oxygen can cause (1) an accelerated hydroxylation of proline bound in the collagen precursor or (2) a less specific stimulation of the growth of granulation tissue. Attempts were made to decide between these two alteratives.

Action of oxygen in the synthesis of collagen hydroxyproline The formation of hydroxyproline appeared to be a special target of oxygen Forty per cent oxygen promoted the formation of hydroxyproline both *in vivo* and *in vitro* (Table VI) The effect of increased oxygen tension on the synthesis of noncollagenous protein was much smaller perhaps insignificant In *in vitro* studies reported elsewhere (Niinikoski *et al* 1967) a maximal proline-hydroxylating activity (Juva 1968) was observed in granulomas removed from rats exposed to 18 % O_2 It is interesting to note that oxygenation during the latent phase seems to be important for certain synthetic activities, especially the synthesis of collagen (Table III)

Retarded synthesis of granuloma collagen resulted from exposure to a hypoxic environment (Fig 16 Table V) A low pO_2 probably caused a relative deficiency of oxygen required in the hydroxylation of proline and, in addition inhibited cellular growth during the first ten observation days (Table V)

The results of the present study agree well with the data of Kao *et al* (1963) and of Lampinho and Kulonen (1967) who found that the synthesis of collagen was stimulated *in vitro* when the atmospheric oxygen concentration was raised from 21 to 95 % A different result was obtained by Chvapil *et al* (1968) collagen synthesis in chick embryo skin slices was promoted by oxygen only up to a content of 20 % v/v

Intermittent exposures to hyperbaric oxygen at 2 ATA tended to reduce the formation of collagen hydroxyproline in granulomas (Table IV) This was possibly due to toxic effects of oxygen such as the inhibition of SH enzymes (Haugaard 1965) to suppression of the growth of fibroblasts (Brosemer and Rutter 1961 Clark 1964 Kenny and Fink 1966) to activation of lysosomal proteolytic enzymes (Sledge 1965) to suppression of glycolysis by oxygen, i.e the Pasteur effect (*cf* Cruickshank and Trotter 1956) or to the changes in pH and carbon dioxide levels.

Action of oxygen in the development of granulation tissue Changes in respiratory oxygen tension affected profoundly the RNA DNA ratio in granulation tissue (Fig 20) The most noteworthy effect of oxygen

was its effect on the incorporation of labeled cytidine into RNA and the availability of oxygen was decisive during the growth of the granuloma *in vivo* (Table VIII) In contrast, an elevated oxygen pressure had no effect whatsoever on the incorporation of labeled thymidine into DNA (Table VII)

Because the finding relating to RNA suggested that the metabolic apparatus for the synthesis of collagen is a special target of the action of oxygen the incorporation of radioactive phosphate into the extranuclear lipid fraction was studied. It was found that the formation of extranuclear phospholipids was enhanced in 40 % O as compared with the formation in 10 % O (Fig 23) *These results indicate an augmented ability of* fibroblasts to synthesize protein as well as an enhanced rate of cellular differentiation at an increased oxygen tension. Supporting evidence was given by Bassot and Herrmann (1961) who noted that variation of the oxygen tension profoundly affects the differentiation of multipotent mesenchymal cells in tissue culture and that collagen fiber formation was favored by 35 % O

The increased activity of fibroblasts in granulomas led to the utilization of more glucose (Fig 21) and to the production of more lactic acid *in vitro* (Fig 22) These findings agree with those of Lampiaho and Kulonen (1967) who found that the consumption of oxygen and glucose and the formation of lactic acid by slices of granulation tissue increased almost linearly during the first 20 days of development of granulation tissue. They observed also that the proliferative phase was characterized by a relative high activity of the pentose phosphate cycle whereas glycolysis dominated during the synthesis of collagen.

In the present study no changes in the total activity or in the isoenzymes of lactate dehydrogenase were observed when the oxygen supply was varied. A different result was obtained by Lindy and Rajasalmi (1966) with chick embryos incubation of the embryos at various oxygen concentrations affected the relative amount of subunit M

To conclude, the action of oxygen in wound healing resolves itself into the following main functions

(1) Oxygen concentrations between 35—70 % at 1 ATA stimulate the synthesis of collagen hydroxyproline, whereas a hypoxic environment retards collagen formation.

(2) A hyperoxic atmosphere (35—70 % O) accelerates both the formation and function of the metabolic apparatus of the fibroblast for protein synthesis.

(3) An elevation of oxygen tension beyond the optimum retards healing probably because of toxic effects on cellular metabolism

contents of hemoglobin were found in granulomas that developed in subnormal oxygen tensions (Fig 12, Table V) High concentrations of oxygen as well as hyperbaric oxygen retarded the accumulation of hemoglobin into the granulomas (Fig 12, Table IV)

The high content of water-soluble hexosamine during the latent phase of the healing originated probably from plasma glycoproteins. The increased contents of hexosamine in 15-day granulomas from rats exposed to 35 and 70 per cent oxygen indicated stimulated formation of mucopolysaccharides (Fig 13) Low pO retarded the formation of hexosamines (Table V)

Adequate respiratory oxygen tension (pO at least 150 mmHg) during the latent phase appeared to be important for the formation of collagen and RNA, but high oxygen concentrations during the latent period retarded the formation of capillaries (Table III)

III. *The mechanism of the action of oxygen in the synthesis of various granulation tissue components*

An increased synthesis of collagen was indicated also by the incorporation of H proline into collagen of incubated granuloma slices. The administration of 40 per cent oxygen either *in vitro* or afterwards during the incubation had effects which were approximately additive. Furthermore a hyperoxic atmosphere appeared to stimulate to some extent the formation of noncollagenous protein (Table VI)

Granulomas that developed *in vivo* in 40 per cent oxygen synthesized more RNA *in vitro* than those which developed in 19 per cent oxygen (Table VIII) The incorporation of ^{3}H thymidine into granuloma DNA *in vivo* was essentially the same in 19 and 40 per cent oxygen (Table VII) The rate of formation of extranuclear phospholipids in granulomas *in vivo* was significantly higher in 40 per cent oxygen than in 19 per cent oxygen (Fig 23)

Granulomas that developed *in vivo* in 35 or 70 per cent oxygen had a greater ability to utilize glucose and produce lactic acid *in vitro* than granulomas that developed *in vivo* in 18 or 12 per cent oxygen (Figs. 21 and 22) No significant variation of the total LDH-activity was observed. Only one LDH isoenzyme typical of a glycolytic tissue was detected.

ACKNOWLEDGEMENTS

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Extrinsic Nervous Control of Colonic Motility and Blood Flow

An experimental study in the cat

BY

LEIF HULTÉN

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CONTENTS

CHAPTER I

General Introduction

I *Morphological considerations* 9

A. Gross structure of the colon in different species 9
B Organization of the colonic wall layers 10
C Vascular supply to the colon 10
D Extrinsic nervous supply 11

1 The parasympathetic supply 11
2 The sympathetic supply 12

II. *Functional considerations* 13

A. The motility pattern of the large bowel 13
B Extrinsic nervous control of colonic motility 14

1. The vagal supply 14
2. The pelvic nerve supply 15
3. The thoracic sympathetic supply 16
4. The lumbar sympathetic supply 17

C Extrinsic nervous control of colonic blood flow 17

1 The vagal supply 17
2. The pelvic nerve supply 18
3 The sympathetic supply 18

III *Summary of earlier findings* 19

IV *The aim of the present study* 19

CHAPTER II

Extrinsic nervous control of colonic motility

L. Hultén and M Jodal

I *Introduction* 21

II. *Methods* 22

A. Operative procedures 22
B Recording of motility 23
C. Nervous stimulations 23

III *Results* 24

A. Patterns of spontaneous colonic motility 24

1 The motility in the non-denervated colonic preparation 24
2. Effects of sympathetic nerve section 24
3. Effects of parasympathetic nerve section 25
4. Volume recordings of spontaneous colonic motility 25

B Effects of graded direct stimulation of the parasympathetic supply 25

1. The vagal nerves 25
2. The pelvic nerves 27

C Effects of graded direct stimulation of the sympathetic supply 28

1 The splanchnic nerves 28
The lumbar colonic nerves 30
3 The hypogastric nerves 31

IV *Comments* 31

CHAPTER III

Extrinsic nervous control of colonic blood flow

L. Hultén M Jodal and O Luxdoren

I *Introduction* 39

II *Methods* 40

A. Operative procedures 40
B Determination of blood flow and blood volume 40
C. Nervous stimulations 41

III *Results* 42

A Effects of graded direct stimulation of the sympathetic supply 42
B Effects of graded direct stimulation of the parasympathetic supply 44

1 The vagal nerves 44
2. The pelvic nerves 44
3 Blood volume determinations 47

IV *Comments* 47

CHAPTER IV

Local and nervous control of the consecutive vascular sections of the colon

L. Hultén M Jonal and O Lundgren

I. *Introduction* 51

II. *Methods* 51

A Operative procedures 51
B Plethysmographic technique 52
C. Determination of the capillary filtration coefficient (CFC) 53

III. *Results* 53

A Infusion of isopropyl-noradrenaline 53

1 Resistance vessels 53
2 Capacitance vessels 53
3. Exchange vessels 54

B Effects of graded direct stimulation of the sympathetic supply 54

1 Resistance vessels 54
2 Capacitance vessels 54
3. Exchange vessels 55

C. Effects of graded direct stimulation of the parasympathetic supply (the pelvic nerves) 58

1. Resistance vessels 58
2. Capacitance vessels 60
3. Exchange vessels 61

IV *Comments* 61

CHAPTER V

Nervous control of blood flow in the parallel-coupled vascular sections of the colon

L. Hultén, M. Jodal and O. Lundgren

I *Introduction* 65

II *Methods* 65

A. Theoretical considerations 65
B Operative procedures 66
C Local injections 67
D Atraumatic labelling 67

III. *Results* 68

A. Resting blood flow of the mucosa submucosa and muscularis 68
B Effects of direct stimulation of the pelvic nerves 68
C Effects of direct stimulation of the sympathetic nerves 72

IV *Comments* 74

CHAPTER VI

Reflex control of colonic motility and blood flow

Leif Hultén

I *Introduction* 77

II *Methods* 79

A Operative procedures 79
B Recording of motility 79
C Determination of blood flow 79
D Nervous stimulations 79

III *Results* 80

A. The intestino-colonic inhibitory reflex 80
B The pelvo-pelvic reflex 82

1 *Effects of mechanical stimulation of the rectal wall* 82
2 *Effects of afferent pelvic nerve stimulation* 85

C Effects of activation of vagal afferents 87
D Effects of afferent somatic "pressor" and "depressor" stimulation 87

IV *Comments* 89

CHAPTER VII

General conclusions and comments

I. *Methodological considerations* 94

II *Main results of the present study* 96

III *Intergrative aspects* 103

Summary 107

Acknowledgment 108

CHAPTER I

GENERAL INTRODUCTION

In many respects the knowledge about the extrinsic nervous control of colonic motility and blood flow is incomplete Around the turn of the century gastrointestinal physiology attracted many investigators, and many studies were conducted not the least concerning the function of the large bowel and its nervous control However the apparent finality of the results obtained in animals seem to have discouraged further investigation as relatively few experimental studies have been reported subsequently In addition, the introduction of the opaque meal greatly stimulated clinical research in the field and much of present knowledge concerning colonic function is therefore based on observations in man. However the experimental conditions in such investigations are of necessity often too complex to allow any definite conclusions with respect to the detailed control of colonic function Furthermore only scant information is available concerning the control of blood supply to the colon and its different wall compartments.

The aim of the present study therefore is to explore in cats the extrinsic nervous control of the colon both with respect to motility and blood supply to the different wall compartments. As an introduction a survey will be given concerning present knowledge of the morphology and physiology of the colon, its blood supply and extrinsic nervous pathways.

I. MORPHOLOGICAL CONSIDERATIONS

A. Gross structure of the colon in different species

One may distinguish between two main types of large bowel, one being typical of carnivores and the other of herbivores with that of omniverous animals falling in between (Garry 1934) In *herbivores* the proximal part, apparently designed for cellulose digestion, is large and sacculated and the longitudinal muscle coat is partially absent The intermediate and distal parts are much narrower have a uniform muscle coat and are devoid of sacculation. In *carnivores* the large bowel is usually short with the outer longitudinal muscles constituting a more or less uniform coat with a vestigeal caecum so that the ileo-colic junction forms a nearly straight tube

In the cat used in this study the colon approximates that of man in form and relative size Thus M'Fadden *et al* (1935) described a fairly characteristic radiological picture of the resting colon of the cat where two main flexures one hepatic and one splenic, made it possible to distinguish a vestigeal caecum, a short ascending colon, a transverse part and a descending part The colon of the cat can however freely alter its position often resulting in the disappearance of the mentioned flexures Therefore it is not fully justified to divide the colon of this animal into segments corresponding to those of human anatomy Perhaps it is better to refer to a proximal and a distal segment and to regard the anal canal as that part encircled by the anal sphincters a nomenclature which will be used throughout this study

B The organization of the colonic wall layers

As is the case with the small intestine, the wall of the colon consists of four layers the mucosa the submucosa the muscular coat and the serosa

The mucosa is covered by a single layer of tall columnar epithelial cells which include goblet cells the source of mucus In contrast to that of the small intestine the colonic mucosa is devoid of villi but its surface is punctuated by closely set epithelial crypts, forming the glands of Lieberkühn which are also lined by abundant mucus-laden goblet cells A thin muscularis mucosa separates the mucosa from the submucosa which contains a rich network of blood and lymph vessels.

Whereas the mucosal organization is largely similar in different species, the muscular coat comprising an outer longitudinal and an inner circular layer differs somewhat In the cat the longitudinal muscle layer forms a continuous enveloping coat while it is condensed into three separate taeniae in man

As in other parts of the gastrointestinal tract the intrinsic nervous system is built up by a submucous (Meissner) and an intermuscular (Auerbach) ganglion plexus. Variations in the general organization of the myenteric plexuses in different colonic parts have been described with respect to the size of the meshes, the thickness of the fasciculi and the size shape and cell content of the ganglia (for ref see Schofield 1965) Auerbach plexus is said to be better developed distally than proximally in the large bowel and in man, where the longitudinal muscle coat is gathered mainly into the taeniae neurons are more numerous adjacent to these Concerning the submucous plexus Müller (1901) found large numbers of neurons especially in distal colonic parts whereas Bielschowsky and Schofield (1962) and Schofield (1962) reported that their number was reduced in these parts.

C. Vascular supply to the colon

The colonic vascular supply in the cat comes from two sources; proximally from the superior mesenteric artery and distally from the inferior mesenteric artery These two arteries are interconnected by a long extramural arcade which extends distally along the dorsal wall and penetrates it tangentially The intra pelvic part of the colon the

rectum and the anal canal are further supplied by small arterial branches from the internal iliac arteries. The veins generally follow the arteries but except for minor collaterals at the anus the colonic blood flow is finally drained via the superior mesenteric vein. There is no venous counterpart to the inferior mesenteric artery

Thorough morphological investigations of the extra and intramural colonic vessels have been performed by different techniques both in animals and in humans Brockis and Moffat (1958) using Neoprene latex in the human pelvic colon described how the arteriae rectae after a subserous course pierce the muscle coat sequentially between the mesenteric and anti mesenteric margins These vessels give off small branches to the serosa and form an external plexus for the supply of the outer muscularis. More deeply the arteriae rectae divide into submucosal branches which continue towards the anti mesenteric border Abundant anastomoses are formed with the arteriae rectae of the opposite colonic side A primary and a secondary submucosal plexus is formed the former one supplying inner muscularis parts and the latter one giving rise to numerous mucosal arteries, which take off at more or less right angles. After piercing the muscularis mucosae these arteries form a plexus from which the rich mucosal supply is derived In general the intramural veins accompany the arteries but the venous plexus in the submucosa is placed closer to the mucosa

Thamm (1940) and Ross (1950) suggested the presence of specific arteriovenous anastomoses in the colonic submucosa. This was more recently investigated by Brockis and Moffat (1957) A few submucosal vessels were suggestive of true anastomoses, according to the latter investigators, but the majority of shunt-like vessels were undoubtedly distended capillaries. Boulter and Parks (1960) studying the submucosal vascular pattern in different gastrointestinal compartments of man demonstrated arteriovenous communications with diameters around 30–35 microns in the stomach while such shunt like vessels were seldom seen in the small and large intestines. As an indication of their relative sparseness 12/cm² were found in the gastric submucosa but only 2/cm² in the colon and rectum. Whether they are truly functional shunts i.e. constituting a non nutritional capillary bypass is not known so far (Wiedeman 1963)

D Extrinsic nervous supply

The autonomic nervous pathways to the colon of cats and rabbits were studied in detail by Langley and Anderson (1895) Trumble (1933) emphasized the accuracy of this work, which was also fully confirmed in a similar study by M'Fadden *et al* (1935) (For ref. see Alvarez, 1948)

1 *The parasympathetic supply*

According to the mentioned investigators, parasympathetic fibres are conveyed mainly via *the sacral outflow* forming *the pelvic nerves* (nn erigentes) which usually

arise from the second and third ventral sacral roots. The pelvic nerve ramifications to the colon have been traced in dogs and cats by Schmidt (1933). He demonstrated that after pelvic nerve section degeneration of nerve fibres throughout most of the colon occurred. Whether or not such fibres run to the most proximal colonic parts is not known for sure. In man parasympathetic fibres have been traced from the pelvic plexus upwards along the hypogastric nerves as far as the origin of the inferior mesenteric artery; from here on the fibres may follow the branches of this artery (Telford and Stopford 1934). In similar studies Mitchell (1935) and Lannon and Weller (1947) observed parasympathetic fibres passing upwards in the mesentery to the promontorial level, from where they obliquely crossed the branches of the inferior mesenteric artery. In dogs and cats, on the other hand, Wright *et al.* (1938), M'Fadden *et al.* (1935) and Mizeres (1955) presented strong evidence to indicate that the sacral parasympathetic fibres, rather than being distributed along the extramural vessels with the sympathetic nerves, enter the colonic wall in its pelvic part and from here on run intramurally in the proximal direction.

There are, however, reasons to believe that parasympathetic fibres to the colon are distributed in *the vagal nerves* as well, though few details are available. A junction in the parasympathetic innervation of the colon was actually first suggested by Cannon (1907). He observed in X-ray studies a ring of contraction dividing the cat colon into two portions, which according to Arendt (1945) was supposed to be caused by an overlap of a cranial and a sacral parasympathetic innervation in man. It would thus appear that the vagal influence in the human colon extends only to proximal parts of the transverse colon at most, a concept which is in agreement with the embryological development (Hunter 1928, Hollinshead 1962 and Schofield 1968). Thus, vagal fibres are supposed to reach proximal colonic parts intermingled with postganglionic sympathetic fibres along the arterial supply. However, convincing histological evidence concerning the extent of vagal fibre distribution to the large intestine seems to be lacking so far. For example, after subdiaphragmal vagal section in cats and dogs Schmidt (1933) could trace degenerated nerve fibres in the ascending colon but not in its distal parts. Jayle and Cabanac (1937) and Wollard (1935), on the other hand, claim that vagal fibres also reach the distal parts of the colon. Mitchell (1935) and Lannon and Weller (1947) found it impossible to trace vagal fibres to more peripheral parts of the colon in dissections on man.

2. *The sympathetic supply*

Little is known whether splanchnic postganglionic fibres reach the colon along the superior vascular supply (Kilee 1913). The main source of sympathetic fibres is therefore supposed to be derived from *the lumbar preganglionic outflow* arising from the second, third, fourth and *sometimes fifth* ventral lumbar roots (Langley and Anderson 1895, Trumble 1933). All or most of these preganglionic fibres pass without interruption through the sympathetic chains and run as *four or five thin strands* to

the inferior mesenteric ganglia forming the spinal rami of the inferior mesenteric ganglia In addition two or more bundles arising from the coeliac or superior mesenteric ganglia pass downward entering the inferior mesenteric ganglia as their coeliac root

The inferior mesenteric ganglia, usually four in number lie close together forming a ring around the inferior mesenteric artery not far from its aortic origin. From these ganglia the postganglionic *lumbar colonic nerves* arise which accompany the inferior mesenteric artery to the colon. Furthermore the paired hypogastric nerves which are also derived from these ganglia run caudally to join the pelvic plexus and give off branches to the ureter bladder internal genital organs and to the sphincter muscles in the anal canal (Learmonth and Markowitz 1929 Trumble 1933)

The literature on the distribution of the extrinsic nervous supply to the large bowel is thus contradictory in several respects especially concerning vagal and splanchnic nerve supply

II FUNCTIONAL CONSIDERATIONS

A. The motility pattern of the large bowel

According to earlier studies the acutely denervated colon in cats exhibits two types of "spontaneous motility rhythmic contractions travelling downwards on which are superimposed shortlasting contractions (Bayliss and Starling 1900) In unanaesthetized cats Cannon (1902) found in X ray examinations that antiperistalsis occurring about every 15 minutes and lasting some 5 minutes constituted a common type of motility in the proximal parts of the colon. The contents were then churned and mixed in a semi-closed sac as the ileocaecal valve prevented reflux Similar observations were made on the proximal portion of the exteriorized colon in dogs by Barcroft and Steggerda (1932) The importance of colonic antiperistalsis was also emphasized by Elliot and Barclay Smith (1904) and it was supposed to arise as a myogenic response to distension These investigators also distinguished a coordinated peristaltic activity moving the contents downwards. This was found especially in middle colonic parts and was supposed to be due to intramural reflexes as a response to distension. The main activity of the distal colon, on the other hand consisted of powerful contraction waves moving downwards and thus emptying the reservoir This latter activity seemed to be largely dependent upon the central nervous system and conveyed by the pelvic nerves. It could be reflexly elicited by distension. According to Cannon (1902) and M'Fadden (1935) defaecation in cat was usually initiated by a powerful wave of constriction starting in middle colonic parts and pulling the shortened bowel towards the pelvis by the recto-coccygeal muscles. This motility pattern was associated with the characteristic defaecation posture contraction of the diaphragm and abdominal muscles, and with relaxation of the anal sphincters.

Colonic motility is known to be affected by activity in more proximal gastrointestinal compartments (Youmans 1949) Thus food intake usually increases colonic activity within few minutes (Hertz and Newton 1913 Welch and Plant 1920 Barcroft *et al* 193?) and initiates a mass peristaltic contraction that often leads to defaecation. The exact pathways of this gastro-colic "reflex" is unknown Douglas and Mann (1930) observed that it was largely unaltered by vagotomy suggesting that neither afferent nor efferent vagal fibres were needed. In fact they suggested that the response might be mediated by a humoral mechanism In experiments on dogs and humans Logan (1967) and Connell and Logan (1967) presented evidence to show that gastrin may play a role in the mediation of the gastrocolic "reflex (For ref see Connell 1968)

It has further been observed that topical stimulation of the lower brain stem close to the vomiting centre causes defaecation (Hatcher 1923 Koppanyi 1930 Wang *et al* 1940) and afferent vagal stimulation is claimed to elicit the same response (Lehman 1913) possibly by activating the bulbar defaecation centres It is also well known that *stress and emotions* considerably affect colonic motility and presumably also colonic secretion and blood flow (e.g Friedman and Snape 1946 Almy *et al* 1949 and Grace *et al* 1951) According to these studies, performed on humans, reduced colonic motility and mucosal pallor occurs in association with overwhelming life situations provocative of abject fear and dejection while situations provocative of conflict with anger resentment and anxiety were associated with increased motor activity and mucosal flushing The sight or smell of food brought about increased motor activity and reddening or engorgement of the mucosa

B Extrinsic nervous control of colonic motility

1 *The vagal supply*

It is still controversial as to what extent vagal fibres reach the colon and certainly as to what an extent they influence motility blood flow and secretion. Thus upon electrical stimulation of the vagal nerves in dogs and rabbits Bayliss and Starling (1900) Elliott and Barclay-Smith (1904) and Klee (1912) were unable to detect any *effects on colonic motility* Carlson (1930) and Wells *et al* (194_) came to the same conclusion in experiments on dogs but the former investigator noted motor effects in proximal colonic parts in monkeys. Gray *et al* (1955) recording motor responses from consecutive sections of the small and large intestines in dogs observed that vagal stimulation regularly caused motor responses in the small intestine and usually delayed responses in proximal parts of the colon and sometimes even in the distal parts. However in these studies the continuity of the intestinal tract appeared to have been left intact implying that a vagally induced motility increase in the proximal intestinal part might have been propagated to the colon along the intrinsic wall plexa

The sympathetic neuro-hormonal influence appears not to have been taken into account in any of the above experiments It is clear from studies by e g Celander (1959) Kock (1959) and Lewenter (1965) that sympathoadrenal influences may markedly affect intestinal motor responses induced by vagal stimulation even to the extent that they can be completely suppressed However only in two recent studies of colonic motility (Fülgraff and Schmidt 1963a, Stavnov *et al* 1963) one of these factors appears to have been considered insofar as the splanchnic nerves had been cut. Vagal stimulation then produced excitatory responses in the proximal parts of the colon in dogs and cats and these responses were blocked by atropine.

2 *The pelvic nerve supply*

The distribution and functional significance of the sacral parasympathetic outflow to the colon (S II–S III the pelvic nerves) is also to some extent debatable even though there is general agreement that these nerves constitute the main excitatory control of distal colonic parts About two thirds of the medullated fibres in the pelvic nerves are efferent and fairly thin (M Fadden *et al* 1935) the remaining third being afferent and containing fibres up to 10 micron in diameter also noted by Langley in his extensive study of the pelvic visceral innervation in cats rabbits and dogs. These latter results, also based on experiments where weak tetanizing induction shocks were used and the motor responses were observed by naked eye suggested that the pelvic nerves produce contraction of the descending colon and rectum These responses were partly unaffected by atropine

Bayliss and Starling (1900) reinvestigated the effect of pelvic nerve stimulation recording colonic motility by means of balloons. They confirmed the results of Langley and Andersson (1895) and further claimed that all parts of the colon are simultaneously affected by pelvic nerve stimulation although this effect appeared to be most pronounced in the distal parts. Preceding the contraction, they often noted transient inhibitory responses which were ascribed to a descending aboral inhibition analogous to the effects of vagal stimulation on the small intestine Furthermore they observed that upon continued stimulation the pelvic nerves appeared to be rapidly fatigued

Garry (1933) studying the nervous control of the caudal region of the large bowel in the cat recorded a drastic fall in tone and some decrease in the rate of rhythmic activity after sectioning the pelvic nerves, indicating a considerable resting "tone in these nerves. This finding could however not be confirmed by M'Fadden *et al* (1935) in a radiological study on cats. With a balloon enterograph and electrical nerve stimulation, Wells *et al* (1942) obtained results in dogs and monkeys suggesting that the pelvic nerve influence was limited to the distal colon only Gray *et al* (1955) on the other hand, concluded that the pelvic nerves affect the motility of the entire colon confirming also Schmidt s dissection studies (1933) Their results thus suggest a considerable anatomical or functional overlap of the vagal and pelvic parasym

pathetics. Wells *et al* (19[illegible]) concluded that the pelvic nerves to the colonic smooth muscles are cholinergic, although they were able to block the responses completely with atropine in only half of the experiments. They also noted that acetylcholine produced a greater circular but a lesser longitudinal response of the colon musculature than that produced by pelvic nerve stimulation and tried to explain this finding by an unequal distribution of the pelvic nerve fibres to the two muscle layers." Garry and Gillespie (1935) showed in a study *in vitro* that the motility response to pelvic nerve stimulation could be abolished by atropine (see also Gillespie 1968) while Fülgraff and Schmidt (1964) concluded that the response was reduced in magnitude but not abolished by atropine. In another series of experiments, these investigators perfused the colonic vessels with Tyrode solution and under these circumstances the motor responses to pelvic nerve stimulation were easily abolished by atropine (Fülgraff and Schmidt 1963 b). After restoration of normal blood flow the same atropine-resistant contractions were again observed. They assumed that the cause of the atropine-resistant contractions was a humoral factor dependent of the blood plasma, but they could not find any evidence for a bradykinin mechanism similar to that found in the salivary glands which here may explain the atropine-resistant vasodilatation (Hilton and Lewis 1955, 1956).

Motor responses in the colon, evoked reflexly by mechanical stimulation of the intestinal wall, have been studied in anaesthetized, decerebrated or decapitated cats by Garry (1933). This extensive investigation suggested that the lumbar sympathetic outflow to the colon is inhibitory in function, that both the afferent and efferent links in the colonic excitatory reflexes are contained in the pelvic nerves and that specific mechano-receptors are located in the mucosa or adjacent parts of the wall of the colon and rectum.

3 *The thoracic sympathetic supply*

The concept th[illegible] splanchnic nerve fibres should be distributed to proximal parts of the colon [illegible] to have little or no experimental support. The only functional [illegible]udies [illegi]al [illegible] this question were performed by Bayliss and Starling (1900), Klee (191[illegible]) [illegible]r (1922). However, they did not observe any effect on colonic motility wh[illegible] splanchnic nerves were stimulated or cut.

The influ[illegible] of these nerves on the motility of the small intestine is, however, well documented (see e.g. Newton 1965). In this study concerning the sympathetic and vagal control of the small intestine it was concluded that distal parts of the small intestine are exposed to a more powerful inhibitory sympathetic control of a "tonic" nature than are the proximal parts. Furthermore, the sympathetic inhibitory fibres appear to exert their intramural effect mainly or perhaps only at the ganglionic level rather than directly on the intestinal smooth muscles. These functional results are in general agreement with the histochemical studies of Norberg (1964, 1965) which suggest that the great majority of adrenergic nerve terminals in the colonic

and gastric walls make contacts with the intramural ganglionic cells. However no clearcut information is so far available concerning the effect of the splanchnic sympathetic outflow on colonic motility and blood supply

4 *The lumbar sympathetic supply*

The lumbar sympathetic outflow the postganglionic fibres of which are named the *lumbar colonic nerves* are known to be mainly inhibitory in their action on colonic motility This is indicated by the effects obtained when these nerves are blocked (Lehmann 1913 Learmonth and Markowitz 1930 Rankin and Learmonth 1930 Garry 1933) or when they are electrically stimulated (Langley *et al* 1895 Bayliss and Starling 1900 Garry and Gillespie 1955 Wright *et al* 1939 Fülgraff and Schmidt 1963 a)

Carlsson (1930) observed on the other hand that stimulation of these nerves could lead to powerful contractions if the colon was relatively atonic, while inhibition resulted if colonic tone was initially pronounced Kuré *et al* (1931) claimed that spinal parasympathetic motor fibres are conveyed to the intestinal tract in the dorsal roots, and these fibres should be cholinergic and run together with the sympathetic inhibitory fibres in the lumbar colonic nerves according to Semba (1956) In fact in recent studies cholinergic fibres have been found to occur so frequently in sympathetic nerves that they may be considered as typical rather than exceptional. The detection of such fibres has been greatly facilitated by the use of reserpine or guanethidine which effectively block adrenergic inhibitory fibres and hence unmask the presence of cholinergic fibres. Thus, Day and Rand (1961) Gillespie and Mackenna (1961) and Boyd *et al* (1962) noted that stimulation of the sympathetic supply to the isolated colon in guanethidine- or reserpine-treated animals sometimes produced contraction instead of inhibition. The origin of these motor fibres could however not be determined but the optimum frequency of stimulation was similar to that of the parasympathetic nerves. It has not been possible to differentiate between the two fibre sets by means of histological analyses. According to M'Fadden *et al* (1935) the postganglionic lumbar colonic and hypogastric nerves contain a small proportion of medullated fibres with a diameter of 2–10 microns. However these might as well represent conventional sympathetic preganglionic fibres which make their synaptic contacts more peripherally

The hypogastric nerves take no obvious part in regulation of colonic motility Learmonth (1931) Garry (1933) but seem to be mainly involved in the control of the anal sphincters, Bishop *et al.* (1956)

C. Extrinsic nervous control of colonic blood flow

1 *The vagal supply*

Besides their gastrointestinal motor control the vagus nerves were previously assumed to contain specific vasodilator fibres. However Kewenter (1965) found no evidence of

such fibres to the vessels of the small intestine in the cat in accordance with earlier observations by Colander and Folkow (1951) Martinson (1965) observed that the high threshold group of efferent vagal fibres produces mucosal vasodilatation concomitantly with gastric secretion and corpus fundus relaxation. This mucosal vasodilatation could however as well be due to the increased production of metabolites in connection with the secretory response or of specific vasodilator agents, such as kinins or histamine It is not known whether the colonic vessels are affected by the vagal nerves and as mentioned above considerable controversy still exists concerning the vagal influence on colonic motility

2 *The pelvic nerve supply*

Langley *et al* (1905) observed flushing of the mucosa in the distal colon upon stimulating the pelvic nerves In an extensive investigation Wright *et al* (1938) studied the effects of pelvic nerve stimulation on the mucosa of the distal colon exposed through a perspex window in decerebrated cats. Stimulation caused a longitudinal colonic contraction a secretory response amounting to about 5 cc/hour and a mucosal vasodilatation Afferent pelvic nerve stimulation produced reflex secretion, which remained after sectioning the spinal cord in the thoracolumbar region. Pilocarpine and acetylcholine after eserine produced similar effects which were blocked by atropine Histological mucosal changes after pelvic nerve stimulation were characterized by increased vascularity around the crypts and a flattening of both goblet and chief cells with a widening of the crypts.

On the whole knowledge concerning colonic blood flow and especially its quantitative distribution to the different wall compartments is scant. The only studies which present quantitative figures of overall colonic blood flow are those of Geber (1960) Delaney and Custer (1965) and Bacaner (1966) while information concerning the capacity for autoregulation of colonic blood flow is presented by Hansson and Johnson (1967) Geber estimated the blood flow of the dog colon to be about 70 ml/min 100 g and Bacaner (1966) presented similar figures in man. Delaney and Custer working on dogs obtained a mean value of 82 ml/min × 100 g employing the radiorubidium distribution method Nothing appears to be known of the normal range of colonic blood flow from intense neurogenic vasoconstriction up to maximal functional hyperaemia

3 *The sympathetic supply*

Langley and Anderssen (1895) noted that stimulation of the lumbar colonic nerves produced blanching of the colon. This was later confirmed and extended by Drury *et al* (1929) Barcroft and Florey (1929) and Wright *et al* (1938) There appears to be no studies concerning the possible effects of stimulation of the splanchnic nerves on the colonic vessels. The vasoconstrictor fibre influence on the stomach and small intestine has on the other hand been studied (see e.g Jansson *et al* 1966 Wallentin

1966) It is well known that the vasoconstrictor fibres in the splanchnic nerves are tonically active, but the gastrointestinal vascular responses to vasoconstrictor fibre activity differ considerably from those in skeletal muscle or skin insofar as e.g the intestinal resistance vessels exhibit an "autoregulatory escape from the vasoconstrictor fibre influence At least partly this escape phenomenon implies a redistribution of mucosal blood flow to submucosal layers (see Wallentin 1966)

III SUMMARY OF EARLIER FINDINGS

Summarizing the results of earlier studies it may be concluded that—analogous to the situation in the small intestine—colonic motility is the result of myogenic activity as modulated and enhanced by the intrinsic ganglionic plexa which is, in turn modulated in both directions by the different types of extrinsic autonomic fibres (Texter 1963 and Kewenter 1965) The considerable degree of autonomy possessed by the large bowel is evident from the fact that even complete decentralization causes only temporary disturbance in motility (Garry 1933) On the other hand the most caudal parts of the large bowel which are directly involved in defaecation appears to be normally controlled by the central nervous system in a similar way as seems to be the case with the reservoir at the other end of the gastrointestinal tract the stomach (e.g Jansson 1969)

Opinions concerning the extrinsic nervous control vary considerably in several respects, e.g concerning the extent and significance of the vagal control of proximal colonic parts, whereas most authors agree that the pelvic nerves affect almost the entire colon. It is therefore possible that proximal parts of the colon might have a double excitatory innervation The significance of this seems obscure.

The lumbar sympathetic outflow exerts an inhibitory influence on colonic motility mainly affecting the distal parts. However this outflow also contains excitatory cholinergic fibres of unknown functional significance There appears to be no experimental evidence of a thoracic sympathetic supply to the proximal colonic parts although this has sometimes been assumed.

Besides their excitatory influence on colonic motility the pelvic nerves induce vasodilatation as well. The mechanism involved is however unknown, as is the extent of the response and its presence in the different wall layers. Whether the vagal colonic innervation also affects the colonic vessels is not known.

Vasoconstrictor fibres in the lumbar sympathetic outflow are on the other hand known to produce mucosal blanching, at least in distal colonic parts but little is known about the vasoconstrictor fibre control in proximal parts or whether the splanchnic outflow is involved here

IV THE AIM OF THE PRESENT STUDY

The anatomical and functional arrangement of the extrinsic innervation of the large bowel and its influence on motility and especially on blood flow is thus in many re

spects poorly understood partly because earlier results are to a considerable extent controversial. The divergent opinions met in the literature may often be explained by different types of interferences between the various sets of parasympathetic and sympathetic nervous supplies. For example a tonic activity in one of the fibre sets may suppress the impact of a nerve fibre blockade or of an electrical activation This is especially true of certain nerve trunks which contain both excitatory and inhibitory fibres.

The aim of the present study is therefore to explore systematically and when possible with quantitative methods the functional organization of the reciprocal nervous control of colonic motility and blood flow which may be unequally balanced in different parts of the colon. Therefore the following aspects of this control were studied

I The extent to which the vagal and pelvic nerves influence motility and blood flow in various parts of the colon.

II The arrangement of the sympathetic nerve supply and its influence on colonic motility and blood flow as compared with that induced by the vagal and pelvic nerves.

III The site of action of the nerve fibres producing colonic vasodilatation and vasoconstriction, both with respect to the different wall layers and the different consecutive vascular sections.

IV The involvement of the various nerve fibre sets affecting colonic motility and vasodilatation in some of the main reflex patterns known to influence function of the large bowel.

It was thought possible that the results might shed further light on the classical concept that the large bowel—apart from its distal sphincteric section—is comprised by two main functionally differentiated compartments. This opinion has so far been mainly based on theoretical and embryological grounds and on some observations of colonic behaviour in the intact organism. However the organization of the involved control mechanisms that may establish such a differentiated function is still little understood in several important respects.

CHAPTER II

EXTRINSIC NERVOUS CONTROL OF COLONIC MOTILITY

LEIF HULTÉN and MATS JODAL

I. INTRODUCTION

Although the nervous control of motility secretion and blood flow in the gastrointestinal tract has been much studied, the very complexity of the system has caused considerable controversy and many important problems remain unsolved. Recently however the efferent vagal control of motility in the stomach (Martinson 1965) and small intestine (Kewenter 1965) has been explored. Thus, gastric motility seems to be controlled by two vagal fibres; first a low threshold group eliciting the well known tonic effect, secondly a high threshold group which induces corpus-fundus relaxation, secretion and mucosal vasodilatation (Martinson 1965). This latter group is involved in "receptive relaxation of the stomach (Jansson 1967). The relaxation thus elicited does not, however necessarily imply any specific vagal fibres. It may be secondary to the secretory response, as a result of the production of tissue metabolites or/and from a local production of vasodilator agents such as bradykinin or histamine. Furthermore while vagal fibres no doubt increase jejunoileal motility there is no evidence of vagal motor fibres to the more distal parts of the gastrointestinal tract.

With respect to the sympathetic inhibitory control of gastrointestinal motility it appears from physiological studies in the cat as if the splanchnic fibres exert their main possibly even their only direct effect at the ileocaecal region (Jansson and Martinson 1966 Jansson 1969). Presumably they also affect the small intestine (Kewenter 1965) These physiological findings are in agreement with recent histo-chemical studies by Norberg (1964), Jodal (1965) and Jodal (1968)

With regard to the colon, it appears from more recent studies, as mentioned in chapter I that vagal fibres may reach proximal parts of the colon via splanchnic sympathetic fibres while the main pathway

from the pelvic nerves. Several physiological studies suggest that there is a vagal excitatory supply to the proximal colon of rabbits cats and dogs, (Langley 1903 Rentz 1938 Starney 1963 and Fülgraff 1963 a) but other investigators (Bayliss and Starling 1900 Carlson 1930 Wells *et al* 1942) could not provide evidence of such an innervation. Lehmann (1913) suggested that the motility response to vagal stimulation might be secondary to concomitant changes in blood flow Studies on this subject have been reviewed by Lannon and Weller (1947) Alvarez (1948) and Truelove (1966)

The functional relations between the suggested vagal supply to the proximal colon and that derived from the pelvic nerves are not fully understood neither are those between this excitatory supply and the sympathetic inhibitory influence, although Norberg s histo-chemical studies (1964 1965) suggest a ganglionic site for the sympathetic influence also in the colon. The functional significance of the excitatory fibres in the sympathetic colonic supply (see chapter I) remains unknown.

Moreover little is known whether the arrangement of the nervous control of the colonic vascular bed differs in the proximal and distal parts. However as earlier reported (Hultén *et al*, 1968) and described in more detail in chapter III the colonic vasoconstrictor fibres seem to be derived from two sources, those to the proximal third being conveyed in the splanchnic nerves and those to the distal two thirds in the lumbar colonic nerves. This finding might be taken as support of the classical concept that the large bowel is differentiated into two main sections. However with respect to involved regulatory mechanisms, this concept is largely unproven. Therefore the present series of experiments was undertaken to investigate whether the extrinsic nervous control of colonic motility is also so organized as to suggest the presence of two functionally different parts.

II METHODS

A. Operative procedures

Experiments were conducted on 115 cats of both sexes, many of which were also utilized for other purposes. Forty animals were utilized for simultaneous recording of colonic blood flow but the results of these will be described in chapter III The animals fasted for 4–48 hours were anaesthetized with chloralose (50 mg/kg) after induction with ether Body temperature was kept constant at 38°C by means of a thermostatically regulated heating pad under the animal. A tracheal cannula was inserted to allow a free airway A plastic tube was introduced via the mouth into the stomach to permit drainage of secretion and swallowed air and another catheter drained the bladder A femoral artery was connected to a mercury manometer for recording of arterial blood pressure. The animals were usually heparinized (3 5 mg/kg).

The abdomen was opened in the midline and the small intestine was extirpated from the duodeno-jejunal flexure to the ileocecal valve except in some animals where the motility of the small intestine was compared with that of the colon In these experiments the proximal 10–15 cm of the jejunum was left *in situ*, separated from the duodenum,

but otherwise left intact. The colonic and jejunal lumina were rinsed from contents for subsequent recordings of motility

B Recording of motility

The jejunal segment and the proximal third of the colon were each connected to a glass tube, which was connected by rubber tubing to an adaptable pressure reservoir coupled to a piston recorder The system was filled with warm Tyrode solution at body temperature. The proximal colonic segment (approximately one third of the total length of the colon) was isolated from the distal one by a loose ligature, so applied that it did not destroy either extra or intramural nervous or vascular connections or the wall continuity The glass tube to the proximal colonic segment was usually introduced from its distal but sometimes from its proximal end through a small longitudinal incision in the antimesenteric wall. By this arrangement the motility of the distal part of the colon could be separately recorded by means of a similar recording system introduced via the anal canal. The nervous or myogenic influences could therefore be freely transmitted along the largely intact wall or along the intact vascular and nervous connections in the mesentery

C. Nervous stimulations

The *splanchnic nerves* on both sides were cut just beneath the diaphragm nd the peripheral ends were mounted on ring silver electrodes. In most experiments the splanchnic nerves of both sides were stimulated simultaneously Supramaximal square wa o pulses (5 t 8 V 3–5 msec) were delivered from a Grass stimulator model 84 E. Generally frequencies between 4 and 8 imp/sec were used, which corresponds to the upper range of the physiological discharge rates. The *lumbar colonic nerves* were dissected free from the inferior mesenteric artery cut and their distal ends were placed on silver ring electrodes. Since these nerve fibres are postganglionic, a stimulation strength of 10–15 V was used. The *hypogastric nerves* were also cut and in some experiments stimulated in efferent direction. The *pelvic nerves* on both sides were dissected free divided as they emerged from the sacral roots, and their peripheral ends were mounted on electrodes as mentioned above. The stimulation threshold of these fibres was first determined by varying the pulse duration while keeping voltage and stimulation frequency constant. The relationship between voltage and pulse duration was then kept high, enough so as to ensure activation of all the nerve fibres. For determination f frequency response characteristics the nerves were stimulated at supramaximal strength (5–8 V 3–5 msec) The *vagal nerves* were usually dissected free in the neck or in some experiments subdiaphragmatically They were cut and their distal ends mounted on ring electrodes and stimulated with supramaximal square wave impulses (5–8 V 5 msec) and at frequencies between 4–16 imp/sec.

In several of the experiments the nerves were cut after the recording of intestinal motility had started. In some experiments a mesenteric nerve pedicle from the extirpated small intestine was prepared for afferent stimulation to induce intestino-intestinal inhibitory reflexes.

The electrodes were insulated from the surrounding tissues with silicone grease

The catecholamine secretion from the adrenal medullae, known to be potent in producing intestinal inhibition, was eliminated by isolating both adrenals from the circulation by encircling ligatures. Hydrocortisone (10 mg/kg) was given intramuscularly to substitute for the loss of cortisol secretion.

Acetylcholine and l-adrenaline hydrochloride could be infused intraarterially usually via a thin polyethylene catheter introduced in a small mesenteric arterial branch of the superior mesenteric artery or in the inferior mesenteric artery

Other drugs were administered i.v.: atropine (Atropine sulphate, Merck) in a dose of 1 mg/kg b.w., ktorsondamon (Ecolid® Ciba) 2 mg/kg b.w., guanethidine (Ismelin® Ciba) 3–5 mg/kg b.w., phenoxibenzamine (Dibenzyline® Smith, Kline & French) 2–5 mg/kg b.w. and alprenolol (Aptin® Hässle) 1 mg/kg b.w.

III RESULTS

A. Patterns of spontaneous colonic motility

1 *The motility in the non-denervated colonic preparation*

Despite the fact that the animals were starved for 24–48 hours, surgical exposure of the colon revealed that it still contained faeces in most cases. In the distal part they were regularly hard and expanded the lumen, whereas in the proximal part they were mostly soft or semisolid. No clearcut motor activity was seen in either part of the colon at this early stage after laparotomy. After gentle rinsing of the lumen the entire colon was as a rule still largely atonic; at the most faint circular contractions were infrequently seen in the proximal part.

2 *Effects of sympathetic nerve section*

Splanchnic nerve section sometimes caused a slight increase of motility but then in the proximal colon only. In contrast, section of the lumbar colonic nerves resulted in a considerable motility increase in the entire colon, although certain differences could be observed between the proximal and the distal parts. Thus, within less than 30 seconds the distal half or two thirds of the colon slowly contracted and shortened lengthwise, as if it was pulled down in the pelvis. The proximal part of the colon also took part in this mainly longitudinal shortening but only to a slight extent. The main effect in the proximal colon was the occurrence of frequent circular contractions, which swept downward along the proximal third. Subsequently these circular contractions often fused into a vigorous and more sustained circular contraction which again only engaged the proximal part of the colon.

After several minutes the colonic contraction gradually subsided whereby the *distal* part increased in length again although it seemed to retain a slightly higher level of tone compared with that before the sympathetic denervation. Infrequent, slight movements appeared in the wall, indicating a feeble resting motility. Also the motor activity in the proximal part subsided gradually although moderate circular contractions moved to and fro over this section for a longer period of time. This sequence of events upon sympathetic denervation occurred regularly and the characteristically different motility pattern of the proximal part never extended to the distal part of the colon. It should further be stressed that these effects of sympa-

thetic nerve section were largely the same whether the vagal and pelvic nerves had been previously sectioned or not Hypogastric nerve section had no obvious effect on colonic motility

3 *Effects of parasympathetic nerve section*

In another series of experiments the effects of vagal and pelvic nerve section were studied in a similar way However these procedures had no significant effects on prevailing motor activity in either part of the colon whether the sympathetic nerves had been previously sectioned or not Thus, neither rhythmic activity nor "basal tone was appreciably decreased by vagal or pelvic nerve section indicating that these nerve fibres were not significantly tonically active in the present experimental situation.

4 *Volume recordings of spontaneous colonic motility in the denervated proximal and distal parts*

When the pressure on the colonic wall was kept at 10–15 cm H_2O a considerable spontaneous motility was usually present in the acutely denervated preparations Although the pattern of colonic motility varied somewhat between animals, it was regularly very different from that in the small intestine as reflected by the volume changes. While the jejunal activity exhibited frequent rhythmic contractions of small amplitude proximal colonic motility was characterized by relatively slow but powerful volume reductions lasting 10–40 sec On the other hand the pattern of "spontaneous activity in the distal colonic part often differed markedly from that in the proximal one with very slow contractions, occurring far less frequently

Both the jejunal and colonic rhythmic activity seemed to occur at fairly constant levels of "basal tone However this basal tone level was in general not so pronounced in the colon, as judged from the relatively smaller increases of colonic volume that could be induced by intra-arterially injected adrenaline As mentioned above neither the rhythmic activity nor the "basal tone was appreciably decreased by vagal or pelvic nerve section, indicating a very low resting" tonic activity in these nerves in the present experimental situation.

B Effects of graded direct stimulation of the parasympathetic supply

1 *The vagal nerves*

As is shown in Fig 1 vagal stimulation at supramaximal strength produced a characteristic motor response in the proximal colon evident both by direct inspection and by the volume recording Superimposed motility waves of increased amplitude and frequency were seen on a slightly elevated tone. This effect was immediately eliminated upon cessation of stimulation. Even intense vagal stimulation had no effects on

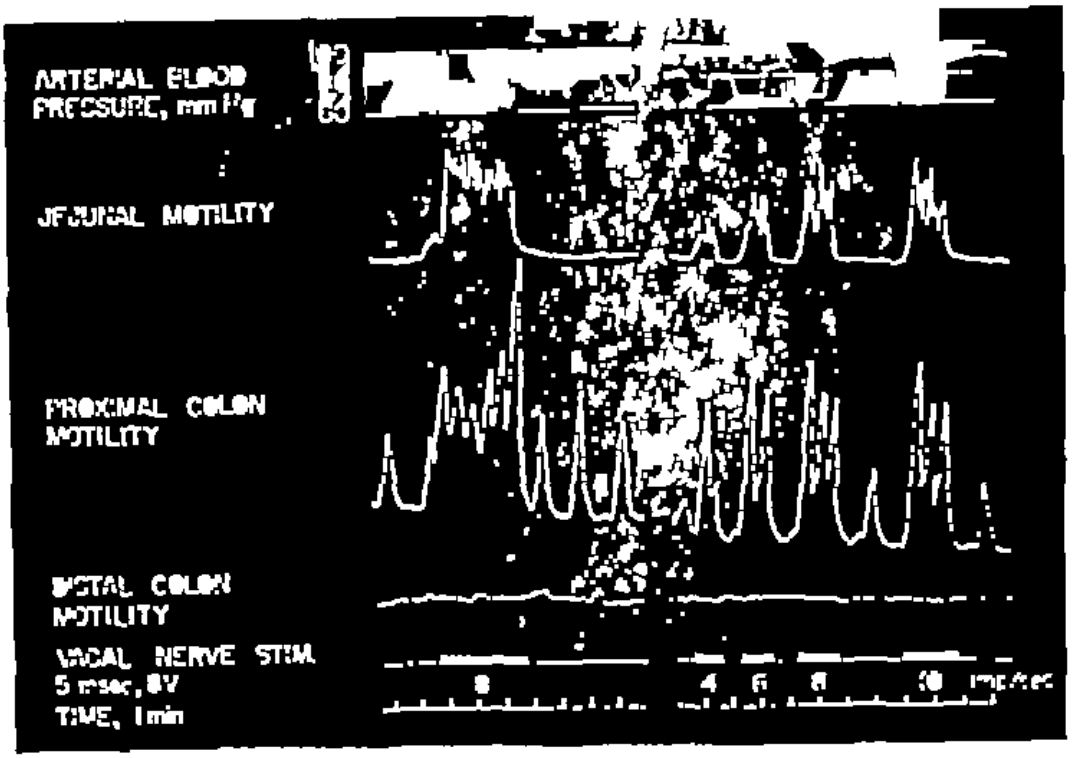


Fig. 1 Cat 3.5 kg Chloralose (Left panel) Effects of efferent cervical vagal stimulation upon proximal and distal colonic motility compared with that of jejunal motility Note the motility waves of increased amplitude and frequency superimposed on the elevated a erage tone. (Right panel) The frequency response relationship for the vagal effect on proximal colon compared with that of the jejunum. The verage volume decreases shown in the figure imply only moderate decreases of the jejunal and colonic lumina.

the distal colon despite the continuity of wall connections. Thus, as is the case with the small intestine vagal stimulation largely intensified the pattern of "spontaneous" rhythmic activity present in the proximal colon, without changing its general characteristics appreciably The same type of motor response was achieved when the vagus nerve (*right dorsal branch*) was *stimulated at the subdiaphragmal level.*

By varying the rate of stimulation, while pulse duration and voltage were kept constant the frequency response relationship for the vagal effect on the proximal colon was found to be similar to that in the small intestine The response increased from low frequencies (1–2 imp/sec) to reach a maximum at 10–16 imp/sec (Fig 1 right panel) Further increases in frequency usually resulted in decreased motor responses but the maximal response was regained once the pulse rate was again lowered to 10–16 imp sec In order to ascertain the stimulation threshold of the vagal excitatory fibres to the colon the pulse duration was varied over a wide range while voltage and frequency were kept constant at 5 volts and 4 imp/sec. A clear colonic response was recorded first when the duration reached 0.8–1 msec. indicating if anything a slightly higher stimulation threshold for these fibres compared to those to th small intestine (see also Kewenter 1965) On further increases of

pulse duration the motor response increased gradually to reach its maximal level for the given frequency at about 3 msec Intravenous administration of atropine (1 mg/kg) blocked the vagal motor response completely There was no evidence of inhibitory vagal fibres to the colon.

2. *The pelvic nerves*

To judge from direct inspection of the colon in situ the characteristics of the motor response to high frequency *pelvic nerve stimulation was very similar to but more* intense than that following section of the lumbar colonic nerves (see page 24) Thus, immediately upon supramaximal pelvic nerve stimulation, there was a sustained contraction of the distal part of the colon which especially shortened in length giving the impression that this part of the colon was forcibly pulled down into the pelvis. Circular contractions here were less prominent The proximal part of the colon also shortened somewhat but the most prominent effect here was a series of circular contractions which rapidly fused into a pronounced and sustained circular contraction limited to this part At the height of this response to high frequency pelvic nerve stimulation the proximal half of colon was transformed into a hard "rod with a largely obliterated lumen and a blanched serosal surface Thus pelvic nerve stimulation evoked an intense and essentially *tonic* contraction of the *entire* colon, provided that the loose ligature separating the proximal and distal parts had not destroyed the continuity of the bowel wall. If, however this ligature was tightened or the wall continuity was broken by cautery the motor response was restricted to the distal segment only even if extramural vascular nervous connections and the colonic mesentery remained intact (Fig 2 right panel)

When colonic motility was studied by means of the volume recorder the motor response to pelvic nerve stimulation occurred within a few seconds in the distal colon segment and in the proximal within another 1–2 seconds. With this type of recording the colonic response to pelvic nerve stimulation was especially characterized by a sustained and powerful reduction of the lumen of both parts, with little sign of rhythmic action, except initially in the proximal part (Fig 2) This response should be contrasted to the vagal response which was characterized by a rhythmicity with only a slightly reduced average volume Furthermore the response to pelvic nerve excitation often declined slowly over several minutes after cessation of the stimulation, again in contrast to the response to vagal stimulation (compare Figs. 1 and 2)

Estimation of the stimulation threshold revealed a motor response to pelvic nerve stimulation with stimuli of 0 08–0 1 msec duration. Frequency and voltage were kept constant at the same levels as during vagal stimulation, i e 4 imp/sec and 5 volt respectively Increasing the pulse duration over a wide range did not provide evidence of any involvement of high threshold inhibitory fibres in the pelvic nerve supply The frequency response characteristics of the pelvic fibres were also

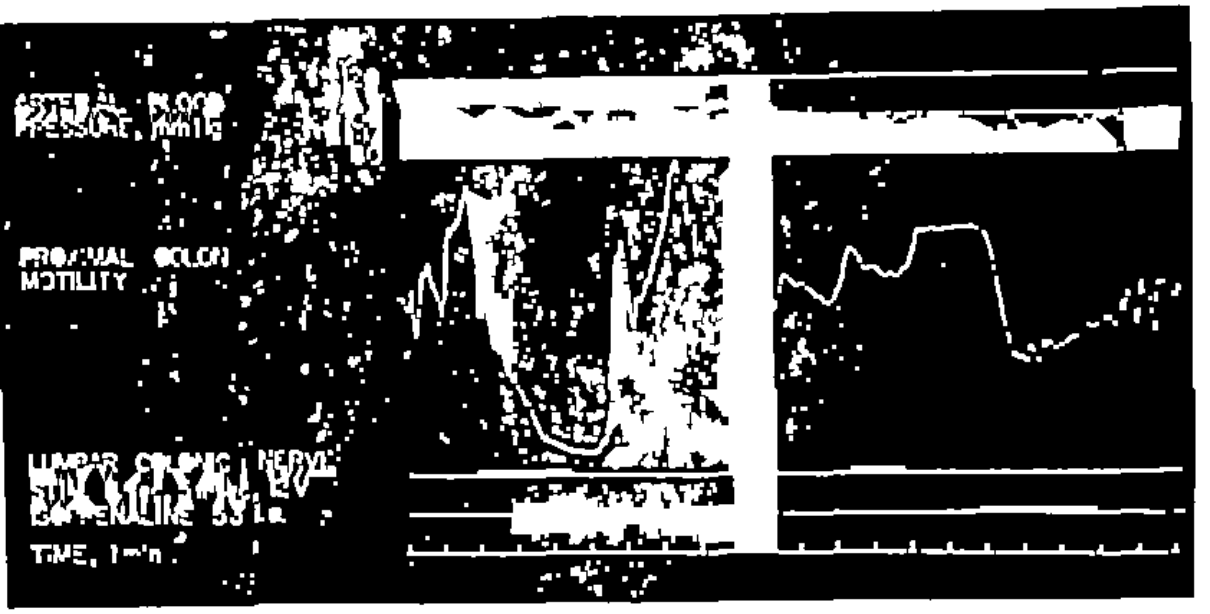


Fig 5 Chloralose Effects of lumbar colonic nerve stimulation upon spontaneous motility in the acutely denervated proximal colon in two different experiments. Left panel shows the prompt inhibitory effect on pronounced spontaneous activity In right panel where "spontaneous activity appeared to be *low* lumbar colonic nerve stimulation had only slight inhibitory effects, whereas intraarterial infusion of isoprenaline relaxed the colonic musculature profoundly

2 *The lumbar colonic nerves*

When spontaneous motility of the proximal and distal colon was weak, stimulation of the lumbar colonic nerves exerted no inhibitory effect until high frequencies were used Then a somewhat delayed inhibition occurred. On the other hand in experiments where spontaneous motility was pronounced indicating a high level of intramural ganglionic activity even low frequency stimulation induced prompt inhibition (Fig 5) Similarly vagally induced motor activity in the proximal colon and the more sustained motor response of the entire colon to pelvic nerve stimulation were both dramatically and promptly inhibited—perhaps less strongly so in the distal colon—by stimulation of the lumbar colonic nerves (Fig 6) This was the case even when low frequencies were used. In contrast colonic motor responses of similar size produced by intraarterially infused acetylcholine were not influenced by stimulation of the lumbar colonic nerves (Fig 7) The inhibitory effect of sympathetic stimulation on the pelvic nerve response was the same before and after atropine administration.

Lumbar colonic nerve stimulation after administration of guanethidine often induced powerful but usually transient contraction of both colonic parts, an effect that was readily blocked by atropine Such effects were never obtained upon splanchnic nerve stimulation after guanethidine

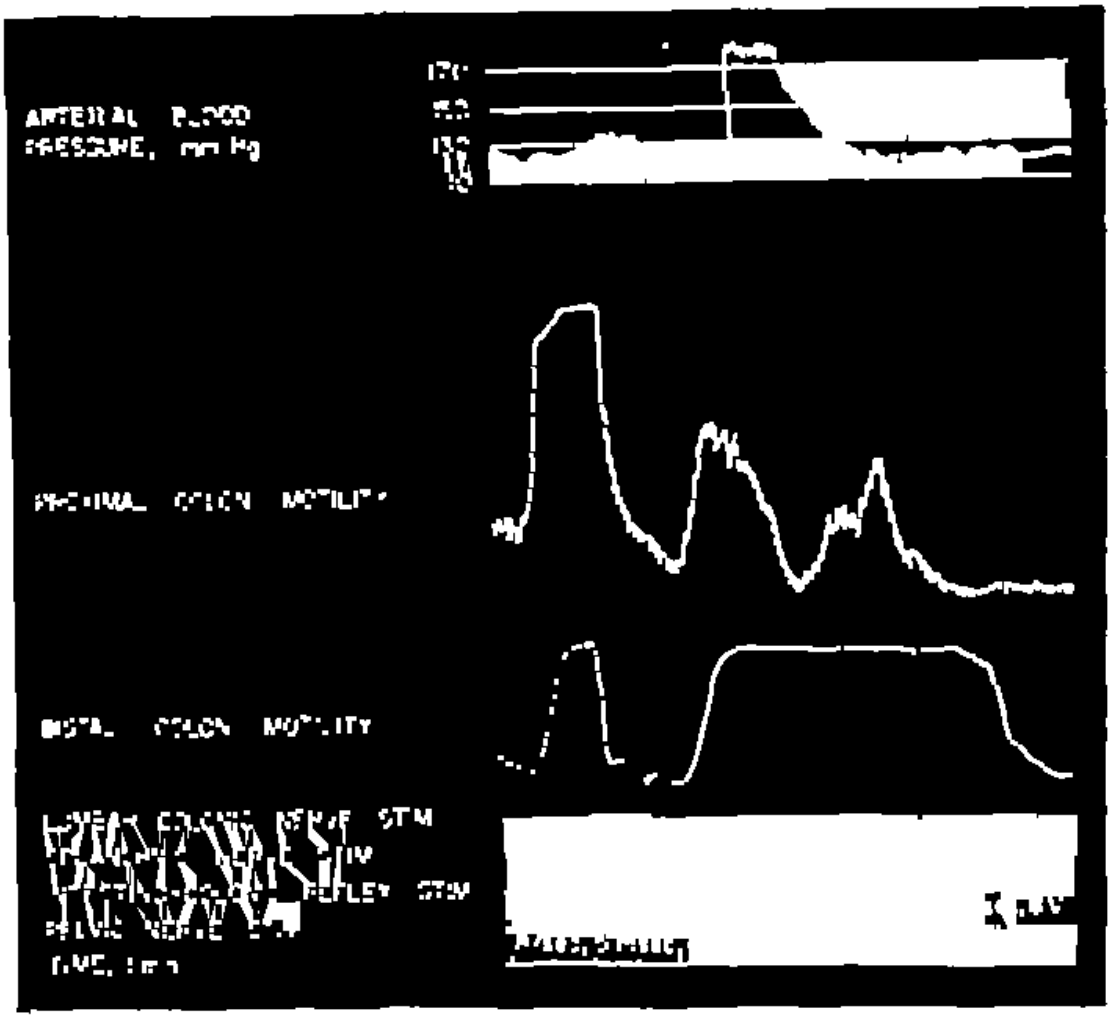


Fig 6. Cat 3.8 kg Chloralose. Effects of lumbar colonic nerve and splanchnic nerve stimulations upon colonic motility induced by pelvic nerve stimulation. Note the sustained contraction of both proximal and distal colonic parts upon pelvic nerve stimulation. The motility is similarly and promptly inhibited in both parts of the colon by lumbar colonic nerve stimulation. In contrast, splanchnic nerve stimulation inhibits only proximal colonic motility N t also that intestino-colonic reflex inhibition occurs readily in the proximal colonic part whereas no effect whatsoever can be traced in the distal part of the colon.

3 *The hypogastric nerves*

Stimulation of the hypogastric nerves in the distal direction had no inhibitory effect on the spontaneous or parasympathetically induced motility of the colon, despite the rate and strength was varied over a wide range Excitatory effects similar to those obtained on lumbar colonic nerve stimulation after administration of guanethidine were never observed

IV COMMENTS

The present results of efferent vagal and pelvic nerve stimulation on the acutely denervated colon of the cat suggest that the vagal nerves affect the *proximal* part of

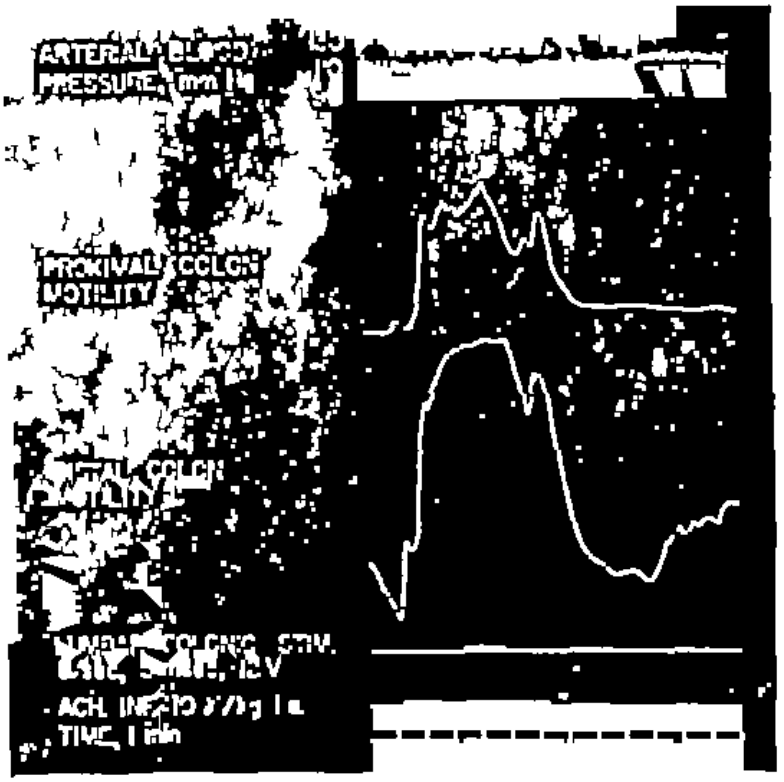


Fig 7 Cat 3.1 kg. Chloralose Effects of lumbar colonic nerve stimulation upon colonic motility induced by intrarterial infusion of acetylcholine N inhibitory effects whatsoever can be seen either in the proximal or in the distal part.

the colon only while the pelvic nerves affect almost the entire colon. This is in accordance with the morphological results of Schmidt (1933) who after vagal section traced degenerated nerve fibres only to the proximal colonic parts whereas this was found largely in the entire colon after division of the pelvic nerves. The opinions as regards a vagal supply to the colon are as mentioned, contradictory Whereas many investigators (Rentz 1938 Stavney *et al* 1963 Fülgraff and Schmidt 1963 a) have reported excitatory responses in proximal colonic parts in rabbits cats or dogs others (Bayliss and Starling 1900 Carlsson 1930 Wells *et al* 1942) denied the existence of an excitatory vagal influence on the colon

The divergent results of these earlier studies might be due to differences in the influence of sympathetic activity whether nervous or hormonal. No doubt sympatho-adrenal activity may markedly even completely suppress motor responses to parasympathetic stimulation in the gastrointestinal tract This is illustrated both in the present experiments and in those of e.g Celander (1959) Kock (1959) and Kewenter (1960) The fact that Fülgraff and Schmidt (1963 a) and Stavney *et al* (1963) did observe vagal motor responses in proximal colonic parts may be due to their experimental condition as they had cut the splanchnic nerves in order to allow for their direct stimulation An alternative explanation may also be that the spinal medulla in one of these experimental series was destroyed This procedure will block the

sympathetic intestino-intestinal reflex inhibition which is spinally mediated (e.g Johansson and Langston 1964) The powerful suppressing effects of a tonically active sympathetic supply upon cholinergic activity in the colon is also clear from the present experiments. This is illustrated by both the drastic release effects of sympathetic section and by the effects of graded stimulations of the cut sympathetic nerves.

The exact morphological distribution of vagal fibres to the colon cannot be determined by stimulation experiments alone However the functional extension of their impact can be shown since extrinsic excitation of only part of the intramural ganglionic plexuses appears to trigger a spread of activity along the entire colonic wall provided that its continuity is not broken. In the present experiments the colonic lumen was separated into a proximal and a distal part without disruption in the continuity of either the wall or the mesentery

According to Wright *et al* (1938) the fibres of pelvic nerves run within the colonic wall This could be confirmed in the present study since the proximal colonic segment no longer responded to pelvic nerve stimulation when the colonic wall was crushed although extramural vascular and mesenteric connections were intact Thus the preparation was shown to allow for free transmission of nervous or myogenic influences along the entire colon Nevertheless, excitatory responses to vagal stimulation were always restricted to about the proximal third of the colon No effects were ever observed in the distal colon. It therefore seems justified to conclude that the vagal supply is restricted to about the proximal third of the colon and that their influence here does not spread along the intrinsic plexuses or by myogenic propagation to distal parts.

The vagal excitatory effect on the proximal colon characteristically enhanced the extent and rate of spontaneous rhythmic activity in a fashion which would greatly improve mixing and stirring of the contents rather than causing any gross expulsion. The average reductions of the colonic lumen were usually small Furthermore these vagal effects were completely blocked by atropine indicating a cholinergic transmission. In contrast the motor response to pelvic nerve stimulation was characterized by a more steady *overall* contraction—although the proximal and distal parts were involved in somewhat different ways as described in Results—a type of activation that would favour expulsion rather than mixing The pelvic nerve response was not significantly reduced by atropine, but became somewhat delayed and occasionally it was preceded by slight motility decline

It is of interest that the different motility responses in the proximal and distal colonic parts to pelvic nerve activation (described in detail in Results) are not necessarily the result of a different mode of action of these nerve fibres in the two parts, although such a difference may well exist Thus even in colonic preparations where all extrinsic nerves had been cut the same difference can be traced in the far less powerful spontaneous motility patterns of the two parts Rhythmicity is a more prominent feature in the proximal part while the distal part exhibits more sustained and generalized contractions. Whether these regional differences in spontaneous motility are

inherent in the functional characteristics of the intrinsic plexuses or in the myogenic activity or both is not known.

It should however also be stressed with respect to the proximal colon that the vagal effect mainly enhances the rate and extent of the inherent rhythmicity while the regional pelvic nerve effect is particularly characterized by a powerful and an ultimately sustained contraction of the circular wall layer which causes almost complete emptying These differences in nerve effects on the same effector suggest that the two types of parasympathetic nerves affect the intramural ganglionic plexuses in different ways This is also suggested by the striking differences in atropine sensitivity between the vagal and pelvic nerve effects. These findings agree with observations in man by Fink and Friedman (1960) who reported a clear difference in the motility response of the proximal and distal colonic parts upon intravenous administration of mecholyl and neostigmine This was taken to support the view that the human colon may be composed of two functionally different segments with the line of division in the transverse part of the colon. The above investigators also suggested that a different concentration of cholinesterase in the two parts of the colon might be a possible explanation for the different effects of drugs on the proximal and distal colonic parts.

The resistance of the pelvic nerve effect to atropine was noted by Langley and Anderssen (1895) and more recently by Fülgraff and Schmidt (1963 b 1964) Garry and Gillespie (1955) however studied an isolated cat colon preparation in an organ bath and showed that the pelvic nerve effect could in such a preparation be completely abolished by atropine. It is possible that both a cholinergic and a non-cholinergic link might be involved in the colonic motor response to pelvic nerve stimulation and that only the cholinergic link can be traced in preparations in vitro In fact the motor response can evidently be abolished by atropine also in preparations in vivo if the colonic vascular bed is perfused with Tyrode solution instead of with blood (Fülgraff and Schmidt 1963 b) On the basis of such experiments Fülgraff claimed that a plasma component might be responsible for the atropine-resistant contraction. Although the extent of the pelvic nerve response was not significantly reduced after atropine in the present experiments it became obviously delayed in onset This might suggest the presence of some indirect excitatory link, resistant to atropine which becomes fully efficient only after a period of stimulation.

Similar findings indicating the release of excitatory factors other than acetylcholine at some parasympathetic neuro-effector junctions have been made concerning the bladder contraction to pelvic nerve stimulation (Eckert 1962 Edwardsen 1967) Furthermore the vasodilatation occurring in salivary glands upon parasympathetic stimulation is at least in part due to such mechanisms (Hilton and Lewis 1956) Lastly the gastric relaxation induced by the vagal high threshold fibres is also uninfluenced by both atropine and guanethidine (Martinson 1965) It is possible that in most perhaps all of these situations a release of plasma kinins might be involved in a similar way as bradykinin (or rather kallidin) release is involved in the

neurogenic vasodilatation in the salivary glands (Hilton and Lewis 1955 1956) Both in the stomach and in the colon (see chapter IV) the secretory responses to parasympathetic stimulation are closely connected to profound and atropine-resistant mucosal vasodilatations as well as to atropine resistant motility changes—relaxation in the stomach and contraction in the colon. As mentioned above the atropine-resistant motility response of the colon might be dependent on the presence of normal blood plasma If this is also the case with respect to the vagally induced gastric relaxations is not known

The inhibitory action of the splanchnic nerves on colonic motility is restricted to the proximal third where it strongly suppresses the excitatory influence of both the vagal and pelvic nerves. The sympathetic lumbar colonic nerves on the other hand suppress both vagal and pelvic nerve effects in the entire colon. It should be noted that the lumbar colonic nerves affect the colonic vascular bed only in the distal parts of the colon (Hultén *et al* 1968) This implies a distinct difference in the distribution of the sympathetic fibres to colonic blood vessels and to the colonic wall proper

Furthermore, the sympathetic inhibitory influence on intestinal motility—whether mediated by splanchnic or lumbar colonic nerve fibres—seems to be predominantly and perhaps only conveyed via an action on the intramural plexuses rather than on the smooth muscles directly Thus even low frequency stimulation of the sympathetic supply profoundly suppresses parasympathetically induced effects throughout the colon, or an inherent activity of the intramural ganglionic plexuses. On the other hand, sympathetic stimulation at "physiological rates has virtually no effect on such motility presumably of myogenic nature which is induced by mild distension. Similarly it has no effect on the motility induced by acetylcholine infusion which causes a muscarine-like activation of the intestinal smooth muscles directly Such a combination of observations appears to be consonant only with the view that the *major* perhaps sole sympathetic effect is exerted at the intramural ganglionic level. This is also suggested by histo-chemical studies (Norberg 1964 Jacobowitz 1965 Schofield 1968)

Inhibition of spontaneous jejunal activity by splanchnic nerve stimulation is according to Celander (1959) essentially due to overflow of the vasoconstrictor transmitter since these inhibitory effects were seen only when "supraphysiological frequencies were used In contrast to this intra-arterial injections of even small amounts of catecholamines brought about immediate and prompt jejunal inhibition. It is likely that in Celander s experiments the jejunal motility studied was largely of a myogenic nature with only little influence from intramural nerve plexa In fact in his preparations these plexa may have been reflexly suppressed via adrenergic connections other than those conveyed in the splanchnic nerves. It is now known that such extra-splanchnic sympathetic connections are present and that they readily convey intestino-intestinal inhibitory reflexes which are indeed often continuously active in experimental preparations of this type (cf. Johansson and Langston 1965) In any

case Celander's results do not support the view that the jejunal smooth muscles should be directly influenced by sympathetic inhibitory fibres to any *significant extent*. The results of Kock (1959) obtained with a similar technique indicate that centrally and reflexly induced inhibition of intestinal myogenic activity is mainly or solely dependent on the *hormonal* component of the sympatho-adrenal system are also consonant with such a view.

However when the intramural plexuses of the colon are active, whether "spontaneously" or due to a superimposed parasympathetic activity such a neurogenic excitatory influence is strongly counteracted by even low frequency activation of the sympathetic fibres. This suggests that there is an adrenergic influence mainly or perhaps only confined to the *ganglionic* level at least in the cat (Norberg 1965 Kewenter 1965 Jansson and Martinson 1966 Jansson 1969). The present results are in full agreement with such a view with respect to the sympathetic control of the colon.

In most of the present experiments where the adrenals were excluded and the splanchnic as well as the lumbar colonic nerves were divided vagal motor responses were nevertheless often difficult to produce until the animal had been left undisturbed for about an hour after the abdominal operation. This suggests that the remaining peritoneal intestinal reflex inhibitions via "aberrant" sympathetic fibre connections to the proximal colon might interfere with the vagal motor response (see chapter VI) as is also the case with the small intestine (Johansson and Langston 1964).

Excitatory apparently cholinergic fibres distributed to largely the entire colon within the lumbar colonic nerves were revealed first after guanethidine administration. This is in accordance with the reports given by Day and Rand (1961) and Boyd *et al.* (1962). The functional significance of these fibres are obscure. Their possible involvement in the reflex control of colonic motility are briefly explored in chapter VI.

Thus there seems to be a clearcut difference not only in distribution, but also partly in function as well between the splanchnic and lumbar colonic nerves as to their suppressing effects on the proximal and distal parts of the colon. The proximal part has in this respect a dual innervation, where the splanchnic supply seems to be involved in "long" intestino-intestinal reflexes while the lumbar colonic supply mediates a tonic possibly centrogenic inhibitory influence (see also chapter VI). Only the latter type of influence effects the distal colon. This again may lend further support to the view that the two colonic parts—despite a considerable overlap—are specialized for different functions as reflected here by this difference in sympathetic inhibitory control. It should also be stressed that the sympathetic vasoconstrictor fibre supply is derived from two sources but then with a fairly distinct borderline between them (see chapter III).

Furthermore the motor response patterns are also different with respect to the vagal influence. Enhanced rhythmic activity is produced in the *proximal* part in a way which would considerably enhance stirring and mixing. On the other hand the

pelvic nerve influence produces strong and sustained contractions favouring expulsion, affecting the proximal and distal segments somewhat differently These observations fit nicely with the classical concept of a differentiated function of proximal and distal colonic parts and indicate by which means this differentiation is achieved

In general the present findings help to explain the establishment of the colonic motility patterns observed in intact organisms, first studied by means of X ray in awake cats by Cannon (1902) and confirmed by M'Fadden *et al* (1935) Their observations suggest that movements characterized as "rhythmic antiperistaltic waves" withhold the contents in the proximal colon until sufficiently dried by absorption. The "ring of contractions of the proximal colon regularly occurring after meals ultimately propelled the contents further down into the descending part of the colon At the same time the liquid contents of the ileum were once again delivered to the caecal part of the colon However this pattern of colonic motility evidently favouring absorption, differed markedly from that during defaecation when extensive contractions pulled almost the entire colon towards the pelvis by a shortening predominantly of the descending colonic part.

The present experiments indicate that the vagi may be concerned with the above mentioned *rhythmic* motility the proximal colonic parts apparently being of importance for the absorption of water and salts by its stirring and mixing effect The pelvic nerves on the other hand are called into action to produce a powerful generalized and sustained colonic contraction specifically designed for expulsion. However the difference in motility patterns between the proximal and distal colonic parts can also be due to their *inherent* wall activities. This of course does not deny that the various parasympathetic nerves in different ways affect the intrinsic plexuses to achieve the mentioned differences of function and extrinsic control in the two colonic parts

Thus, in the cat colon these two functions appear to be controlled by separate sets of reciprocally acting parasympathetic and sympathetic fibres, but they are also dependent on the wall elements themselves. However the two functions obviously overlap somewhat morphologically which is also indicated by the fact that the distribution of the different nerve fibre sets overlaps somewhat in the proximal and distal parts. Thus the lumbar colonic nerves—both their inhibitory and excitatory fibres—as well as the pelvic nerves extend their effects on colonic motility to largely the entire colon, while the splanchnic and vagal nerves are in this respect confined only to the *proximal* third. The vasomotor control is more distinctly separated in the two colonic parts, both with respect to the sympathetic and pelvic nerve influence (see chapter III) It is possible that the spatial overlap of function and motility control is less prominent in man.

Lastly it is interesting to compare the dual vagal control of the stomach with the dual parasympathetic control of the colon, both being reservoir organs but one specialized for rapid reception, the other for rapid evacuation. The stirring and mixing functions appear in both organs to be directed by vagal excitatory fibres, while the

adjustments of the reservoir *per se* are effected by a specific set of nerves in both organs Here the vagal relaxatory fibres (Martinson 1965 Jansson 1969) accomodate the corpus-fundus regions of the stomach for reception, and in a similar fashion the pelvic nerves, by their powerful contractile influence, command the emptying of the reservoir placed at the other end of the gastrointestinal system

CHAPTER III

EXTRINSIC NERVOUS CONTROL OF COLONIC BLOOD FLOW

L. HULTÉN, M. JODAL and O. LUNDGREN

I. INTRODUCTION

There are, as mentioned in chapters I and II, reasons to believe that the colon may be comprised by two functionally different parts, a view that seems to be in agreement also with its embryological development. Thus, according to the classical concept the proximal part would be preferentially involved in water and salt absorption while the distal part would be essentially concerned with storage and subsequent expulsion. Several reports indicate that also the extrinsic nervous control of the colon may be so organized as to suggest such a functional differentiation and some of the findings reported in chapter II concerning the motility control are in agreement with this.

However, despite much effort during earlier years to clarify these problems experimentally, the great technical difficulties involved have in several important respects led to conflicting results and opinions. This is also true with respect to the exact distribution and mode of action of the vagal, pelvic and sympathetic innervation as regards blood flow.

The present chapter deals with the effects on colonic blood flow induced by the efferent sets of extrinsic nerves with particular respect to the localization and gross extent of their vascular actions. Since it is known that electrical stimulation of the lumbar colonic nerves causes blanching of the mucosa in distal parts of the colon (e.g. Langley and Anderssson 1895, Wright *et al.* 1938), it is, for example, tempting to suppose that corresponding vascular effects may be induced in proximal parts by splanchnic nerve fibres, though no such studies appear to be available. It is furthermore known that the pelvic nerves contain fibres which on stimulation induce flushing of the mucosa in distal parts of the cat colon (Langley and Anderssson 1895, Wright *et al.* 1938). On the other hand, it is not known as to what an extent similar

effects are also induced in proximal parts of the colon or whether the vagal supply may here exert such effects. Should such a regional differentiation exist with respect to the nerve fibres, affecting not only motility but also blood flow in the corresponding region this would lend further support to the concept that the colon is functionally comprised by two different parts It is, however evident that the morphological borderlines between the mentioned differentiated functions i e absorption and storage expulsion, can hardly be expected to be distinct a considerable overlap seems likely

II METHODS

A. Operative procedures

Observations were carried out on 73 cats, fasted for 24 hours. After induction with ether they were anaesthetized intravenously with chloralose (50 mg/kg) A tracheal cannula was inserted The abdomen was then opened in the midline and the entire small intestine was extirpated together with the greater omentum and the spleen. The animals were heparinized (3–5 mg/kg b.w) and a femoral artery was connected to a mercury manometer for recording of arterial blood pressure The animals were kept on a heating pad which was thermostatically regulated so that the body temperature was kept around 37–38 C A plastic tube was introduced via the mouth into the stomach to permit drainage of secretion and swallowed air and another catheter drained the bladder

In some experiments (18 cats) the colon was cautiously split with thermo-cautery along its antimesenteric border from the caecum down to the peritoneal deflection border The cut edges were fixed with pins on a cork plate thus exposing the mucosa for direct inspection during stimulation of the different sets of nerve fibres. Great care was taken to keep the exposed colon at body temperature by means of steady superfusion with warm Tyrode solution and with a heating lamp under temperature control.

B Determination of blood flow and blood volume

To establish the magnitude of the overall colonic blood supply the total venous outflow was recorded, either simultaneously with the visual inspection of the mucosa or with an intact mesentery For this purpose a wide-bore polyethylene tube was inserted into the superior mesenteric vein, distally to its confluence with the splenic vein *Since a minor portion of the blood supply to the distal part of the colon is probably* drained by small veins close to the anal region, these small venous connections were ligated so that the venous drainage occurred via the superior mesenteric vein exclusively The venous outflow from the colon was then diverted to a closed perspex drop chamber filled with paraffin oil and returned to the animal via the right external jugular vein An optical drop counter operated an ordinate writer which recorded the flow rate on smoked kymograph paper The height of the ordinates are largely inversely proportional to the rate of the blood flow and by means of a calibration curve the height of the ordinates could be directly converted into flow in ml min. In experiments where the colonic arterial blood flow was simultaneously recorded the left carotid artery was cannulated and connected via a drop recording unit with the superior mesenteric artery By using siliconized tubes of wide lumenmens and keeping them as short as possible the flow resistance of the

recording system was minimized. Peripheral resistance units (PRU_{ss}) were calculated as the ratio of mean arterial blood pressure to blood flow (ml/min × 100 g tissue).

The blood volume contained within the acutely denervated colonic preparation and its mesentery in the steady state was estimated at the end of a number of experiments. A sample of venous blood from the colon was drawn while at the same time the colonic vessels were suddenly clamped. The tissue blood content was washed out with a known amount of dextrane Tyrode solution and the haematocrit and/or haemoglobin content of this sample, as well as the venous sample, were determined. The blood volume could be deduced from the extent of dilution caused by the known amount of dextrane Tyrode solution. A slight underestimation of the blood volume can be expected with this technique as the haematocrit in the small vessels should be somewhat lower than in venous blood.

C. Nervous stimulations

The extrinsic nerves to the colon were in all experiments prepared for subsequent stimulation in the following manner:

The *splanchnic nerves* on both sides were cut just beneath the diaphragm and the peripheral ends were mounted in ringformed silver electrodes. In most experiments the splanchnic nerves of both sides were stimulated simultaneously. Supramaximal square wave pulses (5 to 8 V, 2–5 msec) were delivered from a Grass stimulator, model S4 E. Generally between 4 and 8 imp/sec were used, which corresponds to the upper range of the physiological discharge rates. In some experiments the sympathetic nerves were dissected periarterially for subsequent postganglionic stimulation. The stimulation strength was in these cases raised to 10–12 V.

The *lumbar colonic nerves* were dissected free from the inferior mesenteric artery, cut and their distal ends were placed on silver ring electrodes. Since these nerve fibres are postganglionic, a stimulation strength of 10–12 V was used. This was also the case when *the hypogastric nerve* were stimulated in a few experiments.

The *pelvic nerves* on both sides were dissected free, divided as they emerged from the sacral roots, and their peripheral ends were mounted on electrodes as mentioned above. The stimulation threshold of these fibres was first determined by varying the pulse duration while keeping voltage and stimulation frequency constant. The relationship between voltage and pulse duration was then kept high enough so as to ensure activation of all the nerve fibres. For determination of frequency-response characteristics the nerves were stimulated at supramaximal strength (5 V, 5 msec).

The *vagus nerves* were dissected free in the neck or in some experiments subdiaphragmatically. They were cut and mounted on ring electrodes and stimulated with supramaximal square wave impulses (5–8 V, 5 msec) and at frequencies between 4–16 imp/sec.

In order to prevent the secretion from the adrenal medullae from interfering with the direct nervous effects on the colonic vessels, both adrenals were excluded from the circulation by encircling ligatures. 10 mg hydrocortison was given intramuscularly for substitution.

In 18 cats the respective weights of the proximal and distal colonic segments blanched by splanchnic and lumbar colonic nerve stimulation were determined when the experiments were finished.

Acetylcholine and isopropyl-noradrenaline (INA) could be infused intraarterially to the respective intestinal segments, usually via a thin polyethylene catheter introduced into a small mesenteric arterial branch.

Other drugs were administered i v atropine (Atropin sulphate Merck) in a dose of 1 mg/kg b.w., hexamethonium (Ecolid© Ciba) 2 mg/kg b w., guanethidin (Ismelin© Ciba) 3–5 mg/kg b w., phenoxibenzamine (Dibenzyline© Smith Kline & French) 3–5 mg/kg b w., and alprenol 1 (Aptin© Hässle) 1 mg/kg b w

III. RESULTS

A. Effects of graded direct stimulation of the sympathetic supply

Fig 1 (upper panel) shows typical vascular responses in the colonic mucosa upon splanchnic and lumbar colonic nerve stimulation, respectively Stimulation of the *lumbar colonic nerves* (Fig 1 A) at a high physiological frequency (8 imp/sec) produced a drastic blanching of the mucosa in the *distal* 1/3–3/4 of the colon with a relatively distinct borderline towards the proximal segment which did not show any significant change The mucosa remained blanched throughout the period of stimulation followed by a transient reddening upon cessation of the stimulation.

A similar drastic and sustained mucosal blanching occurred in the *proximal* part of the colon upon stimulation of the *splanchnic nerves* (Fig 1 B) the response showed a borderline zone of overlap towards the caudal 2/3–3/4 of the colon. The distal sections were quite uninfluenced by the splanchnic stimulation. Cessation of the stimulation was also followed here by a transient period of mucosal flushing Simultaneous stimulation of the two vasoconstrictor fibre sets led to a uniform blanching of the entire colonic mucosa (Fig 1 C) No change in colour was observed in the exposed part of the colonic mucosa, when the hypogastric nerves were stimulated in the distal direction, however The proximal colonic portion controlled by the splanchnic nerves and the distal portion controlled by the lumbar colonic nerves comprised some 30 and 70 per cent respectively of the total colonic weight.

The lower panel of Fig 1 illustrates the shifts in venous outflow from the colon induced by stimulation of the different sets of vasoconstrictor fibres. Resting" blood flow of the colon was in most experiments approximately 15–25 ml/min × 100 g tissue As with the case with the small intestine (Folkow *et al* 1964*a*) the neurogenic constriction of the colonic resistance vessels exhibited two phases. First a transient but intense flow reduction was seen reaching its maximum within 30–40 sec and corresponding in time of onset with the mucosal blanching This initial flow reduction subsided within 2–3 minutes and was followed by a second phase of sustained but less intense flow reduction, whereas the intense mucosal blanching was kept virtually unchanged throughout the stimulation period A profound reactive hyperaemia occurred at cessation of the stimulation. Analogous to the situation in the small intestine the decline of the overall neurogenic flow reduction may be called an autoregulatory escape from the constrictor fibre influence A small, almost negligible, increase in resistance occurred in some experiments when the hypogastric nerves were stimulated in the distal direction at high frequencies

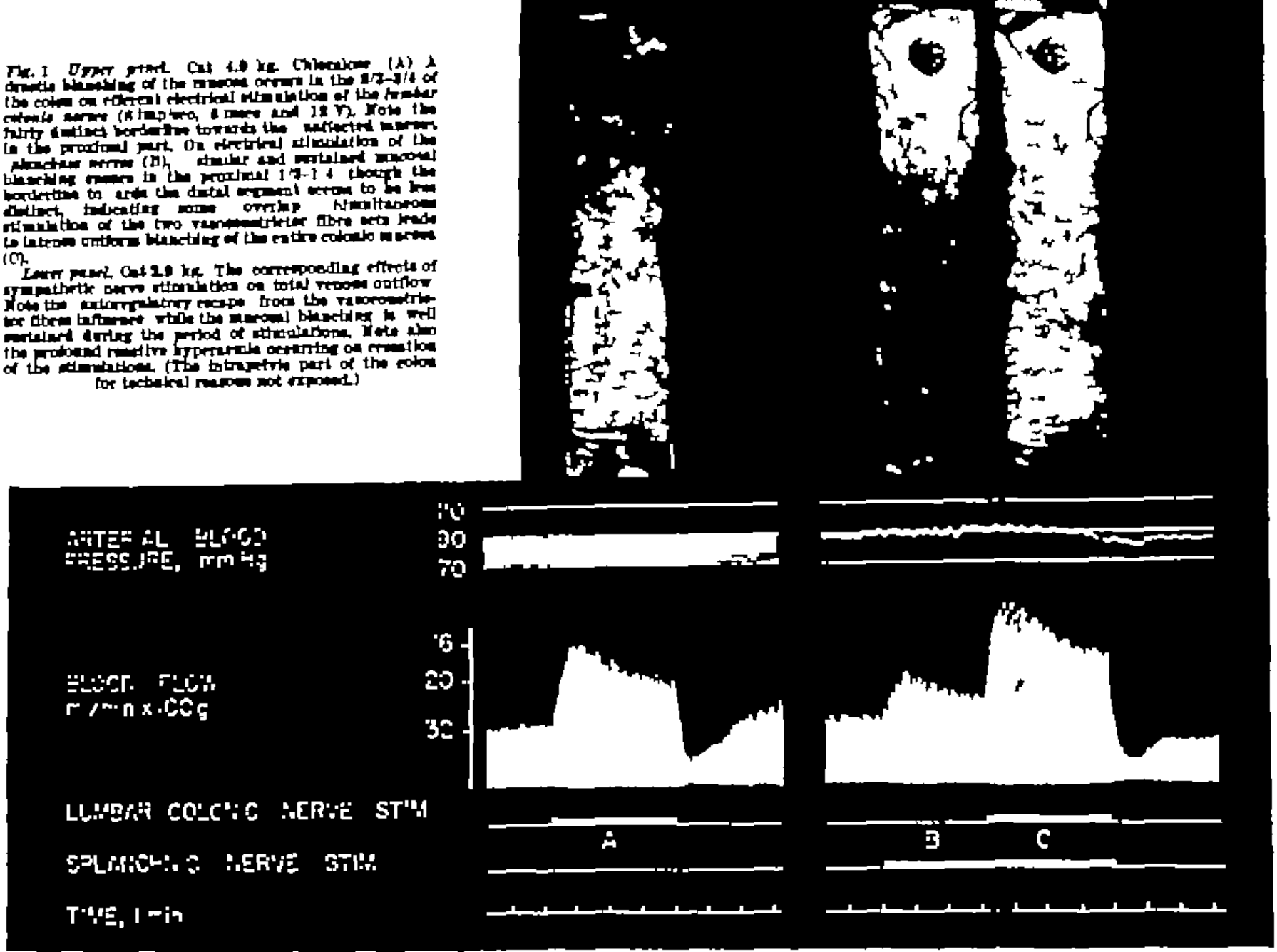


Fig. 1. Upper panel. Cat 4.0 kg. Chloralose (A) A drastic blanching of the mucosa occurs in the 2/3–3/4 of the colon on efferent electrical stimulation of the *lumbar colonic nerves* (6 imp/sec, 6 msec and 12 V). Note the fairly distinct borderline towards the unaffected mucosa in the proximal part. On electrical stimulation of the *splanchnic nerves* (B), similar and sustained mucosal blanching occurs in the proximal 1/3–1/4 though the borderline to ards the distal segment seems to be less distinct, indicating some overlap. Simultaneous stimulation of the two vasoconstrictor fibre sets leads to intense uniform blanching of the entire colonic mucosa (C).

Lower panel. Cat 2.9 kg. The corresponding effects of sympathetic nerve stimulation on total venous outflow. Note the autoregulatory escape from the vasoconstrictor fibres influence while the mucosal blanching is well sustained during the period of stimulations. Note also the profound reactive hyperaemia occurring on cessation of the stimulations. (The intrapelvic part of the colon for technical reasons not exposed.)

In experiments where guanethidine had been administered in doses that entirely block adrenergic transmission, stimulation of the sympathetic nerves had no effect on vascular resistance. Thus no vasodilator effects could be observed on activation of the cholinergic fibres which are suggested to be distributed in the lumbar colonic nerves (see chapter II).

Following activation of either or both splanchnic nerves there was a considerable (20–60 mm Hg) increase in systemic blood pressure evidently due to vasoconstriction and blood expulsion in other abdominal viscera i.e. in the stomach, liver and kidneys. Thus no significant pressure increase resulted upon stimulation of the lumbar colonic nerves or by periarterial stimulation of postganglionic splanchnic fibres running along the superior mesenteric artery and its branches to the colon. However independent of the site of vasoconstrictor fibre stimulation the vascular reactions in the appropriate parts of the colon were essentially the same.

B. Effects of graded direct stimulation of the parasympathetic supply

1 *The vagus nerves*

No obvious change in colour of the colonic mucosa could be traced upon activation of the vagal nerves, whether when stimulated at the cervical or subdiaphragmatic levels. Vagal stimulation did not produce any decrease in colonic blood flow resistance that could be ascribed to any direct dilator action of these fibres. Thus upon vagal stimulation in the neck venous outflow from the colon was somewhat reduced which is explained by a fall in the pressure head. No changes in colonic blood flow were seen when the vagal fibres were activated at the subdiaphragmatic level.

2 *The pelvic nerves*

Bilateral stimulation of the pelvic nerves produced a marked flushing of the colonic mucosa (Fig. upper panel) which seemed to spread in the proximal direction until it engaged some 2/3–3/4 of the colon or largely the portion of the colon that was blanched by stimulation of the lumbar colonic nerves. The remaining proximal part of the colonic mucosa showed no significant change in colour although there was no distinct separating borderline. The colour was first bright red but after about one minute of sustained stimulation the bright mucosal redness in the mucosa seemed to change into a flush tinged colour. Concomitant with the flush a contraction of the colon occurred and a transparent mucoid secretion was obvious, at least in the distal part.

Fig. 2 (lower panel) illustrates the change in total colonic blood flow that occurred simultaneously with the above mentioned mucosal flushing upon pelvic nerve stimulation. Within 5–10 sec after commencement of the pelvic nerve stimulation blood flow increased rapidly to reach maximum in 20–30 sec. However this initial flow increase was transient beginning to vanish after 30–40 sec. and only occasionally

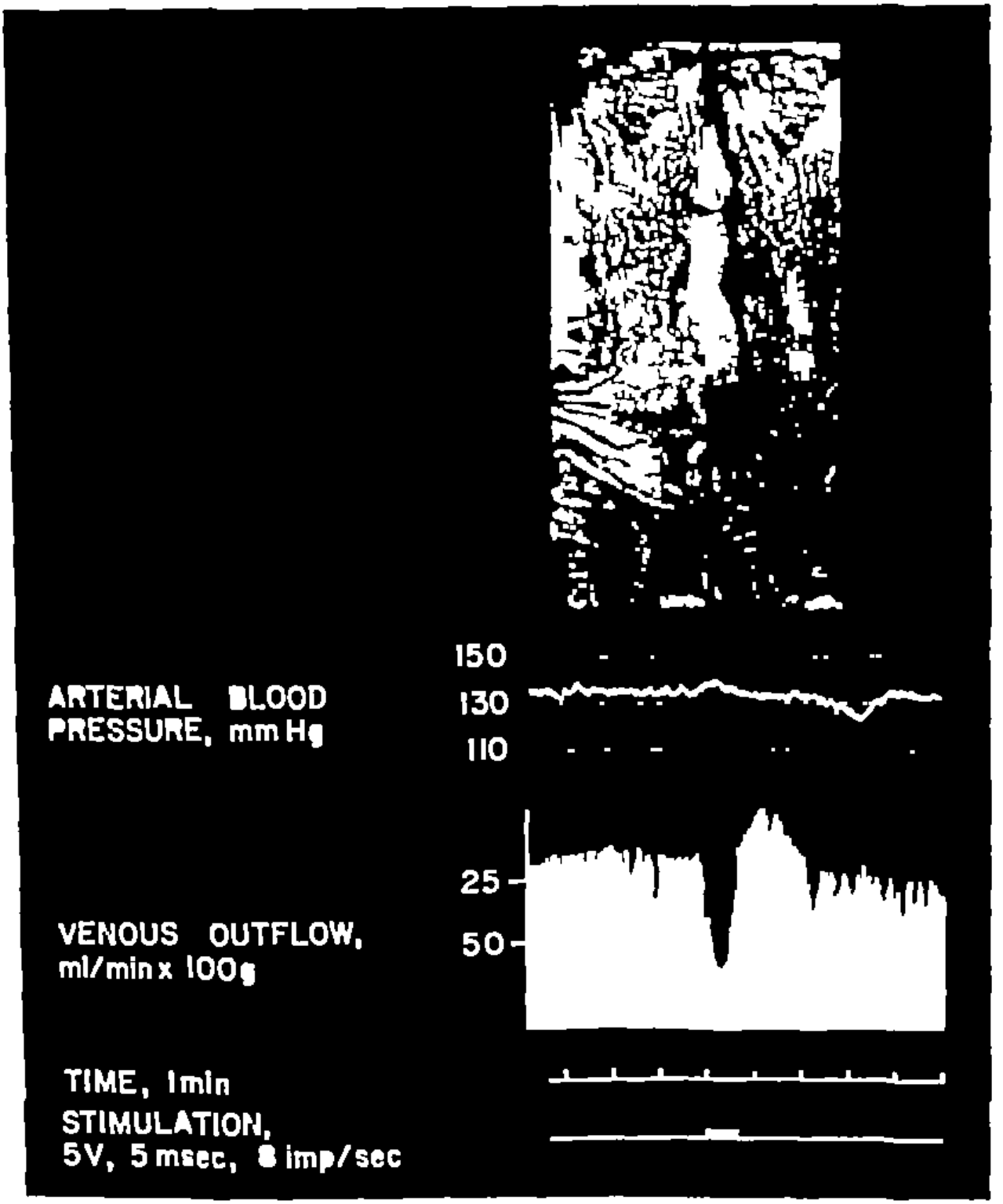


Fig 2. *Upper panel.* Cat 4.0 kg Chloralose A marked mucosal flushing occurs upon efferent pelvic nerve stimulation. This redness is exhibited at least in the distal 2/3–3/4 of the colon while the proximal part seems to be unaffected. Note the motor contraction, especially marked in the distal colonic part.

A corresponding transient blood flow increase is shown in *lower panel* Cat. 4.2 kg Note the phasic reduction of blood flow

lasting more than 1½ min This return towards the resting" blood flow level despite continued stimulation corresponded in time to the change in colour of the hyperaemic mucosa from bright red to bluish.

A blood flow increase was evident already upon stimulation at rates as low as 0.5 imp/sec Maximal values were reached at 8–10 imp/sec when flow values as high as 150–200 ml/min × 100 g tissue could be reached. This corresponded to a nearly maximal dilatation of the colonic vessels, as tested by intraarterial injections of supramaximal amounts of isopropyl noradrenaline

In order to ascertain that the pronounced but transient increase of venous outflow produced by pelvic nerve stimulation, was not to a great extent simply an effect of mechanical squeezing of the intramural capacitance vessels produced by the concomitant motility increase both the arterial inflow and the venous outflow were simultaneously recorded in some experiments. It was then clear that pelvic nerve stimulation evoked an increase of arterial inflow of the same magnitude as the increase of venous outflow (see Fig 3) showing that the venous outflow increase reflected a powerful vasodilatation. In such experiments it was also shown that even during high blood flow levels occlusion of the inferior mesenteric artery did not significantly change venous blood flow as long as the arterial connections to the superior mesenteric artery were intact

In some experiments the continuity of the bowel wall was broken by means of a ligature so applied that the extramural vascular and nervous connections and the colonic mesentery remained intact (see also chapter II) While exclusion of the caecum and the proximal 1/3–1/4 of the colon did not affect the vasodilator response indicating that vasodilation is probably not brought about in this part it was successively diminished in magnitude when the ligature was applied at lower levels and was abolished completely when the continuity of the bowel wall was broken at the peritoneal deflection. As the ligatures did not interfere with the colonic blood supply and drainage this indicates that the fibres mediating vasodilation are distributed intramurally as is also the case with the motor fibres in the pelvic nerves (see chapter II)

In order to ascertain the stimulation threshold of the pelvic nerve fibres, which produce colonic vasodilatation, the pulse duration was varied while voltage and stimulation frequency were kept constant at 5 V and 8 imp/sec A slight vasodilatation occurred at 0 03–0.1 msec, but as a rule a clearcut increase in blood flow was not recorded until durations of 0.8–1 msec were reached As a comparison threshold activation of the preganglionic sympathetic vasoconstrictor fibres was obtained at pulse durations of 0 03–0 1 msec when the same voltage and frequency were applied The dimensions of the nerve trunks stimulated were about the same. Furthermore the stimulation threshold of the motor fibres to the colon was tested in the same way and could not be separated from those producing colonic vasodilatation (see chapter II) However the motor fibres to the bladder which were stimulated in the same way in a few experiments during recordings of bladder volume seem to have a somewhat lower stimulation threshold.

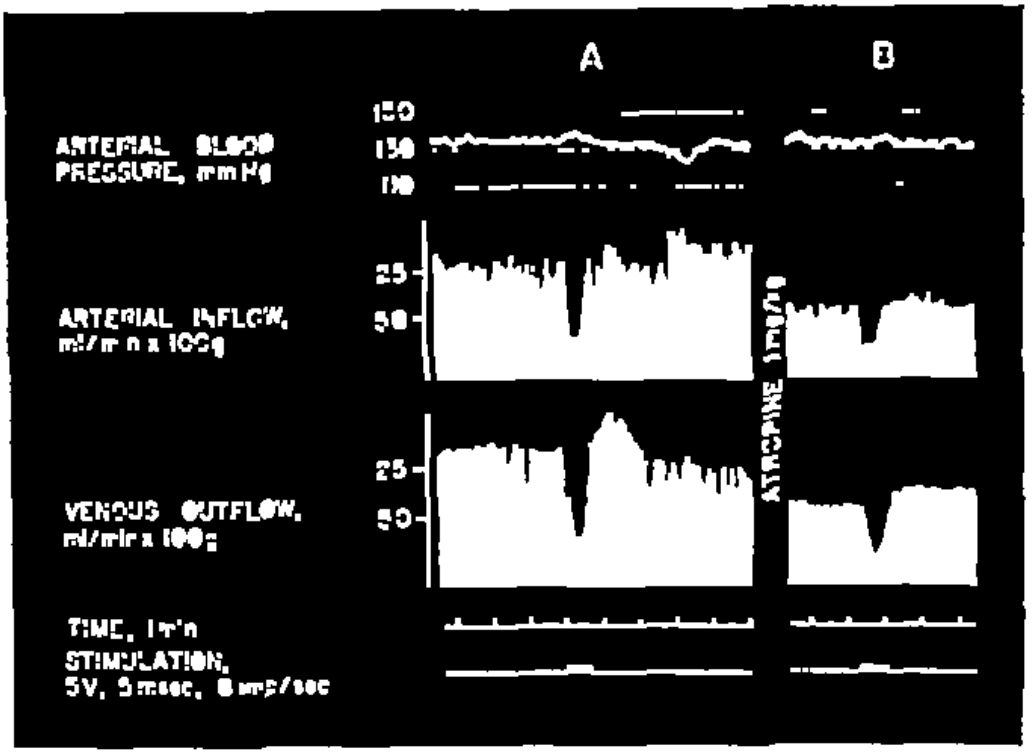


Fig 3. Cat 3 6 kg Chloralose. Effects of pelvic nerve stimulation (8 imp/s, 5 msec 5 V) on simultaneous recording of colonic arterial inflow and venous outflow before (left panel) and after (right panel) atropine administration (1 mg/kg).

In some experiments atropine was given in order to check if the vasodilatation upon pelvic nerve stimulation was mediated by a cholinergic mechanism However as shown in Fig 3 intravenous administration of atropine sulphate in a dose of 1 mg/kg did not interfere with the vasodilator response. Whereas administration of α and β-blocking agents or guanethidine did not affect the response it was completely abolished by a ganglion blocking agent (Ecolid® 2 mg/kg) indicating that a ganglionic synapse is present in the neurogenic pathway

3 *Blood volume determinations*

The regional blood volume of the colon and its mesentery was estimated in five experiments. It amounted to 8–10 ml/100 g tissue

IV COMMENTS

The present findings indicate that the vasoconstrictor fibres to the vascular bed of the colon are supplied from two sources. The thoracic sympathetic outflow innervates the proximal third via the splanchnic nerves while the lumbar sympathetic outflow via the lumbar colonic nerves controls the vessels in the remaining part of

th colon sometimes with a moderate overlap in distribution Both fibre sets produce an intense and uniform blanching of the respective sections of the colonic mucosa The effect produced by the lumbar colonic nerves was observed in the distal colon by Langley and Anderssom (1894) and confirmed by Wright *et al* (1938) However there seem to be no earlier reports concerning the effect of pelvic nerve stimulation Accordingly Hunter (1958) and Hulten (1961) embryologically the distal part of the colon is developed from the hindgut while the proximal part is derived from the midgut which may explain the differences in vasomotor fibre distribution

As in the case with the small intestine (Folkow *et al* 1961a) the neurogenic constriction of the colonic resistance vessels is characterized by a biphasic with an initial peak response followed by a decline to a new steady state of moderately increased resistance despite continued stimulation This autoregulatory escape from the vasomotor fibre influence will be further dealt with in chapter IV However the sustained and dramatic mucosal blanching suggests that the autoregulatory escape with return of the overall blood flow toward control level takes place in deeper wall layers This implies a neurogenic redistribution of blood flow from the mucosa toward submucosal structures also in the colon

Besides their motor influence on the gastrointestinal tract the vagal nerves were earlier assumed to exert widespread vascular effects via specific vasodilator fibres Martinson (1960) showed that vagal stimulation to the stomach produced vasodilatation associated with gastric secretion and corpus fundus relaxation It was further demonstrated by Swan and Jacobson (1960) that stimulation of vagal fibres increased blood flow in the stomach mucosa However this mucosal vasodilatation may well have been metabolic secondary to the increased secretion rather than due to specific vasodilator fibres Brewster (1956) found no evidence of vagal vasodilator fibres in the small intestine In the present study no sign of increased colonic blood flow whether as a mucosal flushing or as a reduced blood flow resistance was observed even upon maximal activation of the vagal nerves It is therefore unlikely that the vagal nerves convey any vasodilator fibres to the colon and this may also be true for the gastrointestinal tract as a whole to judge from the studies quoted also

In agreement with the results of Langley and Anderssom (1894) and Wright *et al* (1938) pelvic nerve stimulation led to an intense flushing of the colonic mucosa It appeared first of this mucosal vasodilatation (see chapter V) spread gradually in the proximal direction It did not however involve at all the most proximal 50 per cent of the colonic mucosa and the borderline zone toward the markedly vasodilated distal parts was indistinct The mucosal vasodilatation was in other words largely restricted to that part of the colon which is also controlled by the sympathetic lumbar colonic nerves perhaps being slightly larger However pelvic nerve stimulation regularly produced increased secretion and a concomitant motor response where the increased motility involved the entire colon (see chapter II)

Thus the vascular response was never obtained alone and the fibres mediating vascular and motor response could not be separated by means of differences in their electrophysiological properties.

The colonic vascular response produced by pelvic nerve stimulation showed a characteristic response pattern when explored by concomitant recording of the venous outflow and of the mucosal flushing. A rather shortlasting but nearly maximal blood flow increase occurred in close parallel with the mucosal flushing. Soon however flow returned towards control, while the mucosa remained hyperaemic but changed in colour from red to bluish. The explanation for the short duration of the initial blood flow increase is not known (cf. chapter IV).

Since pelvic nerve stimulations produced a transient increase of arterial inflow of the same order of magnitude as that concomitantly observed in the venous outflow it is excluded that the phasic venous outflow increase was mainly due to a "squeezing" effect on the venous content of blood due to the motor contraction. It is no doubt a true vasodilator response which the mucosal flush also clearly indicates. Furthermore the regional blood content in the colon during "resting" condition was calculated to be 8–10 ml/100 g tissue and a mere venous squeezing could not possibly be responsible for an increase of venous outflow amounting to more than 100 ml/min × 100 g tissue.

It is thus clear that the pelvic nerves can produce a nearly maximal vasodilatation in the colon probably mainly involving the mucosal vessels (see chapter V). Even though a cholinergic link may be involved it is hardly likely that the vascular smooth muscles are directly affected by cholinergic nerve endings. Even large doses of atropine failed to affect the dilator response while the effect of infused acetylcholine was completely abolished. In this respect the colonic vasodilatation closely resembles the vasodilatation causing penile erection as produced by the pelvic nerves (Dorr and Brody 1967), the vagally induced vasodilatation in the stomach (Martinson 1966) and the salivary gland vasodilatation obtained on stimulation of the chorda tympani (Hilton and Lewis 1955, 1956).

As discussed in the introduction the large bowel may be looked upon as an organ especially designed for mixing the contents delivered by the small intestine and to allow for a final absorption of mainly salt and water. These functions may be dependent mainly upon the proximal colonic parts. On the other hand, storage of the contents and their subsequent expulsion may constitute the main function of distal colonic parts. To facilitate the passage of the often firm faeces lubrication is required calling for a mucus secretion in connection with emptying of the distal colon. The secretory response to pelvic nerve stimulation seems to be mainly limited to the distal part of the colon and is accompanied by a mucosal blood flow increase presumably to supply the glands with raw material for the secretion of mucus. Such a view might explain why the vasodilator and probably also the secretory response to pelvic nerve stimulation are largely restricted to the distal parts of the colon and why the corresponding motility response although involving the entire colon is

different in it characteristics in the proximal (mainly circular contraction) and distal (mainly longitudinal contraction) part (see chapter II)

While the vasodilator effect produced by the pelvic nerves emerges the distal /3–3/4 of the colon the vagal nerve supply to the proximal part of the colon has no influence on the corresponding blood vessel The two sets of colonic vasomotor fibre supply essentially the corresponding part of the colon These result with respect to the differences in vasomotor fibre control may lend further support to the view that the colon is functionally differentiated into two main parts as was also the case with several aspect of the motor innervation to the colonic musculature as described in chapter II

CHAPTER IV

LOCAL AND NERVOUS CONTROL OF THE CONSECUTIVE VASCULAR SECTIONS OF THE COLON

L. Hultén, M. Jodal and O. Lundgren

I. INTRODUCTION

The control of the various systemic vascular circuits have been studied in detail, and considerable differences exist between their functionally specialized series-coupled sections (for a recent review see Mellander and Johansson 1968). Studies of such consecutive vascular sections have been made possible by means of continuous measurements of tissue weight or volume (e.g. the plethysmographic technique) as combined with direct and continuous blood flow recording. This approach originally designed by Pappenheimer and co-workers for their studies of capillary function (see Pappenheimer 1953) was developed for the analysis of the nervous control of the skeletal muscle vascular bed (Mellander 1960) and later applied to the small intestine (Folkow *et al.* 1963). The present study describes the application of this method for the analysis of the consecutive vascular sections of the colon in the cat.

As in the small intestine secretion and absorption are important features in colonic function, both processes being energy consuming and requiring a considerable blood supply. Furthermore the colonic vascular bed is also under the control of a potentially powerful neurogenic control. It was therefore considered of interest to study blood flow during resting conditions and during vasodilatation induced by a drug as well as the range of control exercised by the sympathetic vasoconstrictor fibres and by the vasodilator action of the parasympathetic pelvic nerves.

II. METHODS

A. Operative procedures

Observations were carried out on a total of 46 cats fasted for 24 hours. The operative procedures, the registration of blood flow and the nervous stimulations were with minor modifications (see below) identical to those described in chapter III (see page 40)

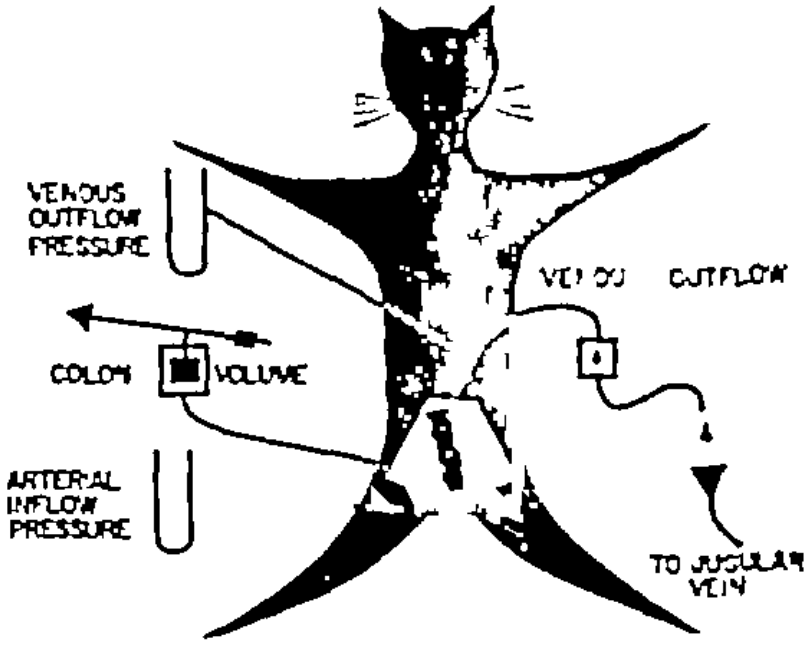


Fig 1 Schematic illustration of the technique used for isolating the rectum with the connected proximal section of the large bowel.

To induce graded vasodilatation close intraarterial infusions of norepinephrine-noradrenaline (NA) were performed by means of a thin polyethylene catheter inserted into a small branch of the superior mesenteric artery Atropin sulphat and l-a lrenaline were administered i v and i.a. respectively

B Plethysmographic technique

To allow a continuous recording of changes in tissue volume the colon was enclosed in a perspex plethysmograph For this purpose the colon was dissected distally in the pelvis beneath the entrance of the pelvic nerves and separated from the bladder Furthermore the colonic mesentery had to be cut up to the superior mesenteric root thereby sacrificing the inferior mesenteric artery In the experiments described in chapter III where the arterial inflow was recorded simultaneously with the total venous outflow it was known that even during maximal flow levels occlusion of the inferior mesenteric artery in the preparation used did not significantly change venous blood flow as long as the arterial connections to the superior mesenteric artery were intact

The colon, with ring electrodes mounted on the planchnic the lumbar colonic and the pelvic nerve was then placed in the plethysmograph in such a manner that the superior mesenteric artery and the cannulated vein passed through a closely fitting opening which was further sealed with plast base (Squibb) or Vaseline grease (see Fig 1). To make the enclosement of the colon easier most of the lymph nodes in the mesentery were often resected The widebore venous cannula was always introduced far enough distally as to be well inside the sealed opening thereby avoiding venous collapse by undue lateral pressure The exact value of the venous outflow pressure was in most experi-

ments recorded by means of a water manometer coupled to the connection between the superior mesenteric vein and the drop recorder The colon was thus enclosed in a perfectly air and watertight way without interference with either arterial inflow or venous outflow and colonic motility and colour could be directly inspected. The plethysmograph was filled with Tyrode solution kept at 38°C and controlled by a thermocouple, leaving only a small air volume in the upper outlet which was connected to a sensitive volume piston recorder

C. Determination of the capillary filtration coefficient (CFC)

After enclosing the colon in the plethysmograph it was, in most experiments, possible to adjust the venous pressure so that an isovolumetric state was attained implying that the net transcapillary movement of fluid was zero This isovolumetric equilibrium could then be deliberately disturbed by temporarily increasing venous outflow pressure by a known amount, usually 7 mm Hg (= 10 cm H O) This procedure evoked a characteristic volume increase in two phases which showed completely different slopes (see e.g. Fig 2) As earlier demonstrated (Mellander 1960 Kjellmer 1965) the first rapid change in volume is caused by vascular distension, mainly of the thin walled veins while the continuous and slower increase of the volume curve the filtration slope reflects the outward filtration caused by the raised capillary hydrostatic pressure The capillary filtration coefficient (CFC) expressed in ml of fluid filtered across the capillary wall per min in 100 g tissue and per mm Hg pressure difference, was calculated assuming a mean hydrostatic capillary pressure of 15 mm Hg at a venous outflow pressure close to zero (cf Folkow *et al.* 1963 Wallentin 1966).

III RESULTS

A Infusion of isopropyl noradrenaline

1 *Resistance vessels*

Intraarterial infusions of supramaximal amounts of isopropyl noradrenaline (INA) increased colonic blood flow from a resting level of about 15–25 ml to approximately 200 ml/mn × 100 g tissue. This corresponded to a maximal vasodilatation. Expressed in terms of *peripheral resistance units per 100 g tissue* (PRU_{100}) the *flow resistance* at rest 4-9 PRU_{100} was reduced to about 0 4–0.5 PRU_{100} during maximal vasodilatation.

2. *Capacitance vessels*

Corresponding in time to the blood flow increase induced by INA, the colonic tissue volume also increased maximally some 3 per cent reflecting a widening predominantly of the capacitance vessels. Since the blood volume of the acutely denervated colon, including the mesenteric vessels was 8–10 ml/100 g of tissue (see chapter III) this change in tissue volume corresponds to a 30–40 per cent increase of blood content.

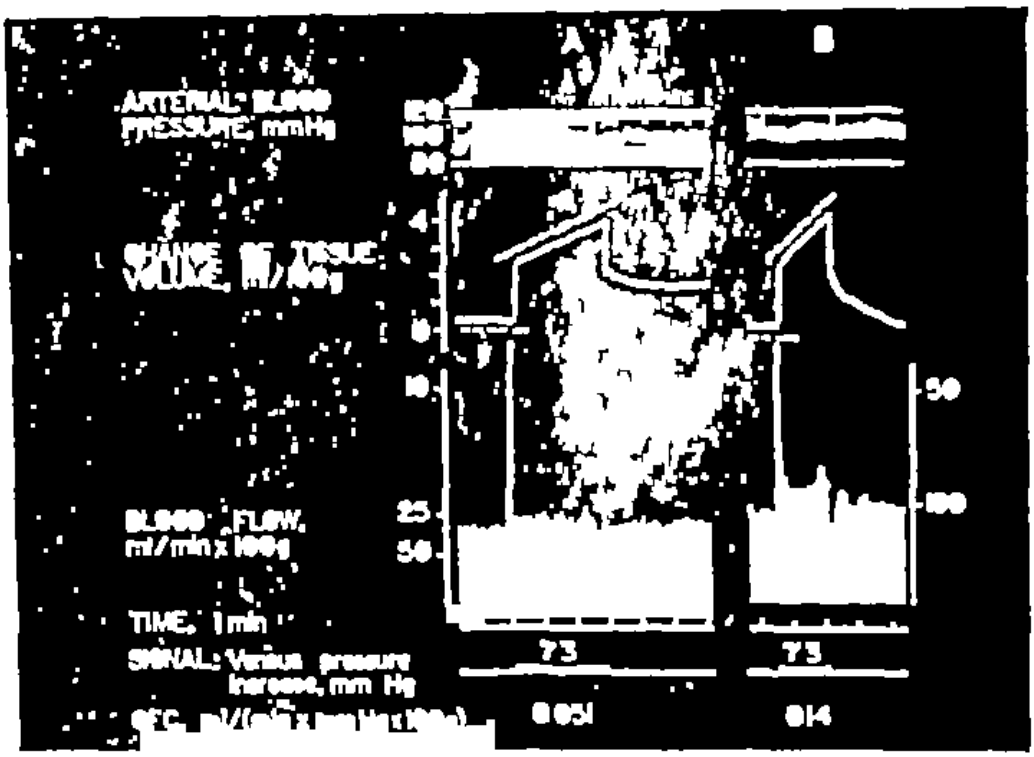


Fig. C t ..8 kg The effect of sudden venous pressure increase (7.3 mm Hg = 10 m H_2O) on colonic tissue volume and blood flow before (A) and during an infusion of norepinephrine (B) The capillary filtration coefficient (CFC) was calculated from the low continuous filtration slope indicated by dotted lines. Note that the blood flow calibration differs in the two panels.

3 *Exchange vessels*

The intestinal capillary filtration coefficient (CFC) reflecting the capillary surface area available for transcapillary exchange and hence varying with precapillary sphincter tone was determined at different levels of vascular tone

Fig illustrates the effects of a sudden rise in venous pressure on tissue volume and blood flow A rapid increase in tissue volume is obtained due to dilatation of the capacitance vessels which corresponds in time with a transient reduction of venous outflow This phase is followed by a second far slower but fairly continuous volume increase reflecting an outward capillary filtration (see Method) At resting colonic blood flow levels CFC was approximately 0.03 ml min 100 g tissue x mm Hg and increased to about 0.15 at maximal vasodilatation when blood flow was approximately 80 ml min 100 g tissue The accumulated data in Fig 3 show the correlation between colonic blood flow and CFC at different levels of vascular tone

B Effects of graded direct stimulation of the sympathetic supply

1 *Resistance vessels*

The neurogenic increase of colonic flow resistance consisted of two phases as was briefly discussed in chapter III After an initial peak flow resistance declined again

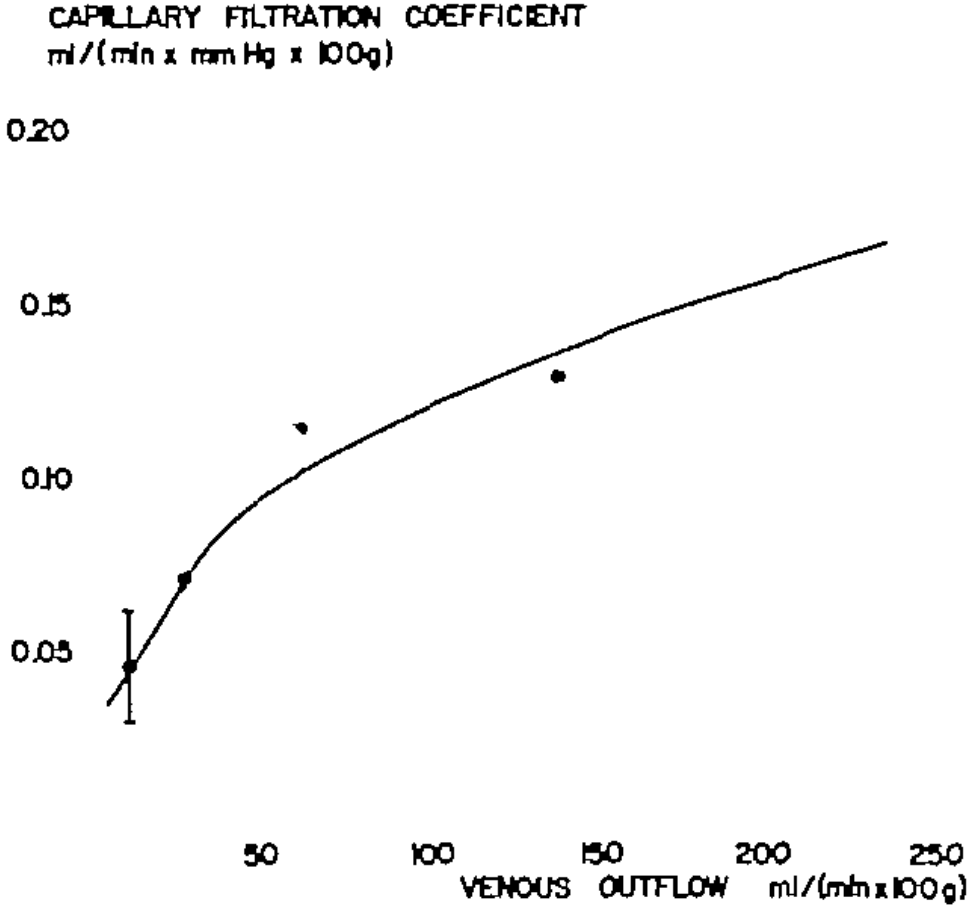


Fig 3. Cumulated data (n = 93) on the correlation between total colonic blood flow and CFC at different levels of vascular tone Vertical lines indicate ± one standard deviation. Curve drawn by inspection. Graded vasodilatations were induced by i.a. infusions of isopropyl-noradrenaline.

within 2 to 3 min, a phenomenon which in the small intestine is denoted "autoregulatory escape from the vasoconstrictor fibre influence (see Fig 4) The peak response increased markedly with increasing rates of stimulation from a "resting value of 4–9 PRU_{100} it reached about 30 PRU_{100} when all vasoconstrictor fibres were simultaneously activated at 8 imp/sec Activation of the lumbar colonic nerves alone increased peak flow resistance to some 15 PRU_{100} but involved as mentioned in chapter III, only about the distal 2/3–3/4 of the colon

The autoregulatory escape was pronounced at high rates of stimulation, but the steady state neurogenic response nevertheless showed an augmentation with increasing stimulation frequencies (Fig 5) The steady state resistance response at high frequencies only exceptionally exceeded control resistance by 100 per cent, cessation of the stimulation led to a reactive hyperaemia (see Fig 6)

2. *Capacitance vessels*

Fig. 4 (panels A, B and C) illustrates the effect of regional vasoconstrictor fibre stimulation on blood flow and tissue volume reflecting changes in blood content and

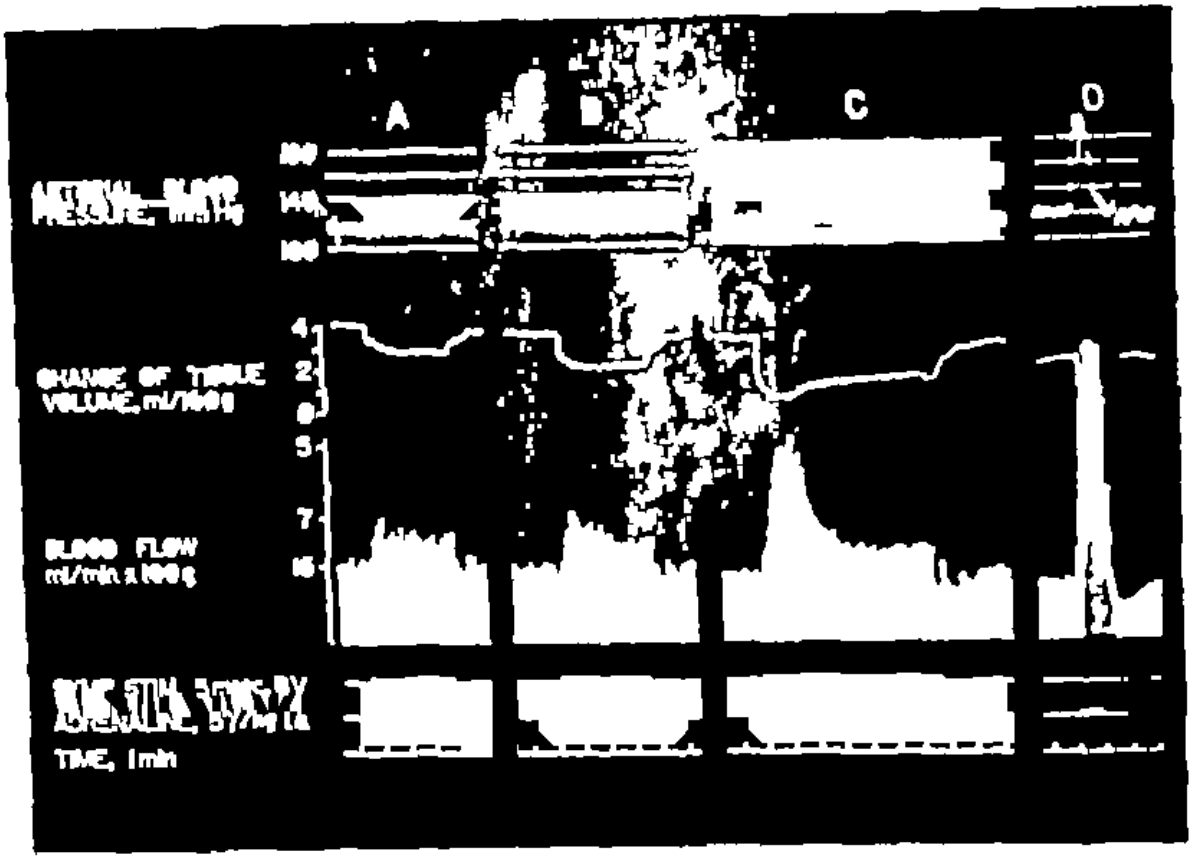


Fig. 4. Cat 3.8 kg. The effect of graded stimulation of the sacral vasoconstrictor fibres on arterial blood pressure, blood flow and tissue volume of the large bowel at normal venous outflow pressure (15 cm H_2O) (panels A, B and C). As a comparison the effect of an i.v. injection of large dose adrenaline is illustrated in panel D.

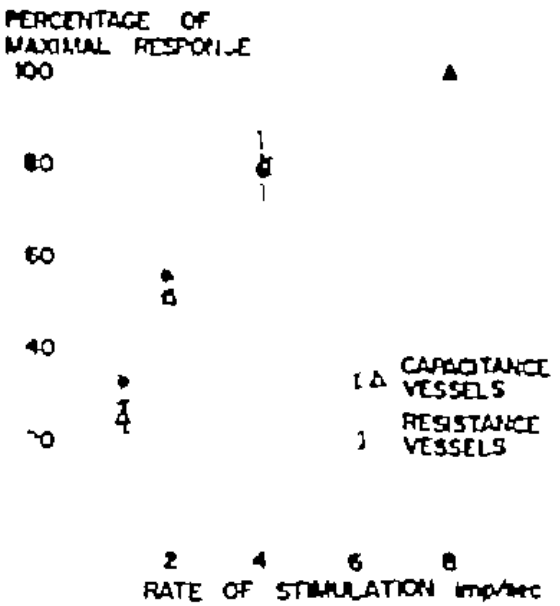


Fig. 5. Frequency response curves derived for the colonic resistance and capacitance vessels. The effects of graded vasoconstrictor activation are expressed in per cent of maximal response obtained in the two vascular sections. The steady state response of the resistance vessels was used in the calculations. The experiments were performed at venous outflow pressure of approximately 15 cm H_2O. Vertical lines indicate ± one standard error of the mean.

tissue fluid Corresponding in time with the drastic resistance increase there is a rapid decrease in volume which was kept far more constant during the stimulation period than the resistance response where the "autoregulatory escape was often pronounced. However when venous outflow pressure was kept low there was often an initial but transient decrease in volume in phase with the peak resistance response This evidently reflects a mere passive-elastic recoil and partial collapse of the venous capacitance compartment in turn due to a transient increase in the pre to post capillary resistance ratio which implies an initially reduced transmural pressure in capillary and venous sections. The largely passive nature of this initial decrease in volume was obvious from the fact that it was largely absent when venous outflow pressure was kept high enough to keep the capacitance vessels distended. This is evident from the experiment in Fig 4 performed at a venous outflow pressure of 15 cm H_2O The volume increase following cessation of stimulation equalled the decrease at the onset of stimulation suggesting that the active capacitance response was well sustained "Resting" colonic blood content estimated separately in five experiments (see chapter III) was related to the volume decrease caused by graded vasoconstrictor fibre stimulation. It was calculated that approximately 20 per cent of the blood content was expelled at a discharge rate as low as 2 imp/sec the maximum of some 30–50 per cent being reached at 8 imp/sec A marked flow reduction caused by adrenaline was accompanied by a capacitance response of a similar magnitude (panel D)

Fig. 5 illustrates the frequency response curve for the capacitance vessels as compared to that of the resistance vessels during the *steady state* phase The response of the resistance and capacitance vessels are expressed in per cent of the maximal values obtained. The figure is based on experiments performed at a venous outflow pressure of 15 cm H_2O It can be seen that the frequency response curves of the two vascular sections are almost identical when in this way the *locally* modulated resistance response to nerve stimulation is used The same result was obtained also when venous pressure was kept low If, on the other hand the *peak* resistance responses were plotted in the same way as in Fig 5 the curve was placed to the right of the capacitance curve

Finally during the steady state of the vasoconstrictor response the volume recording was isovolumetric indicating that the pre-to postcapillary resistance ratio remains largely the same as during the control state i.e. mean hydrostatic capillary pressure was unchanged

3 *Exchange vessels*

Vasoconstrictor fibre stimulation reduced colonic CFC, the approximate maximal effect being reached at 8–10 imp/sec Fig 6 illustrates a representative experiment in which CFC was reduced from 0 065 to 0 045 ml/min × 100 g × mm Hg during a stimulation of 4 imp/sec. As was the case with the capacitance response the CFC reduction was well sustained during the entire stimulation period.

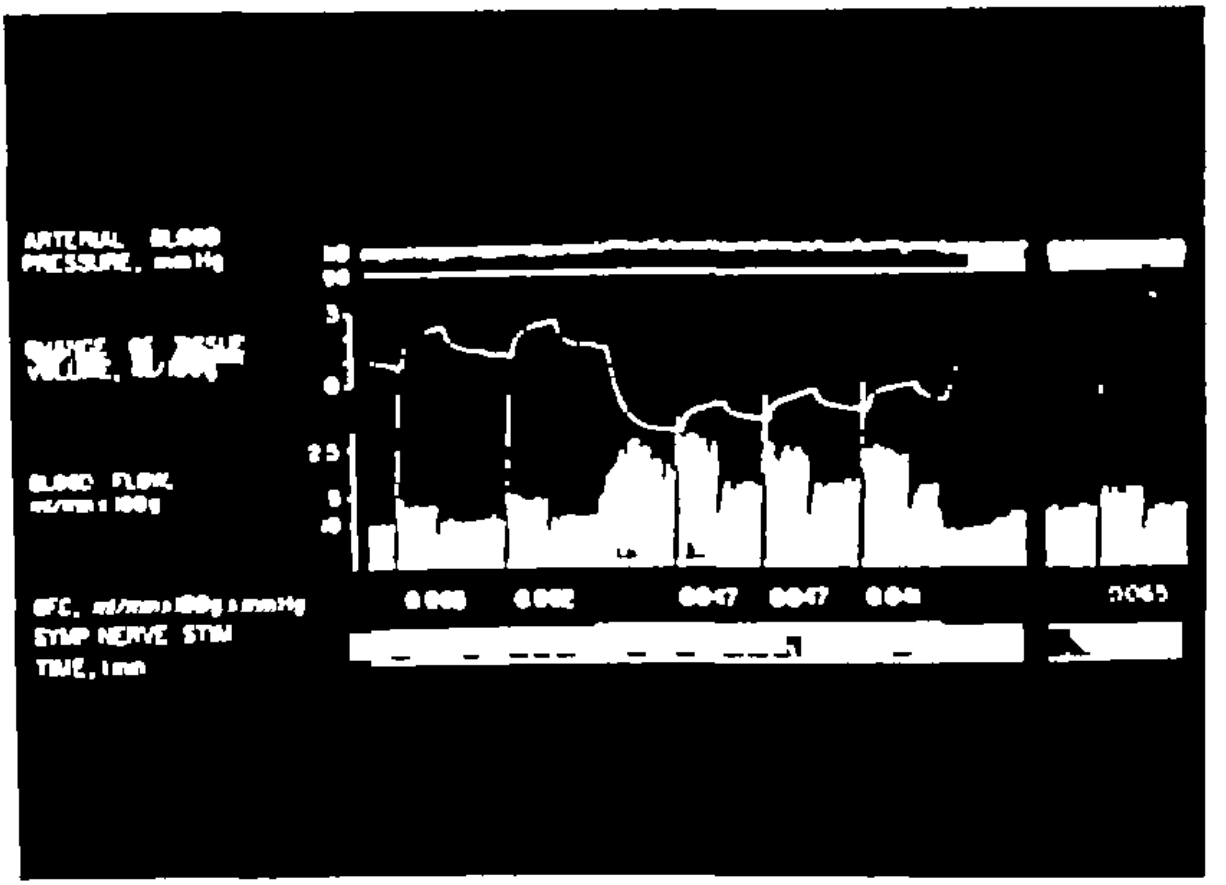


Fig. 4. C. 3.6 kg. The effect of ... vasoconstrictor fibre stimulation at 4 imp/sec on blood pressure, blood flow, tissue volume and capillary filtration coefficient (CFC) of the large intestine. (For details, see text.)

C. Effects of graded direct stimulation of the parasympathetic supply (the pelvic nerves)

1. *Resistance vessels*

Within 5–10 sec after commencement of pelvic nerve stimulation blood flow increased to reach maximal levels in 20–30 sec. This flow increase, in the following referred to as *initial*, usually began to vanish within 30–40 sec, only exceptionally lasting more than $1\frac{1}{2}$ min, despite continued stimulation. In general, the *duration* of the vasodilatation varied inversely with the rate of stimulation, fairly long-lasting flow increases occurring at low frequencies, whereas the magnitude of the blood flow increase was directly graded to the stimulation rate (see Fig. 7).

An initial blood flow increase was evident already at 0.5 imp/sec and maximal values, 150–200 ml/min/100 g tissue, were obtained at 8 imp/sec, with about 80 per cent of the maximum reached at 2–4 imp/sec (Fig. 7). The magnitude of the flow

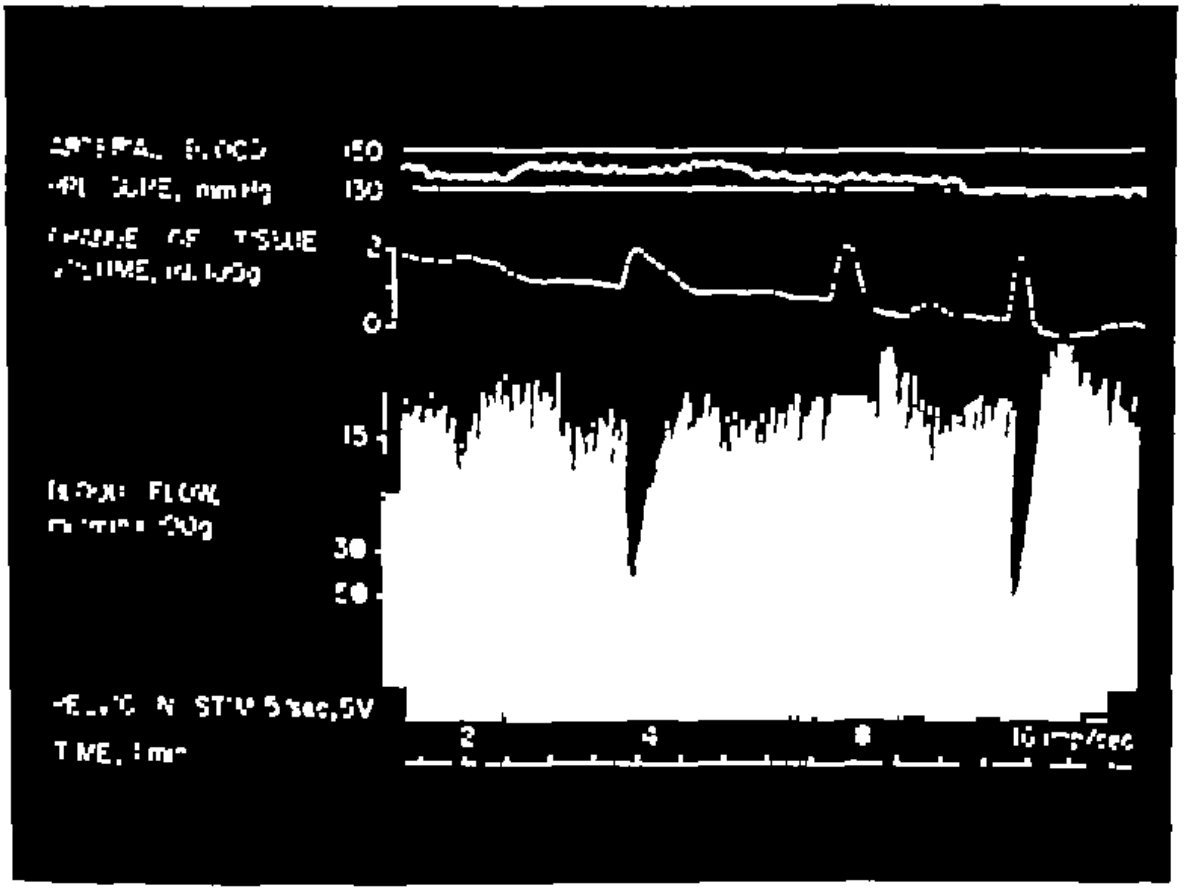


Fig. 7 Cat ~1 kg. The effect of graded stimulation of the pelvic nerves on blood pressure blood flow and tissue volume of the colon. The animal was tropinised (1 mg/kg b.w.) Note the small and transient volume decrease corresponding t the phasic reduction of blood flow

increase was roughly the same whether one or both pelvic nerves were activated indicating extensive overlap in innervation. Expressed as PRU_{100}, flow resistance fell from resting levels of 4–8 to 0.4–0.6 PRU_{100}. Supramaximal amounts of ACh could only exceptionally reduce flow resistance further (see page 53)

Once induced a fully developed initial vasodilator response occurred within 5–10 sec despite interruption of the stimulation. On the other hand this vasodilator response vanished despite continuation of the stimulation and was followed by a phasic blood flow reduction proportional to the stimulation rate and consequently to the magnitude of the vasodilatation. During this transient flow reduction vascular resistance could increase some 50–70 per cent above control at maximal rates of stimulation. In chapter III it was pointed out that this phasic reduction of blood flow corresponded to a bluish colour shift in the hyperaemic mucosa. However as illustrated in fig. 7 (left panel) during *prolonged* pelvic nerve stimulations this resistance increase in the most experiments followed by series of shortlasting blood flow increases until it returned to the resting level upon cessation of stimulation. These oscillations in the following referred to as *recurring* were, however always smaller than the initial blood flow increase, on the average reaching about 40–60 per cent of the

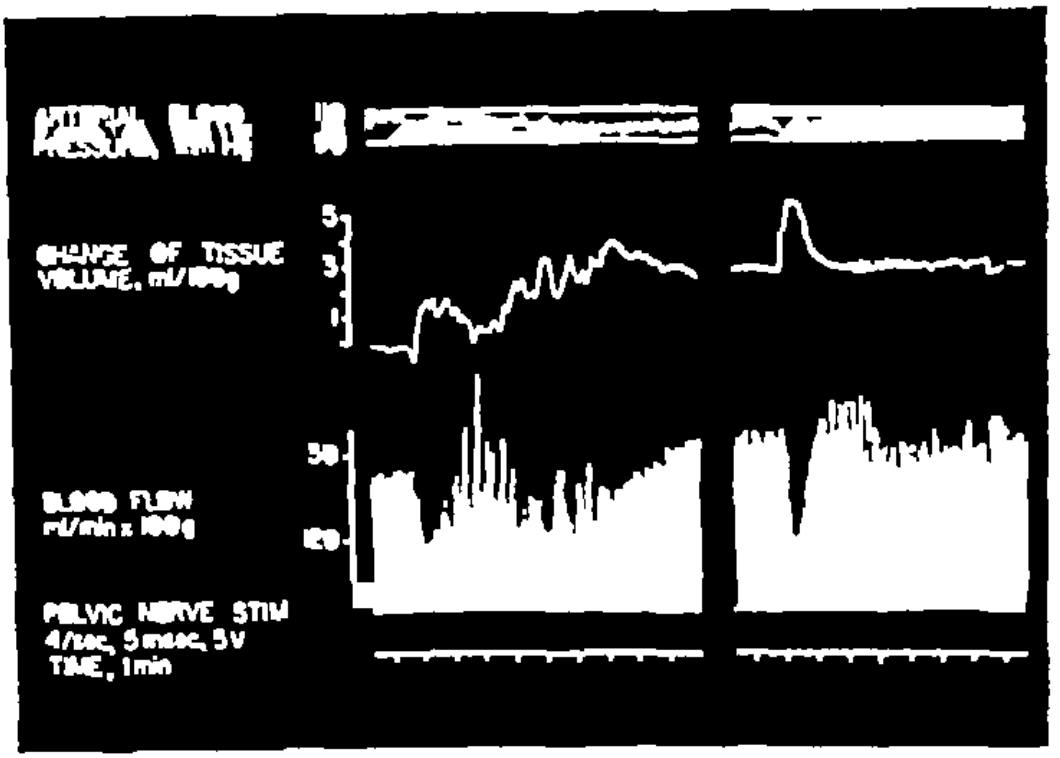


Fig 8 Cat 3.. kg The effect of bilateral pel ic nerv stimulation t 4 imp sec on blood pressure blood flow and tissue volume f the colon bef ee (left panel) and after (right panel) admin tration of atropine (1 mg/kg b w) Note the *initial* and *recurring* blood flow increases, the latter abol shed by troj ne admi istrat n.

The administration of atropine in doses that blocked the vagal effects on heart rate did not reduce the *initial* blood flow increase on pelvic nerve stimulation but aboll hed the recurring blood flow increases seen during prolonged nervous activa tion (Fig 8 right panel)

The pelvic nerves seemed to be more or less fatigued when exposed to repeated stimulations at high rates and the largest initial vasodilatory responses were m stly seen upon th first few stimulations \ high frequency stimulation following one of low frequency often resulted in a smaller initial vasodilator response if a sufficient rest period wa not allowed between the stimulation If one of the pelvic nerves was *stimulat d to fatigue stimulation of the other one resulted in an immediate* and marked vasodilatation despite the evidence mentioned above of a considerable overlap of innervation

° *Capacitance vessels*

Fig 7 taken from an experiment on an atropinized animal illustrates the effect of graded bilateral pelvic nerve stimulation with the characteristic shortlasting initial vascular responses corresponding in time with a rapid increase of tissue volume which is follow l by a somewhat slower volume decline towards control The magni

tude of the volume change followed closely the resistance response increasing with increasing rates of pelvic nerve stimulation At most, volume increased 2 0–2.5 ml/100 g tissue at 8 imp/sec i.e an approximately 25 per cent increase of blood content, with 80 per cent of the maximum being reached at 4 imp/sec Increasing venous outflow pressure from 5 to 15 cm H_2O reduced the capacitance response on pelvic nerve stimulation by some 30–40 per cent. The relation between capacitance and resistance responses was also close with respect to their time courses. Thus as the duration of the resistance response was shortened with increasing rates of stimulation so was the shift of colonic tissue volume Corresponding to the phasic reduction of blood flow a small and transient volume decrease was sometimes observed (Fig 7)

The changes in tissue volume during prolonged pelvic nerve stimulation before and after administration of atropine is illustrated in Fig 8 The response pattern in the atropinized cat (right panel) was similar to that described above (cf. Fig 7) The experiment illustrated in Fig 8 (left panel) shows that before atropine an initial abrupt increase of tissue volume is followed by a comparatively slow irregular volume increase. *Furthermore, upon cessation of stimulation tissue volume never returns to* pre-stimulation control level. For such reasons it seems reasonable to assume that the slow volume increase is to a great extent caused by secretion If this is true the slow phase may reflect a secretion amounting to 0 1 ml/min or 0.5 ml/min × 100 g tissue

3 *Exchange vessels*

Since the initial vasodilator response induced by pelvic nerve stimulation is fairly brief (Fig 7 and 8) lasting at most 1½ min, and accompanied by a transient increase of tissue volume *determination of CFC during that period was not possible. It was* also impossible to determine CFC during prolonged activation of the pelvic nerves in animals not atropinized (see Fig 8 left panel) Determinations made during prolonged pelvic nerve stimulations in atropinized animals and immediately after cessation of pelvic nerve activation gave no conclusive evidence for any change of CFC although in some experiments a slight increase was observed.

IV COMMENTS

A plethysmographic technique which is modified from that developed by Mellander (1960) and also applied to studies of the vessels of the small intestine (Folkow *et al* 1963) was used in the present study for an investigation of sympathetic and parasympathetic control of the colonic vasculature The reactions of the consecutive vascular sections i.e. pre- and postcapillary resistance vessels precapillary "sphincters and capacitance vessels with their secondary effects on capillary exchange, were thus studied at rest" during vasodilatation and at different levels of nervous activity

To facilitate the interpretation of the complex responses within the different vascu

lar sections the effects of sympathetic vasoconstrictor fibre stimulation will be dealt with first. The present results indicate that the colonic vascular responses with few exceptions correspond to those occurring in the small intestine (Folkow *et al.* 1961*a*, *b*). This contrary to the conditions in resting skeletal muscle where the neurogenic increase of flow resistance is fairly well sustained and the precapillary sphincters exhibit a considerable escape as judged from CFC measurement in the colon the resistance response exhibits "escape" while CFC is kept reduced throughout the period of constrictor fibre stimulation. However the escape of the colonic resistance vessels as well as the reduction of CFC was less pronounced than in the small intestine. This perhaps implies that the extent of blood flow redistribution from the outer mucosa to deeper wall layers is not as extensive in the colon as in the small intestine (Folkow *et al.* 1961*a*, *b*). But mucosal low resistance channels allowing for a physiological shunting (but *not* a true low pass of exchange vessels) seem to be involved in this blood flow redistribution (Drost *et al.* 1960). Shunt like vessels have been described both in the stomach and in the small intestine on the basis of morphological studies (Spanner 1953; Thamm 1960; Walker 1961; Wiedman 1963) while such vessels appear to be less numerous in the colon and rectum (Boulter and Parks 1960). It is possible that this difference in the arrangement of the vascular bed in the mucosal-submucosal compartment might explain a less pronounced redistribution of blood flow in the colon when the vasoconstrictor fibres are stimulated. However the sustained mucosal blanching despite the autoregulatory escape of overall flow resistance strongly suggests that flow redistribution does occur in the colon too favouring flow in deeper parts of the mucosa and/or the submucosa.

The type and magnitude of the capacitance response to vasoconstrictor fibre stimulation closely resembled that seen in the small intestine (Folkow *et al.* 1964*a*). Thus the active component of blood expulsion from the venous side was well sustained. However it could be more or less masked by passive recoil effects as venous outflow pressure was kept so low as to allow for partial venous collapse in connection with the peak response of the precapillary resistance vessels. Maximal capacitance responses corresponding to some 40 per cent blood expulsion were obtained at 8 imp/sec. The resistance vessels during the steady state phase exhibited a frequency response curve similar to that of the capacitance vessels. This observation is apparently contrary to the one reported by Mellander (1960) for the skeletal muscle vascular bed where the frequency response curve of the capacitance vessels is placed well to the left of that of the resistance vessels. The observed discrepancy may be explained by the fact that a redistribution of blood flow from superficial to deeper mucosa-submucosal layers occurs in the colon during the autoregulatory escape and this locally induced adjustment is often especially pronounced at high frequency stimulation. The resistance response of the vessels in superficial parts of the mucosa is probably quite similar to that in skeletal muscles being sustained during the period of stimulation. This suggestion is strengthened by the observation that the frequency response curve of the *peak* responses of the resistance increases in the colonic vascular bed was

similar to that of skeletal muscles and placed well to the right of the capacitance curve.

The resting" colonic blood flow after acute vascular denervation largely corresponding to a situation of reflexly inhibited sympathetic activity in the intact organism, ranged between 15 and 25 ml/min × 100 g tissue This is about the same as in the stomach (Martinson 1965) but only about half that of the small intestine when studied under similar experimental conditions (Folkow *et al* 1963 Lundgren 1967) With maximal vasodilatation in the colon induced by infusion of INA the blood flow increased to approximately 200 ml/min × 100 g tissue which corresponds to about 75 per cent of the maximal flow capacity observed in the small intestine (Folkow *et al* 1963) The range of control of the colonic vascular bed as exercised by locally produced vasodilator factors and by the centrally controlled constrictor fibres therefore coincides well with that of the small intestine although the small intestine seems to be in general terms better vascularized

The capillary filtration coefficient (CFC) of the colonic vascular bed amounted to 0 05–0.07 ml/min × 100 g × mm Hg during resting conditions (Fig 2 and 3) and could be increased to approximately 0 15 ml/min × 100 g × mm Hg during maximal vasodilatation induced by isopropyl-noradrenaline (INA) These values are only 1/5–1/3 of the values reported for the small intestine of the cat (Folkow *et al* 1963) This difference is probably explained mainly by a larger capillary surface area in the small intestine reflecting the dominating absorptive function of this part of the gastrointestinal tract

Whereas the vasodilator action of the pelvic nerves upon the colonic vasculature was observed by Langley and Anderssen (1895) no investigations seem to have specifically dealt with the distribution and quantitative effects of these nerves. The results of the present study indicate that the initial dilatation of the resistance vessels observed during pelvic nerve stimulation was accompanied by a widening of the capacitance vessels corresponding to an increase of regional blood volume of about 25 per cent at maximum This value represents in all probability an underestimation since it is calculated on the total weight of the colonic preparation, not taking into account that the vasodilatation occurs mainly in the distal 2/3–3/4 of the colon (see chapter III and V) From the present experiments it cannot be settled whether this widening of the capacitance vessels is only due to a secondary venous distension as a result of precapillary vascular relaxation or if the nerve action causes relaxation of the venous smooth muscles as well Furthermore it was demonstrated that stimulation of the pelvic nerves increased colonic secretion (cf chapter III) with *recurring* increases of total colonic blood flow (Fig 8) The administration of atropine abolished the recurring increases of blood flow and probably the colonic secretion (Fig. 8) while the *initial* marked blood flow augmentation and the motor response were largely unaffected This observation suggests that two vasodilator mechanisms may be involved, one atropine-resistant and one abolished by atropine possibly induced by unspecific metabolites produced during secretion.

The present result indicate that the fibres which mediate the initial marked vasodilatation are distributed with considerable overlap since almost identical responses are induced whether one or both pelvic nerves were activated. The vasodilator effect was easily fatigued but when repeated stimulation of one pelvic nerve had led to elimination of the response an undiminished effect was obtained if the other nerve was stimulated. This finding suggests that the site of overlap is central to the site of "fatigue". The initial vasodilatation which was eliminated by a ganglion blocking drug was not significantly reduced by administration of even large doses of atropine (1 mg/kg) which suggests that a purely cholinergic transmission can hardly be involved (see chapter III). In these respect the mechanism by which colonic vasodilatation is produced closely resembles that involved in parasympathetic vasodilatation in salivary glands (Hilton and Lewis 1955, 1956) or in penile erection (Kerr and Brody 1967).

The general characteristics of pelvic nerve stimulation on colonic function are quite similar to those observed in the stomach during vagal stimulation. Thus there are in both organs three functionally different responses i.e. motility, secretion and vasodilatation which seem to be inseparable. This was the case also when the respective nerve sets were stimulated using different pulse duration or voltages in an attempt to differentiate between fibres with possibly different stimulation threshold. Secretion is accompanied by vasodilatation in both the stomach and the colon while the parasympathetic stimulation produces a widening of the corpus-fundus reservoir sections in the stomach and a contraction of the colon. It seem therefore possible that all these effects in the two organs are induced by one and the same set of nerve fibres.

CHAPTER V

NERVOUS CONTROL OF BLOOD FLOW IN THE PARALLEL-COUPLED VASCULAR SECTIONS OF THE COLON

L. Hultén M. Jodal and O. Lundgren

I INTRODUCTION

It was demonstrated in preceding chapters (III and IV) that efferent stimulation of the pelvic nerves induced a transient increase of total colonic blood flow a nervous effect which persisted after administration of atropine. Concomitantly a marked flushing of the colonic mucosa was observed on visual inspection. Furthermore activation of regional sympathetic vasoconstrictor fibres induced, at moderate and high rates of stimulation a characteristic response pattern, an initial pronounced vasoconstriction followed by increased blood flow in spite of prolonged nervous stimulation to a new steady state flow level. Resistance was then increased moderately as compared to control. Simultaneously a marked blanching of the mucosa was noted which remained unchanged throughout the stimulation period.

In order to investigate in more detail these nervous effects on the different *parallel-coupled* sections of the colonic vascular bed a study was undertaken in which the blood flow in the mucosa-submucosa and in the muscularis layers was studied by means of local injections of minute volumes of radioactive inert gases. The results of these experiments are reported below.

II METHODS

A. Theoretical considerations

The theoretical considerations utilized below are largely based on Kety's approach to tissue-blood exchange of inert gases and the reader is referred to his reviews for more detailed discussions (Kety 1951, 1960).

The elimination of an inert gas from a constantly and uniformely perfused tissue can be

described by the following equation provided that the arterial concentration of the gas is zero or negligibly low during the washout period

$$C_t = C_0 \cdot e^{-kt} \quad (1)$$

where C_t and C_0 denote the tissue concentrations of the gas at times t and 0, respectively, and k denotes the clearance constant. The constant k, which is directly related to blood flow (see below), can be determined from the mathematically derived equation

$$k = \frac{l}{t_{1/2}} \quad (2)$$

where $t_{1/2}$ denotes the half time of disappearance in min. $t_{1/2}$ can be calculated from the straight line obtained when plotting equation (1) on semilogarithmic paper.

If it can be assumed that the equilibration of the tracer between blood and tissue is extremely rapid (i.e. negligible concentration gradients exist in the tissue), blood flow (f) in a tissue can be calculated in ml/min × 100 g tissue from the formula

$$f = k \cdot \lambda \cdot 100 \quad (3)$$

Here k denotes the "clearance" constant (see equation (1)) and λ denotes the tissue/blood partition coefficient of the tracer (divided by the specific weight of the tissue). Experimental data validating the assumption of the blood flow limited washout of inert gases, have been presented for the myocardium (Aukland, Bower and Berliner 1964), the white matter of the brain (Kety 1965), the adipose tissue (Larsen, Larsen and Quaade 1966) and the small intestine (Kampp and Lundgren 1968).

In the present study it was assumed that the washout of the injected inert gases was blood flow limited. This seems to be reasonable since the injected volumes were minute (see below) and a transcapillary diffusion equilibrium for inert gases was demonstrated in the small intestine (Kampp and Lundgren 1968). This conclusion is furthermore supported by the results obtained at experiments carrying an atraumatic labelling technique described below.

B Operative procedures

The experiments were performed on 5 cats anesthetized i.v. with chloralose (50 mg/kg) after induction with ether. The cats had been deprived of food for at least 4 hours.

The operative procedures were similar to those used in previous chapters (III and IV) and were described in detail in chapter III (page 40). Thus, total venous outflow of the colon was registered by an optical drop recorder operating an ordinate writer. Mean arterial blood pressure was recorded from the left femoral artery by means of a mercury manometer. In those experiments where local injections (see below) were made into the mucosa-submucosa, the colon was cut open by thermocautery along the antimesenteric border. It was then mounted with needles on a cork plate to assure constant geometry during the radioactive registrations. The sympathetic and parasympathetic nervous outflow to the colon was prepared and stimulated electrically as described in chapter III (see page 41).

In 18 experiments the muscularis was dissected free from the mucosa-submucosa at the end. From these measurements it was possible to calculate the relative weights of these wall layers. To abolish secretion atropine (Atropine sulphat. Merck) was administered i.v. in a dose of 1 mg/kg b.w. (cf. chapter IV).

C. Local injections

Local injections of a saline solution containing ^{85}Kr or ^{133}Xe (purchased from Radiochemical centre Amersham, England and AB Atomenergi Studsvik, Sweden respectively) were made using a microburette ("Agla micrometer syringe Burroughs Wellcome and Co., England) with a fine-bore glass needle (outer diameter 50–200 μm). 10–20 μl of the solution, coloured with Evan s blue, was injected during 10–30 sec into the intestinal wall, either from the serosal side into the muscularis or from the luminal side into the mucosa submucosa. After the injections the needle was withdrawn and the surface of the colon was flushed with large amounts of bodywarm saline to remove the radioactivity possibly adhering to the colonic surface. The colonic wall at the site of the injections was then covered by Mylar® (1 μm thick; Du Pont) to prevent diffusion of the tracer from tissue to air. The rest of the large bowel was covered by gauze soaked in bodywarm saline.

The disappearance of ^{85}Kr from ~4 local tissue depots was simultaneously recorded by a Geiger Müller end window counting tube (Philips No. 18504, diameter of window 9 mm) completely enclosed in lead (5 mm thick) except for the window. In order to ensure that radioactivity from only the large bowel was recorded, the whole abdomen except the large bowel was covered by a lead plate 5 mm thick. The injections of ^{85}Kr were made from the mucosal side. The window of the G-M tube was placed 2–5 mm from the colonic surface and connected to a linear ratemeter (Packard series 200 A or Philips AW 4242) coupled to an ink recorder (Rika Denki, Kogyo Model 34 B). The time constant of the ratemeter was usually set at 3 or 10 sec. When counts exceeded 5000/min correction was made for coincidence loss, using a value of 100 μsec for dead time of the G-M tube.

The disappearance of ^{133}Xe from 2–4 local tissue depots, usually located in the muscularis, was registered by a scintillation detector with a thallium-activated NaI-crystal (size 1¾ × 2") placed approximately 5 cm from the intestine with the front surface of the crystal parallel to the cork support of the colon. The abdomen was covered by a lead plate as described above. The scintillation detector was coupled to a spectrometer (Packard, Auto-Gamma, Series 410 A) and a linear ratemeter (Packard Model 385) operating an ink writer (Rika Denki or Servogor 60 R Re 511). The time constant of the ratemeter was usually set at 3 or 10 sec. Pulses corresponding to γ-energies between 60 and 200 Kev were recorded.

Background was subtracted from the recorded wash-out curves and the counts per min were plotted *versus* time on semilogarithmic paper. The curves were analysed manually and blood flow was calculated utilising equations (2) and (3).

In order to be able to estimate blood flow from the wash-out curves, the tissue-blood partition coefficient (λ) of the tracer used must be known (see equation (3) above). A tissue-blood partition coefficient for ^{85}Kr of 1, at a haematocrit of 50 per cent, was assumed (cf. Kampp and Lundgren 1968). For ^{133}Xe a partition coefficient of 0.70 at a haematocrit of 30 per cent was assumed (Tönnesen and Sejrsen 1967). The haematocrit of the blood from the mesenteric vein was repeatedly determined in each experiment. The differences in haematocrit were corrected for by utilising the formula of Lassen and Munck (1955) for ^{85}Kr and that of Tönnesen and Sejrsen (1967) for ^{133}Xe.

D. Atraumatic labelling

In a few experiments the tissue was atraumatically labelled using a technique originally described by Sejrsen and Tönnesen (1968). The mucosal or serosal surface of the colon was covered by a Mylar® sheath and the radioactive solution was injected as a gas between the sheath and the tissue. The tracer was allowed to diffuse into the tissue for 30 sec

after which the Mylar was removed and the colonic surface was flushed with large amount of Lyrom saline. The surface was then again covered by Mylar and registration of the elimination of radioactivity was identical to that described here (see section III).

III RESULTS

It was demonstrated in control experiments that the wash-out of radioactive tracer from the minute local injections formed a straight line when plotted versus time on semilogarithmic paper (cf. Fig. 2 and 4). Even in experiments where the registration was continued until a radioactivity level around 5 per cent of the initial one was reached. These control recordings of the tracer wash-out from the mucosa-submucosa and from the muscularis were performed when total colonic blood flow was constant as registered by the venous drop counter. Similar results were obtained in experiments with the atraumatic labelling technique (see Meth. I).

A Resting blood flow of the mucosa-submucosa and muscularis

The regional blood flow of the mucosa-submucosa of the denervated colon amounted to 30–40 ml/min × 100 g tissue (Fig. 3) while the blood flow of the colonic muscularis was calculated to be ~ 14 ml/min × 100 g tissue.

B Effects of direct stimulation of the pelvic nerves

Fig. 1 illustrates the effects of pelvic nerve stimulation in an atropinized animal on total colonic blood flow and on the rate of elimination of ^{85}Kr from a small depot injected *superficially* in the mucosa-submucosa of the distal part of the colon. The nerve stimulation causes a moderate transient increase in total colonic blood flow and a marked augmentation of the wash-out rate of ^{85}Kr reflecting a regional blood flow increase from 35 to 100 ml/min × 100 g tissue during the peak of the vasodilatation. As total blood flow returns to control level despite continued stimulation, there is a simultaneous decrease in the rate of ^{85}Kr elimination and within 2–3 min after the start of the stimulation both recordings have regained the prestimulatory level. Upon cessation of the stimulation no significant changes of the recorded parameters are observed. In a few experiments the wash-out of ^{133}Xe from the mucosa-submucosa was followed during prolonged activation of the pelvic nerves in not atropinized animals. The initial as well as the *recurrent* increases of total colonic blood flow (cf. chapter IV) were accompanied by augmentation in the rate of wash-out of ^{133}Xe.

The response pattern illustrated in Fig. 1 is representative for the effects of pelvic nerve stimulation on the elimination rate from depots of ^{85}Kr injected in *superficial* parts of the mucosa-submucosa of the distal two thirds of the colon. However in some experiments the local injections of the tracer were made into the *deeper* layers

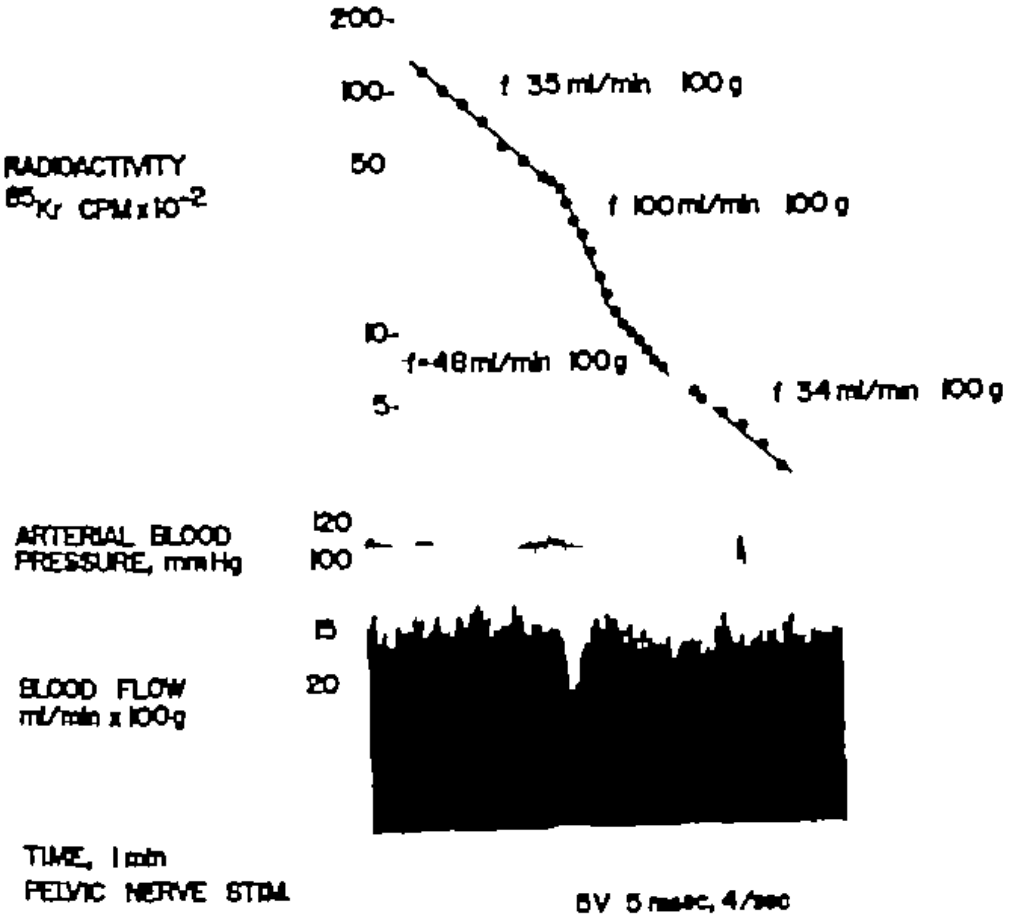


Fig. 1 Cat 2.3 kg The effect of pelvic nerve stimulation on arterial blood pressure total colonic blood flow and wash-out rate of ^{85}Kr from kept the superficial mucosa submucosa of the distal part of the large bowel. The animal was trapanized (1 mg/kg b w) N t the pronounced transient increase of wash-out rat on nerve stimulation kept only moderate increase in the total venous outflow

of the mucosa-submucosa. In these experiments, insignificant increases in the rate of elimination were observed during pelvic nerve stimulation even when marked increases of total colonic blood flow occurred

In a number of experiments two G M tubes were utilized to monitor simultaneously the rate of elimination of the ^{85}Kr from local injections in the mucosa-submucosa in both the *proximal* and the *distal* part of the colon a representative experiment is illustrated in Fig 2 The proximal injections were made in the upper third of the large bowel. As usual stimulation of the pelvic nerves causes a transient increase in regional blood flow of the mucosa submucosa of the distal colon (cf Fig 1) while no significant change occurs in the proximal colon

Fig 3 summarizes the results obtained in those experiments in which injections were made from the mucosal side The wash-out rate of radioactivity expressed in ml/min x 100 g is plotted on a logarithmic scale *versus* the simultaneously registered total colonic blood flow The pelvic nerve stimulations illustrated (filled circles) are

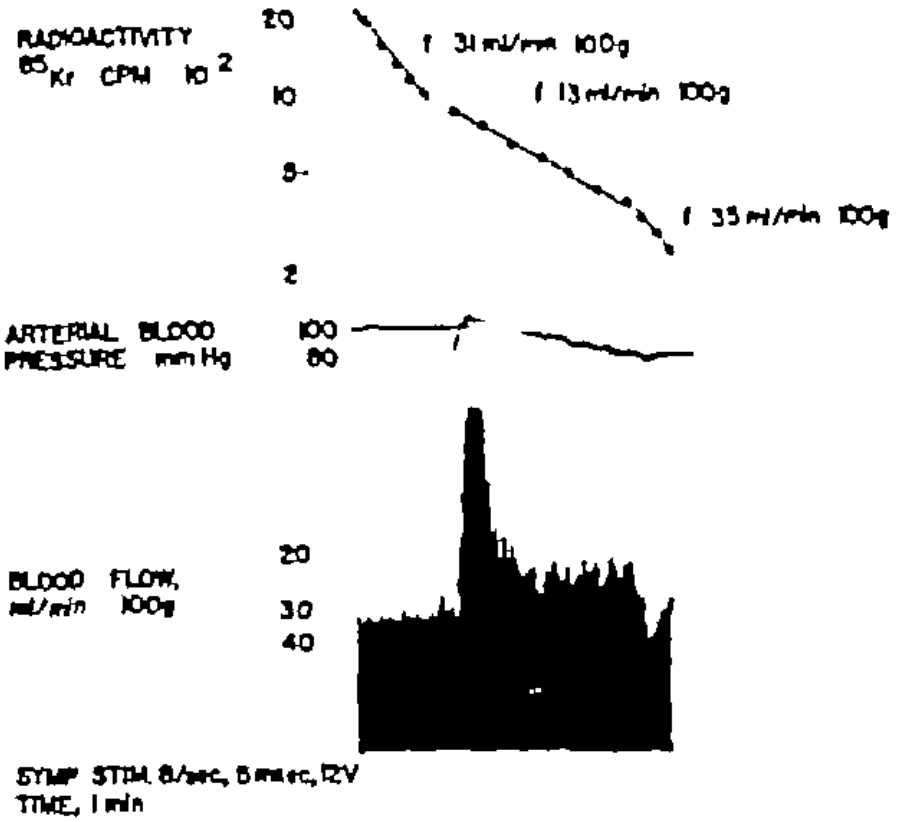


Fig. 5 (cat ~5 kg) The effect of stimulation of the sympathetic vasoconstrictor fibers to the colon (splanchnic and lumbar colonic nerves) on arterial blood pressure (—) colonic blood flow and rate of wash out (^{85}Kr) from a depot in the mucosa submucosa of the large bowel.

C. Effects of direct stimulation of the sympathetic nerves

Fig. 5 illustrates the effects of stimulation of the splanchnic and lumbar colonic nerves on total colonic blood flow and on the rate of elimination of ^{85}Kr from a minute local injection made into the mucosa submucosa of the large bowel. While sympathetic stimulation (splanchnic and lumbar colonic nerves) evokes the characteristic autoregulatory escape phenomenon described earlier (chapters III and IV) the ^{85}Kr wash-out rate remained decreased to approximately the same extent throughout the stimulation period. This corresponds to a blood mucosal flow decrease from 31 to 13 ml/min 100 g. Upon cessation of stimulation a transient hyperaemia occurs reflected both in total colonic blood flow and in the blood flow at the site of the injection. This response pattern is representative of the majority of the sympathetic stimulations but in 7 out of 11 stimulations a biphasic response was observed in the wash-out curve. In such experiments an initial reduction of mucosa submucosal blood flow was registered during the intense initial restriction of total colonic blood flow. As total blood flow increased (autoregulatory escape) regional blood flow also increased in these experiments but to a lesser extent. It cannot be settled

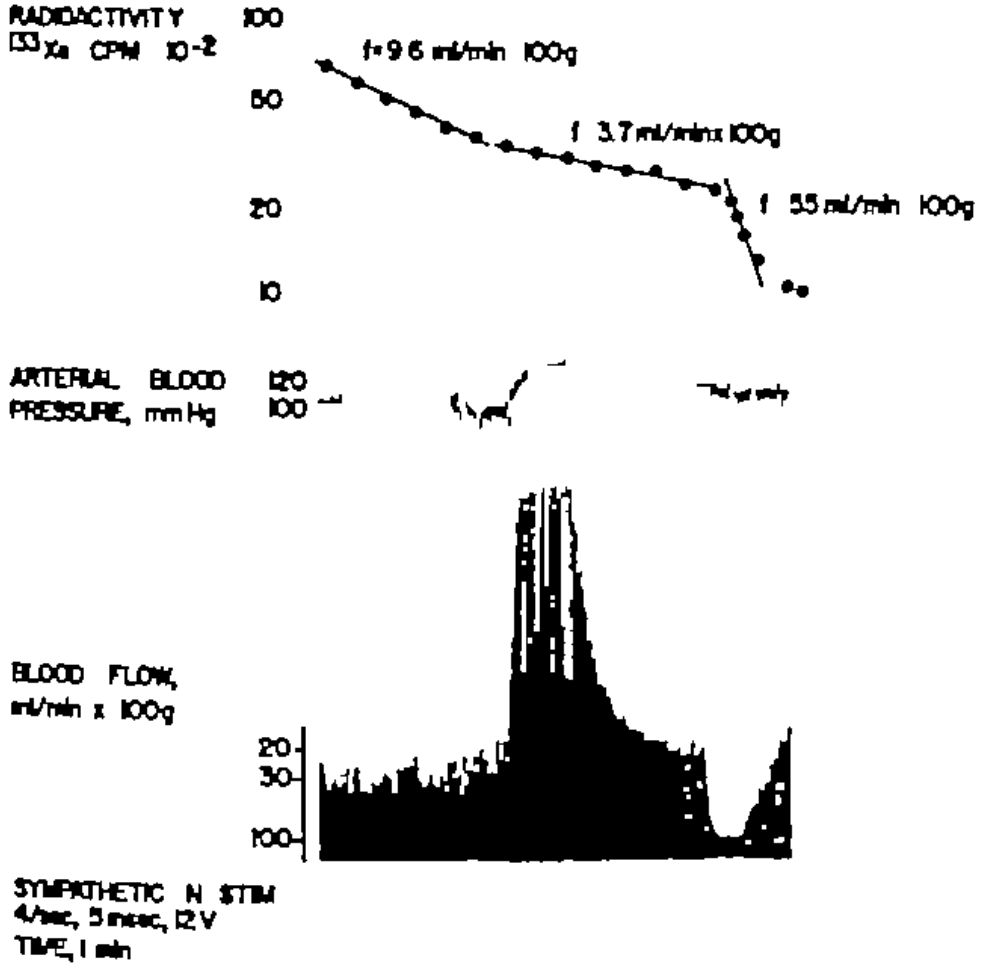


Fig. 6. Cat 2.5 kg. The effect of stimulation of the sympathetic vasoconstrictor fibres to the colon (splanchnic and lumbar colonic nerves) on arterial blood pressure, total colonic blood flow and rate of wash-out of ^{133}Xe from a depot in the colonic muscularis.

whether the tracer might have been more deeply deposed in this group of experiments, so as to reflect also blood flow in the deeper mucosa.

Fig. 3 (circles) summarizes the results obtained during sympathetic stimulation. The illustrated data were obtained in experiments in which the splanchnic and lumbar colonic nerves were simultaneously stimulated at rates of 2, 4 and 8 imp/sec and represent values recorded during the steady state phase of vasoconstriction. It can be seen that the rate of mucosal wash-out is in the lower range of, or below the control values observed at a similar total colonic blood flow.

The effect of sympathetic stimulation on the rate of elimination of ^{133}Xe from the muscularis was studied in a few experiments and is illustrated in Fig. 6. There is a reduction of rate of wash-out (upper panel) during the nerve stimulation, the decrease corresponding to a reduction in blood flow from 9.6 to 3.7 ml/min × 100 g at a stimulation rate of 4 imp per sec. Upon cessation of stimulation a marked transient increase of both total and muscularis blood flow is observed.

IV COMMENTS

The results of the present study strongly suggest that the increase in total colonic blood flow observed on pelvic nerve stimulation mainly reflects a vasodilatation in superficial parts of the mucosa submucosa restricted to the distal two-thirds of the colon at the most (Fig 1 2 and 3) These observations are in accordance with the results obtained on visual inspection reported in chapter III which also suggested that the blood flow increase predominantly occurs in the colonic mucosa Since pelvic nerve stimulation causes an increase in colonic secretion (cf chapter IV) one may assume that blood flow is augmented mainly in the secretory part of the mucosa when pelvic nerve activation is induced This conclusion also reached by Wright *et al* (1038) who provided histological evidence of an augmented vascularization around the colonic crypts and of increased secretory activity of the colonic epithelium during pelvic nerve stimulation

The present experiments also suggest that blood flow may sometimes become moderately increased in some part of the colonic muscularis during pelvic nerve activation. Thus, a transient small increase of muscularis blood flow was seen in 3 out of 17 measurements Possibly this increase is secondary to the marked activation of the colonic smooth muscles that always occurs upon pelvic nerve activation (cf. chapter II)

Quantitatively regional blood flow amounted to 7–14 ml/min × 100 g (Fig 4 and 6) in the colonic muscularis and to 30–40 ml/min × 100 g in the mucosa (Fig 1 2 and 3) in the resting" colon. These values are in good agreement with those reported for the resting small intestine of the cat (Lundgren 1067 Kampp and Lundgren 1068) The relative weights of the mucosa submucosa and of the muscularis in the colon were determined to be 60 and 40 per cent respectively (see Method) The total colonic blood flow can therefore be deduced from the regional flow figures being then approximately 30 ml/min × 100 g (Table I left panel) which is in close agreement with direct measurements of total colonic blood flow This indicates that blood flow of the mucosa submucosa of the denervated colon may be fairly homogenous (cf. Kampp and Lundgren 1068) It can furthermore be calculated that about 80 per cent of total colonic blood flow is distributed to the mucosa submucosa during rest the remaining 20 per cent being diverted to the muscularis (Table I)

During pelvic nerve activation blood flow in superficial mucosal portions is increased transiently from 20–40 up to 300 ml/min × 100 g (Fig 3) with a less pronounced augmentation of total colonic blood flow which in this series of experiments was increased only to about 60–70 ml/min × 100 g However in other experiments (see chapters III and IV) total colonic blood flow could at a maximum increase to 150–200 ml/min × 100 g Assuming that the regional blood flow increase during pelvic nerve stimulation is augmented proportionally to total colonic blood flow it can be calculated that the maximal blood flow in the superficial mucosa submucosa amounts to approximately 400 ml/min × 100 g tissue In this connection it should be pointed out however that the increase of total colonic blood flow in-

Table I

	Total blood flow at rest — ml/ml × 100 g		Moderate increase of total blood flow by pelvic nerve stimulation 60 ml/min × 100 g		
	Mucosa submucosa	Muscularis	Mucosa-submucosa Superficial	Deep	Muscularis
Regional blood flow	30	10	150	30	20
Relative weight (per cent of total)	60	40	68	32	40
Calculated blood flow distribution (per cent of total)	82	18	78	17	13

Calculated values are given in *italics*

duced by pelvic nerve stimulation represents an underestimation of the "true" blood flow increase since it is calculated on the total weight of the colonic preparation including regional lymph nodes, mesentery etc The results in chapter III and IV and those in the present series of experiments clearly indicate that the blood flow increase induced by pelvic nerve stimulation is restricted mainly to the distal 2/3–3/4 of the colon. Thus the maximal increase of blood flow in the superficial mucosa-submucosa may be even higher than calculated above

In Table I (right panel) the flow distribution has been estimated during pelvic nerve stimulation which increased total colonic blood flow to 60 ml/min × 100 g It is assumed that the muscularis blood flow is increased to 20 ml/min × 100 g and blood flow in the superficial mucosa-submucosa to 150 ml/min × 100 g (Fig 3) Blood flow in deeper parts of mucosa-submucosa is presumed to equal control. This agrees with the observations reported above. It can then be estimated that approximately 70 per cent of total colonic blood flow is diverted to the vasodilated part of the mucosa which constitutes only part of the mucosa-submucosa. Concomitantly a smaller proportion of total blood flow is distributed to the muscularis as compared to control. The calculated relative weights of the different blood flow compartments are also given in Table I

Stimulation of the regional sympathetic vasoconstrictor fibres induces an initial reduction of total colonic blood flow causing a decrease in regional blood flow in the mucosa-submucosa as well as in the muscularis (Fig 5 and 6) During prolonged stimulation total colonic blood flow again increases and reaches a new steady state level within 2–3 min. (cf chapter III and IV) while in most experiments there is no change or only small changes in regional blood flow of the mucosa or of the muscularis as registered with the techniques of the present study These findings suggest that a redistribution of blood flow occurs within the colon during the autoregulatory

escape This conclu ion is also supported l y the foll wing bservation *First* the mucosa remained blanched on vi ual in pection throughout the stimulati n period (chapter III) *Second* the reduction of regional blood flow in the mucosa and mucosa during the st ady state phase wa greater than could be explained by the concomi tant reduction of total c l mic l lood flow (Fig 3)

A similar blood flow red tribution during nervou vasoconstriction ha been described in the small intestine (Folkow *et al* 1961*b* Wallentin 1966) From the present experiments it i not possible to draw any conclu ion exactly where the blood flow increase occurs during the autoregulatory escape It i proposed however that blood flow is augmented around the base of the crypt and in a jacent parts of the submucosa since autoradiographic studies in the *small intestine* (Lundgren *unpublished* observation) suggest such a localization

CHAPTER VI

REFLEX CONTROL OF COLONIC MOTILITY AND BLOOD FLOW

LEIF HULTÉN

I INTRODUCTION

The reflex changes of colonic motility in man, elicited from mechanical stimulation of the rectal wall, are relatively well known from previous investigations summarized by Hurst (1910) Alvarez (1948) and recently by Mendeloff (1968) With respect to animal experiments, studies on cats (e g Cannon 1902 Garry 1933 Schmidt and Fülgraff 1963) show that the motility is increased in the entire colon during defaecation with peristaltic contractions occurring even in the caecum.

Most investigations indicate that this defaecation reflex" is dependent on the integrity of the pelvic nerves and with at least the main bulk of afferent as well as efferent fibres running in these nerves. The exact nature of the stimulus for this pelvo-pelvic reflex response is however still debated (Zimmerman 1909 Hertz 1911 Cannon 1902 Garry 1933) and it is also discussed whether other pathways and mechanisms may be involved Thus it has been suggested that vagal efferents may be involved in the reflex activation of proximal colonic parts in association with defaecation (Schmidt and Fülgraff 1963)

The expulsive motility in the descending colon and the rectum seems to be governed also by autonomic centres in the cerebral cortex and the brain stem (Davenport 1966) A medullary area close to the vomiting centre has thus been claimed to control defaecation (Hatcher and Weiss 1923 Koppanyi 1930 Langworthy and Rosenberg 1939) and inhibitory hypothalamic centres have also been suggested to exist (Wang *et al* 1940 Sheehan *et al.* 1941) The normal dominance of supraspinal excitatory influences may explain why transection of the cervical or thoracic cord in man acutely leads to suppression of colonic motility but later reflex hyperactivity develops (White *et al* 1940) presumably a result of sensitization after partial denervation of the spinal reflex arcs. In chronically spinal man it has further been observed that passage of faeces through the colon seems to augment its motility presumably as a result of pelvo-pelvic reflexes. Moreover stimulation of the skin

around the anus or activity of the bladder facilitates reflex defaecation in man (Denny Brown and Robertson 1933).

In anaesthetized cats rectal distension results in enhanced defaecatory responses but they become obvious first when the excitatory sacral reflex influence on the colon is released from the suppressing impact of the sympathetic lumbar colonic nerves (Garry 1933) an effect presumably exerted at the intramural ganglionic level. While the pelvo-pelvic autonomic reflex mechanism involved in defaecation thus seem to be relayed at the sacrospinal level the somatic" defaecation responses are dependent on centres cranially to the spinal cord itself for their full expression (Garry 1933).

Another important type of colonic response ultimately often leading to expulsion the clinically well known gastro-colic reflex was previously supposed to be mediated in vagal efferents and afferents (Hertz 1913 Lehmann 1913 Larsson and Bargen 1932). More recent investigations have however cast doubt upon such a nervous basis for this reflex" response (Logan 1967 Connell 1969 see also chapter I).

Whereas the general principles of reflex colonic activation in connection with defaecation are fairly well documented information concerning the corresponding effects on colonic blood flow is scant. Wright *et al.* (1938) described a marked mucosal vasodilatation occurring concomitantly with an increased secretion and motility as a result of afferent pelvic nerve stimulation in the cat while Cuber (1964) claims that defaecation is accompanied by a diminution of colonic blood flow. No systematic and quantitative studies concerning reflexly induced changes in colonic blood flow whether pelvo-pelvic or of other nature seem to have been performed apart from the apparently contradictory studies already mentioned.

As partly outlined in chapter II a sympathetically mediated spinal reflex—the so called intestino intestinal inhibitory reflex (Kuntz and Saccomanno 1944) studied in detail with respect to the small intestine by Johansson *et al.* (1964)—can strongly inhibit gastrointestinal motility exerting its effect mainly or perhaps only on the ganglionic level (see Norberg 1965 Kewenter 1965 Jansson 1969). To what an extent and in which situations, a corresponding type of reflex also affects colonic motility is unclear although the results of chapter II suggest that such a tonically active inhibitory reflex might be in part responsible for the suppression of vagal excitatory responses in proximal colonic parts.

As the so-called gastro-colic and defaecation reflexes are essential for bringing about appropriate colonic emptying it is the aim of the present investigation to explore further some of the major reflex effects on colon motility and blood flow to examine whether the reflex organization is different in proximal and distal colonic parts and explore to what extent sympathetically mediated intestino-intestinal reflex inhibition is involved in the colonic motility control. It was also considered of interest to explore if the excitatory apparently cholinergic fibers within the lumbar colonic nerves (cf. chapter II) were possibly involved in the reflex control of colonic motility.

II METHODS

A. Operative procedures

32 cats, fasted for 24 hours, were anaesthetized intravenously with chloralose (50 mg/kg) after induction with ether A tracheal cannula was inserted to allow a free airway T permit drainage of swallowed air and secretion a plastic tube was introduced orally into the stomach. Another catheter drained the bladder The arterial pressure was recorded from a femoral artery by means of a mercury manometer The animal was kept on a heating pad which was thermostatically regulated keeping the body temperature around 37–38°C. The abdomen was opened in the midline and the small intestine was extirpated from the duodenum to the ileo-caecal junction together with the greater omentum and the spleen In some experiments a small jejunal segment or a mesenteric nerve pedicle from the extirpated small intestine was isolated for subsequent intestino colonic reflex activation (see below) Great care was taken to avoid any interference with the vascular and nervous connections to the colon. The secretion from the adrenals was eliminated by isolating the glands from the circulation by means of encircling ligatures; hydrocortisone 10 mg/kg was given intramuscularly for substitution

B Recording of motility

The proximal half of the colon was used for recording of reflex changes in colonic motility while the distal part was utilized for activation of the appropriate receptor mechanisms. For this purpose the lumen of the proximal colon was connected to a glass tube as described in chapter II, and was by way of a rubber tube and an adaptable pressure reservoir connected to a piston recorder The proximal colonic segment was isolated from the distal one by a loose ligature, so applied that it did not destroy either extra or intramural nervous or vascular connections or the wall continuity to distal colonic parts. Motility of the distal part was sometimes separately recorded in a similar way via a tube inserted into the anal canal especially when pelvic afferents were stimulated electrically Thus nervous or myogenic influences, overbridging the artificially separated colonic parts could be freely transmitted along the largely intact intestinal wall. The recording systems were filled with Tyrode solution at body temperature The volume reservoir was arranged in such a way that the intracolonic pressure could be set and continuously adjusted to a level of 10–12 cm H_2O

C. Determination of blood flow

For quantitative recordings of colonic blood flow the total venous outflow was, after heparinization (3–5 mg/kg), diverted through a closed photoelectric drop recorder (see chapter III) and returned via the external jugular vein. The recorder operated an ordinate writer where the heights of the ordinates are inversely proportional to the flow rate, allowing conversion of the recording into ml of flow/min and 100 g tissue The peripheral resistance unit per 100 g of tissue (PRU_{100}) were calculated as the ratio of mean arterial blood pressure (mm Hg) to blood flow (ml/min × 100 g tissue)

D Nervous stimulations

Sympathetic *intestino-intestinal reflex* activation was induced either by graded distension of a small jejunal segment or by afferent electrical stimulation of a mesenteric pedicle.

In some of these experiments all sympathetic nerves connected to the colon were left intact; in others the existence of an inhibitory sympathetic connection was explored by section of the major sympathetic trunk. In other experiments the cut peripheral ends of the sympathetic nerves were placed on ring electrodes for frequent electrical stimulation described in Chapter II. The colon could also be investigated for its sympathetic inhibitory control pharmacologically by intravenous administration of guanethidine (3 mg/kg).

Reflex activation of the pelvic nerves was achieved by mechanical stimulation of the colonic mucosa either by a functional stimulus, namely by distension of balloons placed in the rectal lumen. In a few experiments electrical stimulation was applied to proximal colonic parts or small pieces of cotton attached to a silk thread was introduced through the anal canal and placed in the proximal part of the colon. By means of the thread the cotton could be dragged along the intestinal lumen in an attempt to initiate an adequate mechanical stimulation of the wall. Reflex activation of the pelvic nerves was also studied by means of graded electrical stimulation of afferent fibres in one of the pelvic nerves, the other one being left intact.

The presence of a *vagal reflex control* of the colon was examined by distension of a balloon within the stomach when the vagal fibres were left intact. In some experiments the ventral vagal trunk was dissected out at the subdiaphragmatic level with its central end mounted on a silver ring electrode for afferent stimulation. The pelvic nerves in these experiments were dissected free but otherwise left intact.

For *somatic afferent stimulation* the hamstring nerve was dissected free, cut and its central end mounted on a silver ring electrode.

The nerve stimulations were delivered from a Grass stimulator S4 L. For afferent stimulation the nerves were stimulated at 4–50 pulses/sec at a voltage of 15 and a pulse duration of 0.05–3 msec. In four experiments the spinal medulla was transected either initially or in the course of the experiment at the cervical or upper thoracic level (Th I–V).

The following drugs were administered i.v.: atropine (Atropine sulphate, Merck) in a dose of 1 mg/kg b.w., guanethidine (Ismelin® Ciba) 3–5 mg/kg b.w. In some experiments gallamine triethiodide (Flaxedil® May & Baker) was used to prevent disturbing somatomotor reflex movements. Artificial respiration was then maintained by a respirator pump.

III RESULTS

A. The intestino-colonic inhibitory reflex

Activation of this sympathetically mediated reflex either by distension of a small intestinal loop or by electrical stimulation of afferent mesenteric nerve fibres usually had no effect or only a weak inhibitory effect on the sparse colonic motility recorded in the parasympathetically denervated colon. Inhibition was first observed when high frequency afferent stimulation (above 10 imp/sec) was utilized; then a delayed and slight increase in the proximal colonic volume could be induced in such a preparation.

In contrast, a prompt and marked inhibition of the vagally induced motility in the proximal colon occurred, even upon slight intestinal distension (Fig. 1, upper panel) or upon low frequency stimulation of mesenteric afferents (Fig. 1, lower panel).

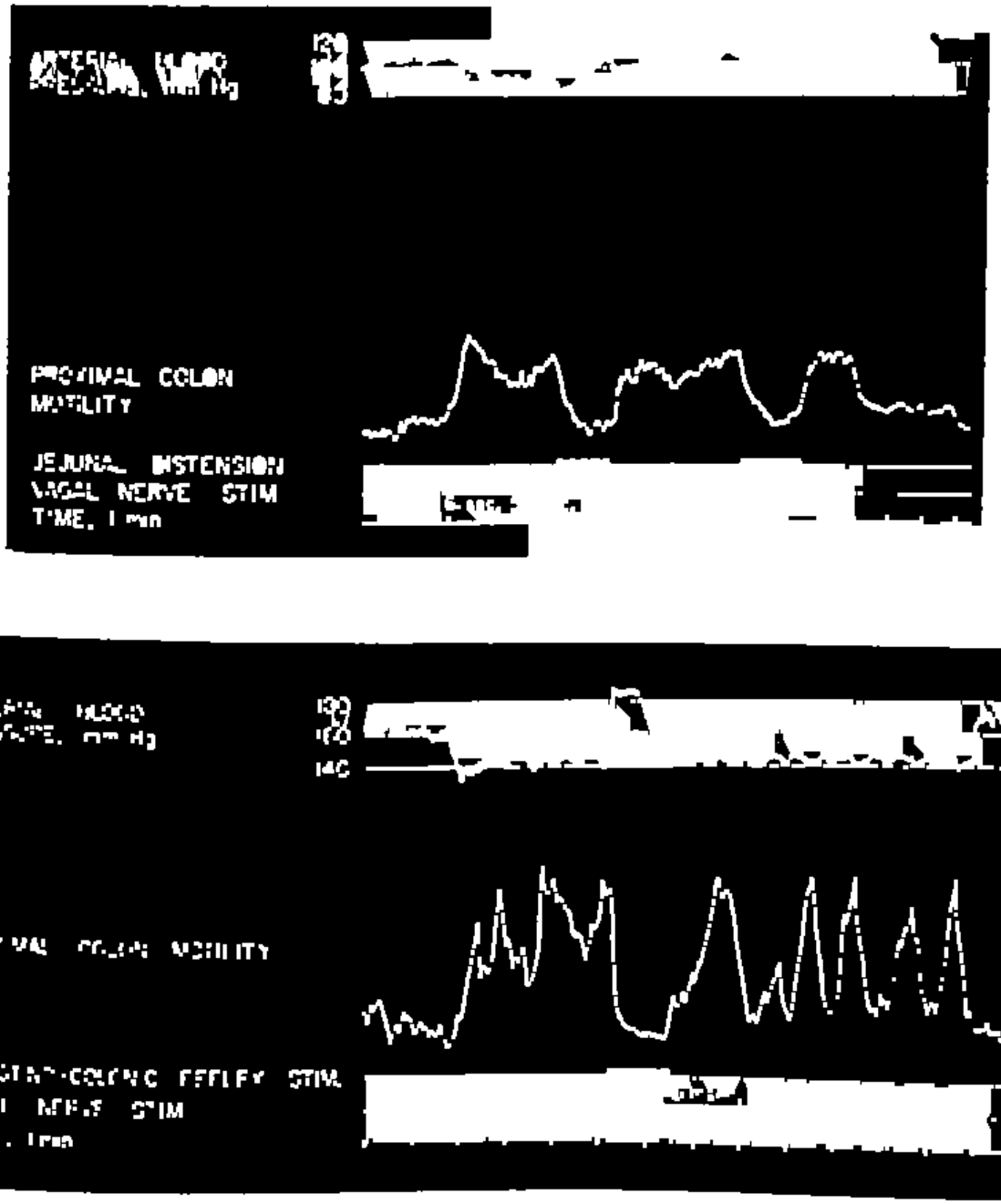


Fig. 1. *Upper panel.* Cat 2.8 kg. Chloralose. Intestino-colonic reflex inhibition of vagally induced proximal colon motility produced by jejunal distension (60 cm H_2O). *Lower panel.* Cat 2.1 kg. A similar and prompt reflex inhibition of vagally induced motility is achieved by stimulation of visceral afferents. Not the vagally induced rhythmic activity superimposed upon a moderate increase of tone.

Similarly a drastic inhibitory effect was exerted also when a motor response of the proximal colon was induced by pelvic nerve stimulation. In contrast the excitatory response to pelvic nerve stimulation in the distal part of the colon was largely unaffected even with afferent stimulation at high frequencies (10–16 imp/sec) (Fig. 2.)

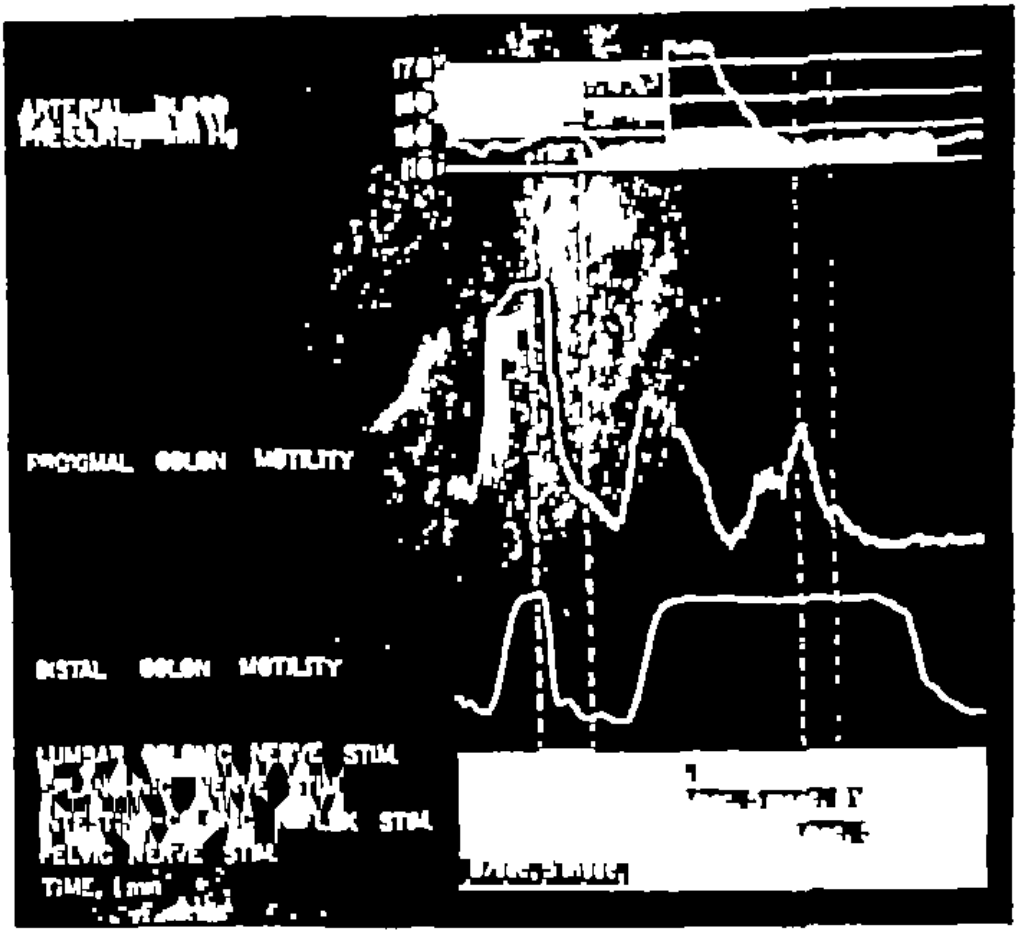


Fig. — Cat 3 kg Chloralose The effect of intermittent colonic reflex activation by high frequency stimulation of rectal efferent pelvic nerve induced colonic motility as compared with the inhibitory effect of efferent splanchnic nerve stimulation. Note the sustained contraction of both segments after pelvic nerve stimulation. Inhibition of motility of the proximal part of the colon is only achieved as a result of direct or reflex activation of the sympathetic nerves whereas the motility of the distal part is not affected whatsoever

B The pelvo-pelvic reflex

1 *Effect of mechanical stimulation of the rectal wall*

Since in these experiments reflex increases of colonic motility and blood flow were achieved either by a frictional stimulus applied to the rectal wall or by distending a balloon in the rectal lumen colonic motility was recorded only in the proximal part

As is shown in Fig 3 mechanical stimulation of the rectal wall led to a strong reflex motor response and a concomitant transitory increase in colonic blood flow The vasodilator response had qualitatively and quantitatively the same characteristics as that induced by longlasting electrical stimulation of the efferent pelvic nerve fibres, as described in chapter III and IV although the motor response sometimes seemed to be less sustained Cutting the vagus nerves in the neck did not affect the extent of the response

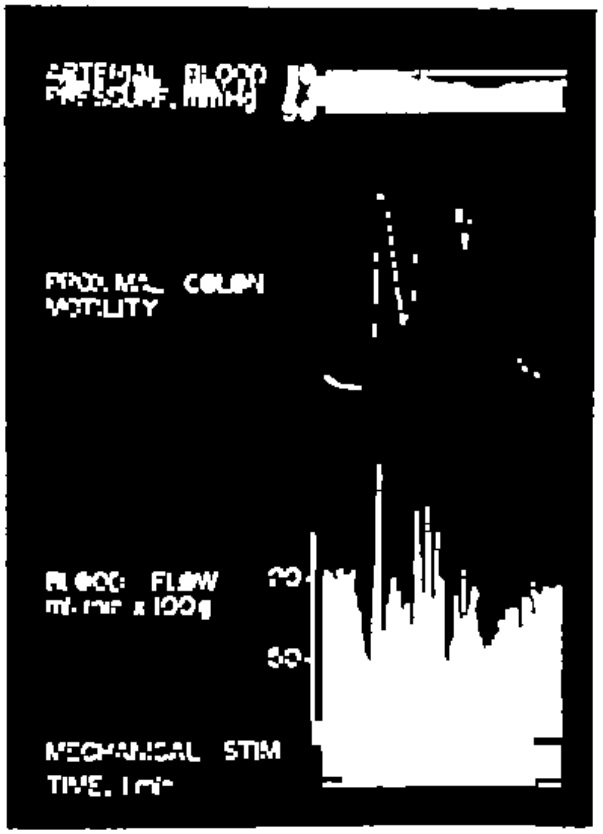


Fig. 3. Cat 3.1 kg Chloralose. Effects of frictional stimulation by means of to and fro movement in the rectum, on proximal colonic motility and total venous outflow. The sympathetic supply to the colon was cut in this experiment. Note the *initial* blood flow increase followed by *recurring* increases on prolonged stimulation.

Straining movements were seldom seen in association with this frictional stimulation, although such somatomotor reflex effects were sometimes noted when strong rectal distension was applied. When straining occurred the reflex vasodilator response was preceded by a brief decrease in blood flow and was consequently somewhat delayed. A small reflex decrease of arterial blood pressure was sometimes noted upon frictional stimulation of the rectum but it was far more pronounced when rectal distension was performed. Frictional stimuli or distension applied to the colon proximally to the rectal part had little or no influence on colonic motility or blood flow.

The reflex responses described, both with regard to motility and blood flow were often difficult to reveal until the sympathetic colonic nerve supply had been divided or the sympathetic influence blocked by guanethidine (3 mg/kg). This indicates the presence of considerable sympathetic tonic discharge which suppresses the parasympathetic reflex effects. This inhibitory sympathetic influence could also be demonstrated after sympathetic section by the fact that both the reflex motor and vasodilator responses in the colon could again be suppressed when either of the cut sympathetic supply (splanchnic and lumbar colonic nerves) were directly stimulated at low rates (see Fig. 4 A, B and C).

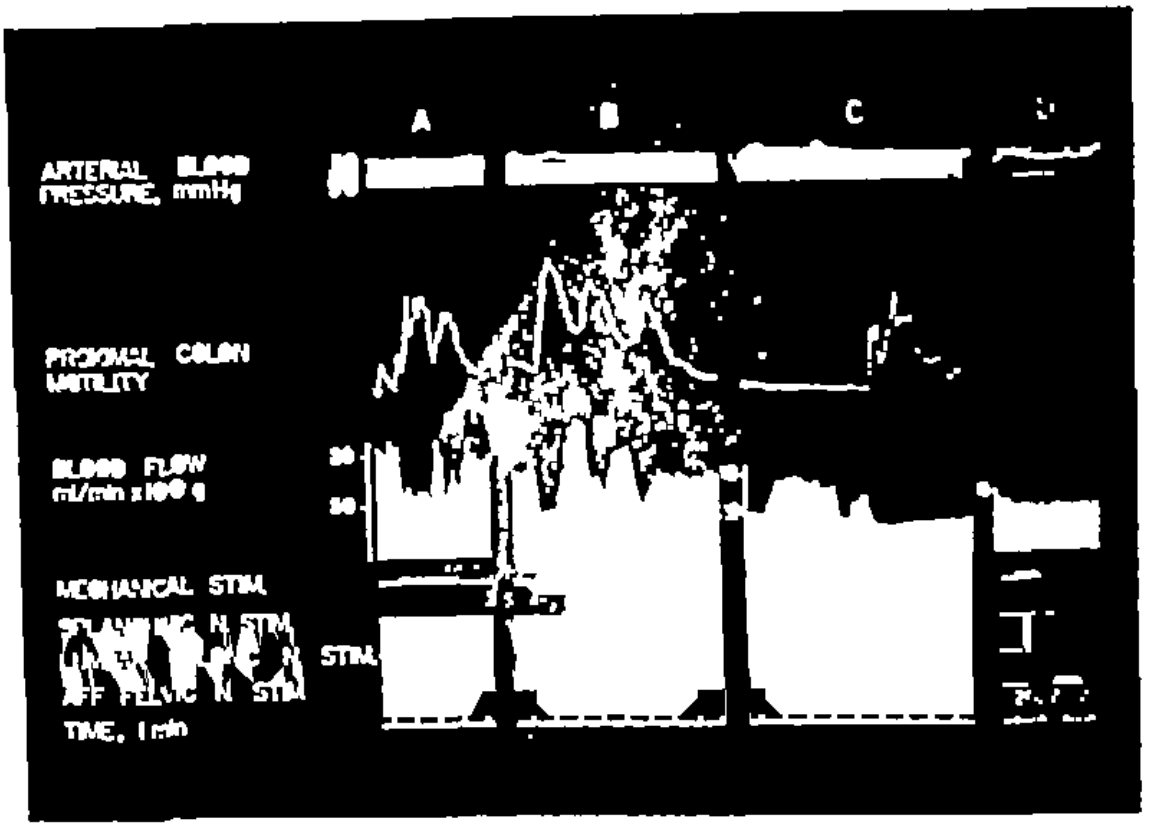


Fig. 4. Cat 4.4 kg. Chloralose. The effect of rectal frictional stimulation on motility and blood flow is shown in (A). In (B) is illustrated the effects of efferent sympathetic stimulation on the reflexly induced increase of colonic motility and blood flow. Note the immediate resistance increase of blood flow and simultaneous inhibition of motility. The effects of frictional stimulation on continuous increased sympathetic tone is illustrated in (C). Note that vasodilatation occurs readily whereas motility increase is not induced until cessation of the sympathetic stimulation. The pelvo-pelvic reflex responses are abolished when both pelvic nerves are severed (D).

Furthermore the pelvo-pelvic reflex responses often became pronounced if the spinal medulla was cut at the upper thoracic-lower cervical level despite the fact that the efferent sympathetic supply was left intact. This indicates that the suppressing tonic sympathetic discharge may be of *supraspinal* origin rather than a spinal intestino-intestinal reflex (see below).

The magnitude of the blood flow increase reflexly produced via the pelvic nerves by mechanical stimulation of the colonic or rectal wall was about two- or threefold from a resting blood flow of about 15–20 ml/min 100 g tissue. On repeated attempts to elicit these reflex responses they regularly faded and ultimately vanished only to return again after a resting period. In experiments where frequently repeated mechanical stimulation had caused the response to vanish *efferent* stimulation of the pelvic nerve still evoked a powerful motor and vasodilator response indicating that an adaptation of the receptors in the rectal wall, or/and of the reflex centres was mainly responsible of the fading reflex effect rather than any fatigue or adapta-

tra of t. efferent nervous links or of the effect wa. At the end of these experi
ments the colon contained rich amounts of muco especially in the distal sec
tion.

As mentioned, vagotomy never affected the reflex res ponse and it was also un
affected by severing the pudendal nerves or one of the pelvic nerves. Cutting both
pelvic nerves on the other hand abolished the response completely indicating that
both the afferent and the efferent fibers are confined to the pelvic nerves (see Fig.
4 D). This was the case even when the vagal and sympathetic supply was left
intact and the adrenergic fibers were blocked by guanethidine. However, mechan
ical stimulation of the rectal mucosa could sometimes result in a slight vaso-
dilatation, probably of local origin as it was also seen in a completely denervated
colon. To check whether the reflex effects described were dependent on a cholinergic
transmission mechanism, atropine in a dose of 1 mg/kg was administered intraven-
ously. Neither the motor nor the vascular response were substantially reduced by atro-pine.

Effects of afferent pelvic nerve stimulation

In another series of experiments the reflex arrangement of the pelvic nerves was further explored by graded afferent stimulation of one of the pelvic nerves, the other one being left intact.

As seen in Fig. 5 stimulation of *low* threshold afferent fibers in the pelvic nerves (3 volt, 0.05–0.2 msec) regularly produced a marked fall in blood pressure. This fall became gradually more enhanced with increasing rates of stimulation to reach a maximum at about 10 imp/sec and could be as great as 40–60 mm Hg. If the sympathetic nerves were left intact a moderate increase of colonic blood flow could occur concomitantly with the depressor effect, whereas no increase of motility was seen. The increase of colonic blood flow vanished when the sympathetic nerves were severed and instead a small reduction of flow was seen in connection with the reflex reduction in blood pressure, at the same time as a slight increase of colonic motility was sometimes recorded.

Afferent pelvic nerve stimulation at *high* nerve strength (8 volt, 5 msec) often induced a complex somatic defaecation reflex pattern including powerful contractions of the muscles in the abdominal wall during which colonic blood flow appeared to be temporarily reduced. This was evidently a result mainly or solely of the straining as the blood flow increase could often be traced in the venous outflow recording between the mechanical artifacts produced by the straining movements (see Fig. 4 C). When these were eliminated by administration of Flaxedil[®] the usual and typical increase of colonic blood flow and motility *often* appeared as a result of this type of afferent stimulation.

As is seen in Fig. 6 (left panel) *the reflex fall in blood pressure* as well as the pressor straining movements were *eliminated by transection of the spinal cord* and the reflex motility increase and *the characteristically phasic vasodilatation* became

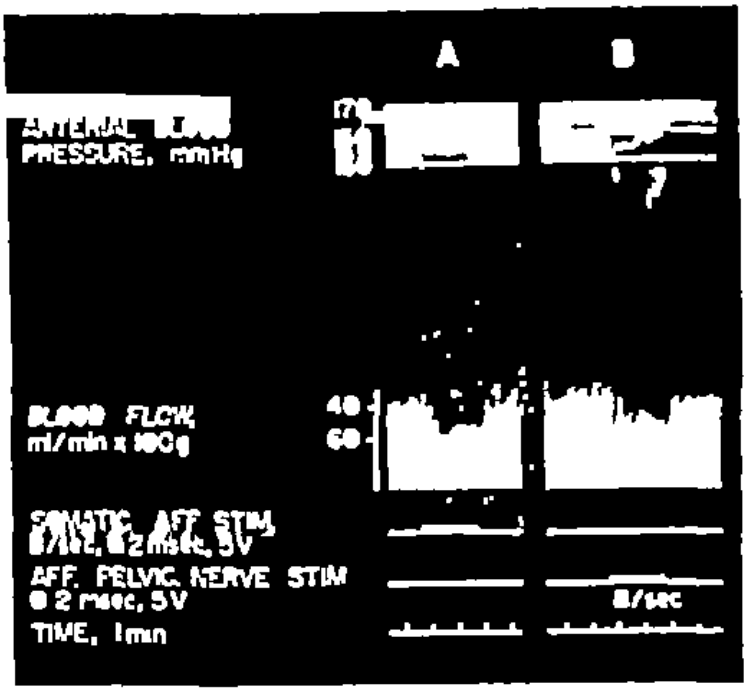


Fig. 5. Cat 4.4 kg. Chloralose. Effects of low threshold somatic depressor fibre activation (hamstring nerve) (A) compared with stimulation of low threshold afferent fibres in the pelvic nerves (B) on systemic blood pressure and colonic blood flow. Note the constant increase in blood flow. Effect of this type is abolished by guanethidine.

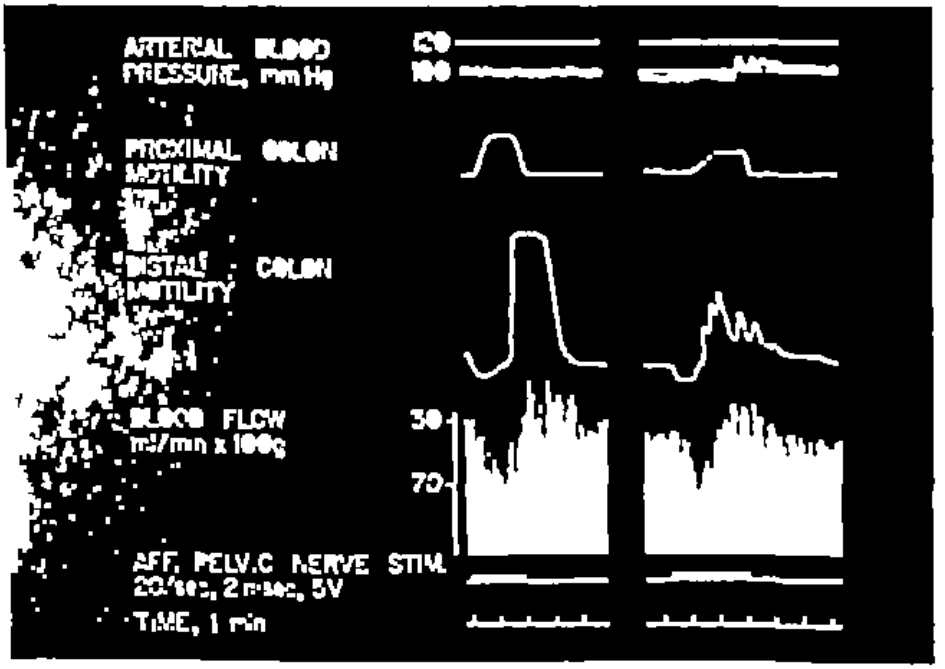


Fig. 6. Cat 3.6 kg. Chloralose. Effect of high strength afferent pelvic nerve stimulation on colon motility and blood flow before (panel left) and after (panel right) atropine administration 1 mg/kg. The spinal medulla had been cut in the upper thoracic level. Note the increase of blood flow and the readily occurring motility increase in proximal and distal colonic parts even after atropine treatment. Note also the transitory increase in colonic volume preceding the motor contraction, sometimes occurring in this situation.

The reflex vasodilator response to high strength afferent stimulation of the pelvic nerve was in a given animal largely comparable in size and general characteristics to that produced by frictional stimulation of the rectal wall when both pelvic nerves were intact. The presence of a superimposed sympathetic activity could however cause considerable differences between animals, but after blockade of this sympathetic activity intense vasodilator responses were readily obtained in almost all animals and they were sometimes comparable in magnitude to those induced by efferent pelvic nerve stimulation (chapter III and IV) Atropine (1 mg/kg) failed to abolish the reflex response (see Fig 6 right panel) and although guanethidine eliminated the reflex blood pressure fall upon pelvic nerve stimulation the colonic vasodilator response was unaffected or even enhanced

Cutting the remaining pelvic nerve abolished the reflex completely even if the vagal and sympathetic supply to the colon was left intact but the adrenergic fibres blocked with guanethidine This indicates that the pelvic parasympathetic fibres constitute the sole *efferent* pathway and that the cholinergic excitatory fibres in the lumbar sympathetic supply (see chapter II) or the vagal fibres are not involved in the defaecation reflex as induced from the rectum anus region.

C. Effects of activation of vagal afferents

To test the validity of the hypothesis that the gastro-colic "reflex may be mediated by nervous connections in the vagal nerves, a small series of experiments were performed where vagal afferents were activated either by means of gastric distension or by graded electrical stimulation of the central end of the cut left vagal nerve.

Neither separate distension of the fundus and antrum of the stomach nor afferent vagal stimulation, procedures known to produce e.g a profound reflex gastric relaxation (Jansson 1969) had no effect whatsoever on colonic motility despite the fact that afferent vagal stimulation was varied over a wide range and though the pelvic nerves were intact and the inhibitory influence exerted by the sympathetic adrenergic supply was blocked with guanethidine This indicates that neither the vagal fibres to the proximal colon nor the cholinergic fibres in the lumbar sympathetic supply to the colon or the pelvic efferents are reflexly excited by vagal afferents.

Sometimes however afferent vagal stimulation produced a complex response pattern with vomiting and a pronounced fall in blood pressure but colonic motility was never enhanced

D Effects of afferent somatic "pressor" and "depressor" fibre stimulation

As a comparison to the intestino-colonic and pelvo-pelvic reflexes, somatic" pressor and depressor afferents (see Johansson 1962) were stimulated in some experiments to see whether such afferents in a more *specific* way might reflexly affect colonic motility and blood flow

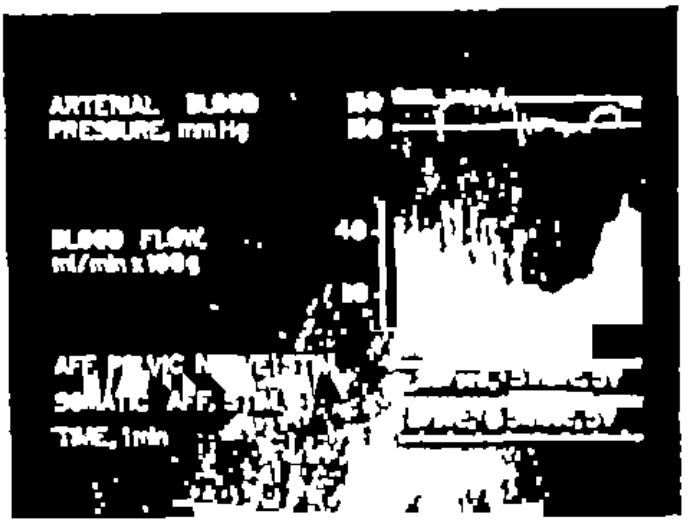


Fig 7 Cat 5.1 kg Chloralose Effect of superimposed activation of somatic *depressor* afferent (hamstring nerve) on the reflex response upon a *high strength* stimulation of pelvic nerve afferents. Note that the blood flow increase can be traced behind the mechanical artifact produced by straining movement which are eliminated by depressor activation at the same time the blood flow increase is somewhat enhanced

Activation of the fairly *low* threshold somatic depressor fibres produced similar reflex effects on the colonic blood flow as did the *low* threshold afferent fibres in the pelvic nerves Thus a moderate increase in colonic blood flow occurred associated with the reflex fall in blood pressure (Fig 5) The blood flow increase was abolished when the sympathetic fibres to the colon were cut indicating that it was due to a reflex inhibition of sympathetic vasoconstrictor fibre activity and not due to pelvic nerve activation Colonic motility was not essentially changed again indicating that the parasympathetic supply was not reflexly activated The same seems to hold for the cholinergic motor fibres running in the lumbar sympathetic supply If however a reflex activation of the pelvic nerve supply was produced by afferent *high*-strength pelvic nerve stimulation producing straining associated with a partly masked pelvo-pelvic reflex vasodilatation a superimposed activation of somatic depressor afferents had a dramatic effect (Fig 7) The straining movements became largely inhibited while the pelvo-pelvic reflex vasodilatation was enhanced This finding may indicate that the somatic depressor fibres besides suppressing to some extent somatomotor reflex activity produce an overall sympathetic inhibition eliminating also the tonic sympathetic suppression of the pelvo-pelvic reflex

The somatic pressor fibres produced largely the same reflex effects as in other tissues (see Johansson 1967) with no evidence of any excitatory involvement of the colonic parasympathetic supply Thus the reflex pressor effects were associated with a moderate reflex vasoconstriction in the colonic vascular bed with no significant change of colonic motility It should here be stressed that the adrenal glands were in all experiments denervated so that any reflex release of bloodborne catecholamines

could not occur which might otherwise have affected the motility somewhat (cf Kock 1950)

IV COMMENTS

To facilitate the discussion of the results the reflex activation of the pelvic nerves will be dealt with first

In the present study it was shown that a motor contraction of largely the entire colon as well as a pronounced vasodilatation of the colonic vascular bed can be brought about by reflex activation of the pelvic nerves. These reflex activations of the specific parasympathetic supply could be induced only from the rectum anus region This seems to be the case also in man, whereas distension of the rectum produces an immediate desire to defaecate (Zimmerman 1900 Hertz 1911 and Golligher and Hughes 1951) this is not the case when distension is applied at proximal colonic levels, which would indicate that receptors specifically involved in the defaecation reflex are restricted to the rectal part of the large bowel Exactly where in the wall these specific" receptors are localized is not known. Painting the mucosa with anaesthetic solutions (Garry 1933 Schmidt and Fülgraff 1963) is said to block the reflex response but as the anaesthetic solution might affect the muscle coat as well, the suggestion that the receptors are located to the mucosa cannot be taken for granted.

Frictional stimuli were in this study by far the most efficient ones in bringing about reflex colonic activation. Pure distension was less effective in this respect at least until spinal transection was performed. Moreover distension appeared—when powerful—to superimpose another type of reflex response involving mainly a generalized reflex sympathetic inhibition with a consequent blood pressure fall coupled to a slight colonic vasodilatation. These two types of reflexes could often be better distinguished by graded afferent pelvic nerve stimulation where the fibres producing the depressor effects had distinctly the lowest stimulation threshold.

Cannon (1902) came to the conclusion that the adequate stimulus for propulsive activity and emptying of the large bowel was not likely to be mere distension only He suggested that frictional movements of colonic contents might supply a most essential stimulus. In fact, a frictional stimulus applied to the urethra by the movement of a catheter or of fluid is in a similar way suggested to facilitate reflex micturition responses in the cat, whereas pure distension in the same region had no effect whatsoever (Barrington 1921) It was pointed out by Garry (1933) that the effective stimulus for eliciting a colonic reflex response in anaesthetized cats was friction and he tried to explain the inadequacy of mere distension of the rectum as a stimulus by the acuteness of the experiments and consequent shock to the nervous system.

The reflex parasympathetic responses, especially the pelvic nerve effect on colonic motility were often suppressed by tonic sympathetic activity This evidently emanated mainly from supraspinal centres rather than being preferentially a spinal intestino-

colonic reflex as the suppression seemed to be much reduced by high spinal section. This was the case also after section of the sympathetic supply directly or by guanethidine blockade (3 mg/kg). Intestino-intestinal inhibitory reflexes are if anything known to be enhanced by high spinal section (Johansson *et al.* 1966, 1968). After sympathetic blockade or especially when high spinal section was performed the reflex vasodilatation and motility increase mediated by the pelvic parasympathetics could sometimes be almost of the same magnitude as those produced by maximal efferent pelvic nerve stimulation (see chapters II and III). The often particularly marked pelvo-pelvic reflex responses after high spinal section might indicate that also the sacrospinal reflex centres become released from a supraspinal inhibitory influence and not only the synapses in the colonic wall. This was also illustrated by the fact that rectal distension produced a clearcut increase of colonic motility and blood flow only when the spinal cord was cut at the upper thoracic level, which also eliminated the earlier mentioned reflex depressor response. The pelvo-pelvic reflex effects were usually not substantially decreased and certainly not abolished after atropine administration.

The autonomic part of the defaecation reflex was shown to be solely dependent on the integrity of the pelvic nerves confirming the results of Garry (1933) and Wright *et al.* (1938). Both the afferent and efferent paths for this reflex appear to be confined to these nerves and there was no evidence that either the vagal nerves or the cholinergic motor fibres running in the lumbar sympathetic colonic supply were involved.

The term gastro-colic reflex introduced by Hertz was originally thought to be a specific reflex mechanism activated by distension of the stomach during feeding and resulting in a reflex activation of the colon preceding defaecation. Afferent electrical stimulation of the vagal nerves or distension of the stomach has in some experimental series been observed to elicit a response of this general nature (Lehmann 1913, Galapeaux and Templeton 1937). Recent investigations have however failed to confirm the presence of such a specific reflex arrangement and it has been suggested that other mechanisms are involved (Pearcy and van Liere 1956, Douglas and Mann 1940, Logan 1967, Connell 1968, see also chapter I).

In the present study neither gastric distension nor afferent vagal stimulation which produce powerful reflex effects on the stomach (Jansson 1969) influenced colon motility although all parasympathetic pathways were intact and the sympathoinhibitory influence blocked in the course of the study. Thus neither the vagal and pelvic nerves nor the cholinergic sympathetic fibres to the colon appear to be involved in this type of response. Furthermore, there was no evidence that the afferent vagal stimulation might enhance colonic motility by inhibiting reflexly the suppressing effect of prevailing adrenergic activity. These findings cast further doubt on the existence of a strictly nervous basis for the gastro-colic response.

This does by no means imply that the vagal nerves which certainly influence proximal parts of the colon (see chapter II) should not be engaged in the reflex control of colonic motility. They are evidently not involved in either the gastro-

colic "reflex" or in the defaecation reflex but they may well be engaged along with the excitatory vagal supply to the stomach and small intestine in connection with e.g meals, thus enhancing the mixing type of motility in these parts favouring digestion and absorption It is likely as discussed in chapter II that the proximal colon is mainly involved in absorption events where a neurogenic enhancement of mixing movements seems rational

With respect to the spinal sympathetic inhibitory reflexes of the intestino-intestinal and intestino-gastric type these are generally *enhanced* by the elimination of a tonic bulbospinal activity that suppresses their spinal reflex coupling These sympathetic reflexes exert an inhibitory action on gastro-intestinal activity mainly perhaps only at the cholinergic ganglionic level at least in the cat (Kewenter 1965 Jansson and Martinsson 1966) In the present study intestino-colonic reflex inhibition induced either by graded jejunal distension or by afferent mesenteric nerve stimulation, produced a clearcut inhibitory effect on the parasympathetically induced motility in the proximal colonic part—whether induced by the vagal or the pelvic nerves. However it appears as if the sympathetic lumbar colonic nerves are far less perhaps not at all, activated in this particular type of spinal inhibitory reflex. The effects produced by high spinal cord transection also agree with such a view since this procedure appeared to eliminate a tonic inhibitory effect conveyed by the lumbar colonic nerves rather than enhancing such an influence which is the case when the intestino-intestinal type of reflex is released from supraspinal control. Thus the pelvo-pelvic motility and vasodilator response occurred more readily after a spinal transection while the reverse is true for reflex vagal excitations of the stomach and the small intestine (Johansson *et al.* 1965 1968 Jansson 1969)

As a comparison to the described parasympathetic and sympathetic reflexes, "specific" in the sense that they were elicited from the colon itself or from adjacent gastrointestinal parts, reflex effects emanating from somatic pressor and depressor afferents (see Johansson 196") were also explored to some extent. These types of afferents—most of which seem to mediate "superficial and deep pain, respectively —apparently did not directly engage either the parasympathetic fibre supply to the colon or the cholinergic fibres supposed to run in the sympathetic colonic supply As in other tissues the somatic pressor fibres produced reflex vasoconstriction and the depressor fibres reflex inhibition of vasoconstrictor fibre tone in the colonic vascular bed In this respect the somatic depressor afferents thus produced effects similar to those of the low threshold afferents present in the pelvic nerves (see above) In the "resting colon neither type of somatic afferent fibres produced any significant effects on motility However as is the case with the stomach (Jansson 1969) activation of somatic depressor afferents under circumstances of a prevailing reflex activity in the parasympathetic supply enhances the excitatory effect of this supply This was in the present experiments clear from the "release of the reflex vasodilatation induced via the pelvic nerves (Fig 8) This seems to be the result of the well known generalized inhibitory effect on sympathetic tonic activity that these fibres exert (e.g Johansson

1962 Jansson 1969) This overall reflex reduction of a tonic sympathetic inhibitory influence, which in the colon is exerted mainly via the intramural ganglia will enhance the impact of the pelvic nerves on colonic motility and blood flow It is not a result of any reflex excitation of the pelvic parasympathetic fibres induced by the somatic depressor fibres because the mentioned enhancement was entirely abolished when the sympathetic fibres were cut or blocked by guanethidine

Summarizing the results in the present study it appears evident that the motor and vascular responses as induced by efferent stimulation of the pelvic nerves (see previous chapters) are also achieved by reflex activation of these nerves, which only seems to be produced by specific pelvic afferent This pelvo-pelvic reflex constituting the autonomic part of the defaecation reflex involves the motility of largely the entire colon and appears to be activated by specific mechano-receptors These are most easily activated by frictional stimuli but also by distension and they are situated in the rectal anal section It also appears as if there exist supraspinal inhibitory mechanisms which adjust and regulate the activity in the micro-spinal reflex arc both at the spinal relay and at the parasympathetic ganglionic level in the colonic wall in the latter case conveyed by the inhibitory action of the lumbar colonic nerves. Neither the vagal fibres nor the cholinergic fibres in the sympathetic supply are involved in the defaecation reflex

The vagal fibres to the proximal colon are probably reflexly involved along with those to the small intestine to facilitate digestion and absorption The functional significance of the cholinergic sympathetic fibre supply remain unknown Neither fibre type is involved in the gastro colic reflex nor are the pelvic nerves.

Intestino-colonic inhibitory reflexes probably of spinal nature and via splanchnic efferents suppressing the vagally or pelvically induced activity in the proximal colon appear to be of the same nature as the intestino-gastric and intestino-intestinal inhibitory reflexes The lumbar colonic nerves supplied to largely the entire colon, appear to be less, perhaps not at all activated in this particular type of reflex indicating a somewhat different functional role for the sympathetic inhibitory control of proximal and distal colonic parts They seem to convey a centrally induced inhibitory influence at least in the present experimental situation

Thus, the results in the present reflex study agree with the view outlined in earlier chapters that the colon may be composed of two functionally different parts. It seems probable that the proximal part though relatively small in the cat and apparently of importance mainly for the absorption of water and salts possesses a nervous control different from that of the rest of the colon but closely related to that of the small intestine as it is supplied by vagal parasympathetic and splanchnic sympathetic fibres. These nerve fibres, distributed to the proximal colon only are apparently involved in similar patterns as are the nerves to the small intestine but are not engaged directly in the reflex responses that mainly involve the distal parts of the colon. Here the nervous control is exercised by the pelvic parasympathetic nerves and the lumbar sympathetic colonic nerves although these nerves partly extend

also to the proximal colon but only with respect to the motility control. The distal colon and its nervous control is mainly involved in the evacuation mechanism and associated with this there occurs in this part a reflex production of protective and lubricating mucus and a mucosal vasodilatation. All effects are conveyed by *pelvic* efferents.

However as pointed out in chapter II the difference in motility patterns of the proximal and distal colonic parts—so obvious in the reflex patterns—can also be traced in the inherent activity of the colonic wall. This does by no means deny that the mode of action of the vagal and pelvic parasympathetic fibres may in somewhat different ways affect the intrinsic plexuses. Thus the vagal effects on the proximal colon differ from those of the pelvic nerves since the vagal effects are easily blocked by atropine whereas the pelvic effects are not.

CHAPTER VII

GENERAL CONCLUSIONS AND COMMENTS

The aim of the present study was to shed further light on the classical concept that the large bowel may be comprised of two functionally different parts. This concept is based mainly on theoretical and embryological ground but only incompletely supported by functional analyses as clear from the most recent reviews of the field (Connell 1968, Gillespie 1968, Schuster 1968, Wakeloff 1968). As a matter of fact current opinions about the nervous control of colonic motility are in many respects controversial and incomplete and little is known about the nervous regulation of colonic blood flow and secretion (see Chapter I). The main interest was therefore focused upon the distribution mode and extent of action as well as reflex involvement of the cranial and sacral parasympathetic supply (vagal and pelvic nerves) as compared with the sympathetic supply (splanchnic and lumbar colonic nerves) with respect to colonic motility, blood flow and secretion.

In the preceding chapters these different aspects were sequentially dealt with and discussed. The present chapter will therefore be devoted to a brief review of these earlier chapters and to a general discussion of the functional significance of the results. However it is justified in this context to first consider some general methodological problems even though the experimental procedures are described in connection with each chapter which should be consulted for detail.

I METHODOLOGICAL CONSIDERATIONS

In order to analyse in more detail the autonomic nervous control of a visceral organ like the colon the effects of nervous section or blockade and of graded efferent stimulations of the out nerves should be sequentially explored together with the various types of reflex and central involvement of the intact nervous pathways. Furthermore since the parasympathetic and sympathetic effects on the colon can in various ways interfere with or even entirely mask each other it is also necessary to perform analyses of their respective influence both when the antagonistic pathway is intact and when it is blocked. These different steps have all been utilized in the present series of studies. As however several autonomic neuroeffector systems are

involved and closely intermingled—visceral smooth muscles glands, blood vessels—their separate analyses calls for quantitative recordings of appropriate effector responses rather than of the nerve fibre activity for exploration of the general organization of the neurogenic patterns involved Electrophysiological recordings alone can hardly provide full insight into all these aspects, although such techniques are of course of greatest importance when aspects like synaptic organization occlusion facilitation etc in the autonomic nervous system are to be studied (cf Job and Lundberg 1952 Oscarsson 1955)

In the present study colonic motility and various aspects of the vascular responses have been separately recorded in the proximal and distal parts of the colon. As far as possible not only qualitative but also quantitative aspects of the responses have been dealt with. This type of recording implies a "randomization" of the neurogenic influences on the respective effector cells. The frequency response characteristics of the different effectors were also studied furthermore it is known that there is a marked degree of convergence and divergence in autonomic fibre supply to *functionally* homogeneous effector groups. It appears therefore as if such groups are more or less, neurogenically directed as "functional units" For such reasons this type of recording may provide good insight into the general characterization of the viscero-motor control if certain precautions are taken (see below) This methodological approach is, in fact related to that used by Sherrington in his classical studies of the spinal-supraspinal somatomotor control.

The equivocal and divergent results often obtained in previous studies of colonic function can partly be explained by the fact that unphysiological rates and strengths of electrical stimulations of the nervous supply have mostly been utilized, which—when partly antagonistic nerve supplies are involved—may lead to erroneous information. For example stimulations of this type can imply such a massive impact on the effectors that it may entirely mask a normally wellgraded reciprocal control Many earlier workers were also aware of these facts and have therefore preferred to study only the effects of nerve section Again however the information arrived at in this way depends on whether the fibres cut were tonically active or not and in this respect anesthesia, surgical trauma with consequent pain fibre activation, etc. may greatly influence the situation Thus, the surgical trauma often leads to increased sympathetic activity (e.g Jansson 1960) which may in various ways greatly affect other regulatory mechanisms, e.g by way of the spinally relayed intestino-gastric intestino-intestinal and intestino-colonic reflexes. Furthermore during anesthesia the resting parasympathetic discharge is usually low or even absent and supraspinal control mechanisms may of course be grossly affected.

Recently considerable insight has been gained into the normal discharge range of the autonomic nervous system, both during "rest and in various situations of excitation and inhibition and also into some of its more important patterns of reflex and central nervous involvements (e.g Folkow 1955 Uvnäs 1960 and Folkow *et al* 1965) Therefore also in acute experiments where anesthesia and trauma is m

volved such new information somewhat reduces the difficulties in exploring the mode of action and the involvement of the extrinsic nerves to a visceral organ. For example, the various links involved can now more easily be activated in a well graded fashion or selectively and temporarily blocked, which facilitates the analysis of e.g. the reciprocal relationship between parasympathetic and sympathetic nerves. Moreover, the greatly improved knowledge concerning the various types of smooth muscle aids in studies of their nervous control.

For evaluating the colonic motility in the present study a device was utilized (chapter II) which made a double set of volume recordings. Even though this simple technique only allows for a semiquantitative estimation of the average volume changes in the two colonic parts, it no doubt reflects directly the important reservoir function of the colon. Colonic pressure changes, which have often been utilized in earlier studies, especially as regards studies on colonic function in man, will less well reflect this essential function of the colon. Furthermore, it randomizes the individual effector responses which, as mentioned, is of advantage for the present analysis of the main principles of neurogenic control. This does, of course, by no means deny some obvious shortcomings of the recording technique. Thus, more detailed studies of the individual smooth muscle layers, etc., call for quite different techniques. Concerning the recording techniques used for the analysis of the colonic vascular responses, these have been widely used in this laboratory and have been critically discussed, e.g. by Mellander (1960), Kjellmer (1965), Wallentin (1966) and Lundgren (1967).

II. MAIN RESULTS OF THE PRESENT STUDY

In *chapter I* a survey of the literature was presented and the aim of the present study was summarized.

In the experiments described in *chapter II* it was studied whether and in which ways the innervation of the colonic musculature is differently organized in proximal and distal part, and whether the sympathetic inhibitory influence is exerted mainly at the parasympathetic ganglionic site, as suggested by histochemical studies (Norberg 1964, Jacobowitz 1965, Schofield 1968). The results strongly suggest that both the parasympathetic excitatory and sympathetic inhibitory control of colonic motility are in fact organized to form two different but partly overlapping antagonistic fibre systems. Thus, the vagal and splanchnic nerve supply affects only the proximal 1/3–1/4, while the pelvic and lumbar colonic nerves affect almost the entire colon, i.e. also that part to which the vagal and splanchnic sympathetic supply is restricted.

However, the consequent double control of the proximal colonic part appears to be functionally different in important respects. Thus, vagal activation induced a response here that was characterized by motility waves of increased amplitude and frequency superimposed on a slightly elevated average tone, a motility pattern that would favour mixing of contents rather than their expulsion. Pelvic nerve activation

on the other hand excited the entire colon in a way that was different both with respect to its effect on the proximal and distal colonic sections and especially in comparison with the vagal influence on the proximal section. Thus the volume recordings showed that the pelvic nerves produced intense and apparently sustained luminal reductions in both colonic parts favouring expulsion. However direct inspection revealed that the response in the proximal part started as pronounced and frequent annular contractions but soon these fused into a sustained overall contraction that greatly reduced the lumen, such effects were never seen when the vagal supply was activated. The distal two-thirds of the colon exhibited from the start of the pelvic nerve activation a sustained and intense contraction which could virtually abolish the lumen. Another difference between the two types of parasympathetic supply which in terms of frequency response characteristics were fairly similar was that the vagal effects were easily blocked by atropine while this drug had little or no influence on the motor response to pelvic nerve stimulation.

The sympathetic supply appeared to exert its inhibitory influence on colonic motility at the intramural ganglionic level. The splanchnic fibre supply was almost exactly identical with the vagal supply while the lumbar colonic nerves took the same distribution as the pelvic nerves. Thus the proximal colonic part also has a double supply of sympathetic fibres, with respect to the motility control.

Chapter III was devoted to the distribution and type of vascular effects that were produced by graded stimulation of the various extrinsic nerves to the colon. Simultaneous stimulation of the two sets of sympathetic vasoconstrictor fibres the splanchnic and lumbar colonic nerves, led to intense and uniform blanching of the entire colonic mucosa which remained strongly blanched throughout the period of stimulation. Selective activation of the splanchnic vasoconstrictor fibres blanched the mucosa of the proximal third only and the remaining two thirds were similarly affected when the lumbar colonic nerves were subsequently activated usually with only little overlap between the two fibre supplies. Thus the lumbar colonic nerve fibres controlling the vessels do not overlap with the splanchnic fibres to the proximal third in contrast to the situation with respect to their influence on colonic motility.

In contrast to the well maintained blanching of the colonic mucosa upon vasoconstrictor fibre stimulation such stimulation did not produce any sustained marked increase of overall colonic blood flow resistance. Thus the initially intense flow reduction decayed within few minutes of continued stimulation until a new steady state level of moderately increased flow resistance was reached. Analogous to the situation in the small intestine this phenomenon was referred to as an autoregulatory escape from the vasoconstrictor fibre influence and the concomitant maintenance of the constriction in superficial mucosal parts may suggest a secondary intramural redistribution of blood flow probably to deeper mucosa-submucosal layers.

With respect to the parasympathetic supply even intense vagal stimulation over a wide range of frequencies had no effect whatsoever on colonic blood flow resistance or on the mucosal vessels suggesting that these nerves do not convey any specific

vasodilator fibres to the colon or that they in other ways significantly affect the blood vessels. This finding is in agreement with that of Lewenter (1965) concerning the small intestine.

In contrast pelvic nerve stimulation produced an intense flushing of the colonic mucosa but mainly in its distal two thirds i.e. largely corresponding to the part supplied with vasoconstrictor fibres via the lumbar colonic nerves. Concomitantly with this mucosal vasodilatation and largely restricted to the same parts a mucoid secretion appeared while the entire colon contracted as described in chapter III. Corresponding in time with this mucosal flush there was an intense but transient increase of venous outflow often followed by a phasic flow reduction.

The phasic reduction of colonic resistance was also reflected in the arterial inflow and pelvic nerve stimulation could produce blood flow figures as high as 150–200 ml/min×100 g tissue which is close to maximal vasodilation. Therefore the greatly increased venous outflow induced by pelvic nerve stimulation no doubt reflects a true and powerful colonic vasodilatation and cannot be ascribed to any squeezing of the intramural capacitance vessel produced by the simultaneous colonic contraction.

Intravenous administration of atropine in a dose of 1 mg/kg did not significantly interfere with the vasodilator mucosal motility responses to pelvic nerve stimulation indicating that both may have been brought about by some non-cholinergic mechanism. Administration of a ganglion blocking agent (Ecolid® 2 mg/kg) abolished all responses to pelvic nerve stimulation suggesting that a ganglionic synapse is present in the neurogenic pathway. The apparently identical level of stimulation thresholds for the pelvic fibres which produced colonic contraction and vasodilatation and their atropine-resistance might indicate the involvement of some indirect mechanism e.g. the release of kinin like substances as shown to be the explanation *of the atropine resistant vasodilatation in salivary glands (see below). However* an important difference was demonstrated in the distribution of the pelvic nerve effects producing vasodilatation and motor contraction (see Fig. 1).

The observation that two sets of colonic vasoconstrictor fibres control different parts of the colonic vascular bed does not mean *per se* that these parts should be functionally differentiated. It could as well be explained by the embryological development of the colon, the proximal part being derived from the midgut and consequently supplied by fibres from the thoracic sympathetic outflow and the distal part emanating from the hindgut and controlled by the lumbar sympathetic outflow. However the absence of vagal vasodilator influences combined with the fact that the pelvic nerves produce mucosal vasodilatation which is essentially confined to distal colonic parts, suggests that the two parts may be functionally differentiated. In general the blood flow control of the gastro-intestinal tract appears to be closely linked to the functional characteristics of its different compartments. This is also obvious when the stomach is compared with the small intestine (see chapter I). No doubt the colonic mucosal vessels in the proximal and distal parts are controlled

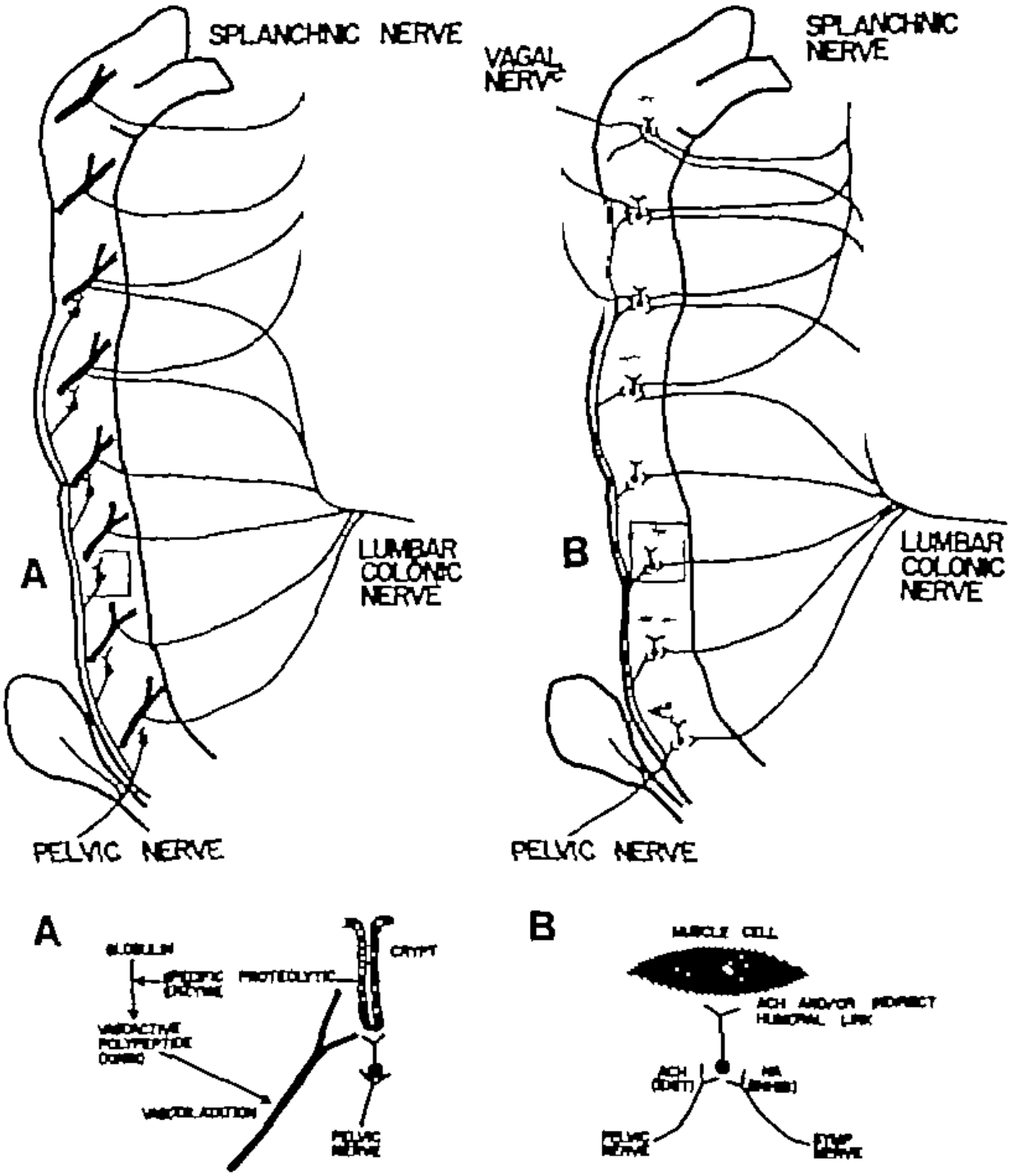


Fig. 1 Schematic drawing of the nervous control of colonic blood flow (left panel) and motility (right panel). A, shows the possible background of mucosal vasodilatation B that of intramural motility control. As described in text, the functional significance of the reciprocal vagal-splanchnic nerves control and the partly overlapping pelvic-lumbar colonic nerves control seems to be different.

differently In this respect they resemble the vessels of the small intestine and the stomach respectively

The aim of the experiments presented in *chapter II* was to study the effect of vasoconstrictor fibre and pelvic nerve activation on the consequtive sections of the colonic vascular bed The vasoconstrictor fibre effects were shown to be largely similar to those occurring in the small intestine in corresponding situations (Wallentin 1966). Thus stimulation of all colonic vasoconstrictor fibres at high physiological frequencies caused an initial increase of regional vascular resistance from 4 0 PRU_{100} "at rest to about 20–30 PRU_{100}. This was followed by a second phase a decrease in resistance which after 2–3 min attained a new steady state level still well above the control. As mentioned above a redistribution of blood flow appeared to take place in connection with this autoregulatory escape" with a fairly well maintained constriction of the vessels in the outer mucosa as was further tolled in the subsequent chapter The capacitance response of the colonic vessel which could expel at least a third of the regional blood content was well sustained during the entire stimulation period. These observations suggest that the escape involves mainly precapillary resistance vessels and not at all the venous capacitance vessels. As a result of the precapillary "escape the pre/post capillary resistance ratio remain largely unchanged during prolonged vasoconstrictor fibre activity implying that mean capillary pressure is hardly altered Furthermore neurogenic blood volume mobilization from the colonic part of the splanchnic vascular bed will be sustained

Compared with the small intestine both resting and maximal blood flow levels and CFC values were lower in the colon which might be expected as its mucosa is thinner devoid of villi and appears to be less abundantly involved in absorption Furthermore while CFC in the small intestine can be markedly reduced by vasoconstrictor fibre activation despite a considerable escape of the resistance vessels CFC in the colon was hardly reduced more than the corresponding reduction in blood flow during the escape period

The vasodilatation induced by longlasting pelvic nerve activation gave rise to an initial volume increase corresponding to the peak flow increase but was then followed by a second slower phase of steady volume increase concomitant with recurring periods of vasodilatations. After atropine administration the *initial* phasic increase of blood flow and tissue volume was largely unaffected while the second phases were both eliminated The second volume phase may therefore reflect a neurogenic colonic secretion. Since increase of the venous outflow pressure only partly reduced the magnitude of the capacitance response occurring concomitantly with the *initial* vasodilatation a neurogenic effect acting on the smooth muscles also of the veins cannot be excluded.

Chapter V deals with a study of the blood flow in the different wall layers of the colon i e the mucosa-submucosa and the muscularis respectively as analyzed both at "rest and during changes induced by the vasoconstrictor fibres and the pelvic nerves. For these measurements, the technique of recording the wash-out of topically

injected ^{85}Kr or ^{133}Xe was used while total colonic blood flow was concomitantly recorded

At least 80 per cent of the total blood flow at rest appears to be distributed to the mucosal-submucosal sections, and even when total colon blood flow was only moderately increased by pelvic nerve activation very marked increases of blood flow occurred in the superficial mucosa submucosa but hardly any change occurred in the deeper part of these sections Evidently certain parts of the mucosa perhaps those located at the base of the secretory crypts appear to be especially well perfused with huge increases of blood supply occurring here upon pelvic nerve stimulation. By indirect calculation maximal blood flow in the superficial mucosal parts may be of the order of 400 ml/min × 100 g of tissue or even higher

At most 20 per cent of the total blood flow appears to be diverted to the muscularis at "rest being of the order of 7–14 ml/min × 100 g of tissue Even supramaximal activation of the pelvic nerves which greatly increased mucosal-submucosal blood flow usually had insignificant effects on the blood supply to the muscularis. This suggests that the pelvic nerves essentially affect only the vessels of the secretory absorptive parts of the colonic wall their vascular action being closely associated with the secretory response in the colon

Vasoconstrictor fibre activation led to an initially drastic reduction of mucosal submucosal blood flow as well as a reduction of flow in the muscularis In connection with the autoregulatory escape" from the vasoconstrictor fibre influence described above the blood flow reduction appeared to be relatively well maintained in the outer mucosa and in the muscularis, compared with the escape reflected in the recording of total colonic blood flow This observation suggests that the autoregulatory escape is due to an intramural blood flow redistribution presumably towards some sections in the deeper mucosa-submucosa.

Chapter VI deals with the reflex control of colonic motility and blood flow An intestino-colonic reflex inhibition was found to influence preferentially the motility of the proximal colon being conveyed by the splanchnic nerve supply to this region. No clearcut intestino-colonic reflex inhibition, conveyed via the lumbar colonic nerves was observed however not even after high spinal transection which is known to facilitate the appearance of intestino-intestinal inhibitory reflexes. Again, these findings suggest a somewhat different organization of the control in proximal and distal colonic parts. The intestino-colonic inhibitory reflex in the proximal colon conveyed via the splanchnic supply and localized to proximal parts only appeared to exert its effect mainly and perhaps only at the parasympathetic ganglionic level as it could be clearly demonstrated only when the colonic muscles were excited via their intramural ganglia This is analogous to the effects produced by direct sympathetic stimulation.

High strength afferent pelvic nerve stimulation produced a reflex increase of motility in largely the entire colon and a mucosal blood flow increase, in a similar way as when efferent pelvic nerve stimulation was performed The reflex response

also involved an increased mucus secretion as evidenced by a huge amount of mucus content within the colonic lumen at the end of these experiments. As long as at least one of the pelvic nerves was left intact this reflex response pattern was also induced by mechanical stimulation of the rectum. Gentle to-and-fro movements were more effective than even pronounced distension.

This pelvo-pelvic reflex response favouring colonic emptying also involved a somatic component in the form of straining activity. However this latter component appeared to be dependent on supraspinal centres for its full expression—presumably in the form of descending facilitatory influences as it was completely eliminated by high spinal transection a procedure which usually enhanced the autonomic part of the pattern. Furthermore the autonomic pelvo-pelvic reflex response was considerably facilitated by elimination of prevailing sympathetic tone. This sympathetic inhibitory influence could be abolished either by guanethidine administration by peripheral section of the sympathetic supply or at least in part reduced by high spinal transection. This latter observation indicates that supraspinal structures exert an inhibitory influence on colonic motility via the lumbar colonic nerves at least in the present experimental situation. In addition supraspinal structures might also exert an inhibitory influence upon the parasympathetic reflex centres" in the sacral medullary parts. If so higher centres may damp the pelvo-pelvic reflex both by an intraspinal inhibitory influence and by means of the adrenergic nerves, at the intramural ganglionic level conveyed via the lumbar colonic nerves.

Activation of vagal afferents either by distension of different parts of the stomach or by direct stimulation of such afferents—which causes prompt reflex activation of the vagal relaxatory fibres to the stomach (Jansson 1969)—did not excite either the vagal efferents to the proximal colon or the pelvic efferents to the distal colon. Therefore in agreement with other recent studies these results suggest that the clinically well known gastro-colic reflex is not mediated by any bulbo-spinal reflex coupling between gastric vagal afferents and vago-pelvic efferents to the colon. The pelvic nerves could be reflexly excited only from the lower colon itself and particularly from the rectum but it was impossible to also activate reflexly the vagal efferents to the proximal colon. Presumably these latter type of fibres are engaged in connection with situations where the vagal excitatory supply to the small intestine is involved. In fact little is known as to which gastro-intestinal receptor mechanisms may produce reflex activation of this group of efferent vagal fibres, although they are well known to be involved during e.g. meals; central autonomic structures seem to be to a great extent responsible of such patterns (see e.g. Eliasson 1960). For example topical stimulation of the "hunger centre" in the hypothalamus of the cat produces a vagal activation of intestinal motility (Folkow and Rubinstein 1965).

III INTEGRATIVE ASPECTS

As briefly outlined above the nervous effects exerted on the musculature and on the vascular bed of the colon are somewhat differently organized in the proximal and distal colonic parts both in terms of the efferent pathways and their reflex involvement. Some functional implications of these observations will now be briefly discussed

To understand the rather complex and differentiated nervous control of colonic motility and blood flow it must be related to the functions known to be exhibited by the colon viz absorption storage secretion and expulsion It is generally held that the colon in carniverous and omniverous animals as in the cat and man is involved in absorption of water and salts. However compared with the small intestine this absorptive function appears to be small and no doubt the colonic surface area is far smaller due to the villous arrangement of the jejuno-ileal mucosa In man by far most of the fluid (intake and secretion amounting to 6–8 l per day) is reabsorbed in the small intestine It has on indirect grounds been calculated that in man perhaps 500–800 ml of approximately isotonic chyme enters the proximal colon daily (Smiddy et al. 1960 Davenport 1966) With an average faecal water content of some 150–200 ml colonic fluid absorption would amount to about 300–700 ml per day This figure has however been challenged by Levitan (1962) who suggests that the daily water absorption in the human colon is at least three times larger and equally efficient in both the proximal and distal colonic parts On the other hand both in cat and in man the colonic contents are as a rule solid or semi-solid when reaching the distal colon. Thus normally most of the liquid seems to be absorbed proximally which of course does not deny that a substantial absorptive capacity may be present but seldom utilized in the distal colon as well. Such a view is supported by the clinical observation that even extensive resections of the colon have but little effect on the absorptive function In any case the proximal part appears to be normally involved in the final water electrolyte absorption, a process which would be enhanced by the characteristic churning and mixing or even antiperistaltic motility observed in this part of the colon in intact organisms The present findings show that this type of motility pattern is to some extent basically inherent in the proximal colon and greatly facilitated by the vagal innervation which is restricted to this part.

The vagal innervation is on the other hand evidently not involved in the defaecation reflex. It seems likely that these fibres which are reserved for the absorptive part of the colon, are involved in a similar way as the vagal innervation of the jejuno-ileum appears to be involved, in the motility control and hence indirectly in absorption in this compartment.

In contrast to this vagal colonic supply the sacral parasympathetic nerves seem to be specifically involved in the expulsive function of the colon, whereas the associated secretion and mucosal vasodilatation in the distal parts mainly appear to serve protective and lubrication purposes. Defaecation is normally preceded by a general increase of gastro-intestinal activity including the proximal colonic parts, a response that is often so obviously associated with feeding that the concept of a gastro-colic

reflex has been widely accepted However as mentioned in chapter I and VI recent investigations indicate that the gastro-colic reflex may be mediated by a humoral factor perhaps gastrin thus constituting a neuro-hormonal activating mechanism—where even the afferent link may be strictly intramural—rather than a classical reflex arc It may be consistent with such a view that complete vagotomy in man if anything increases colonic motility following feeding and may hence accentuate the defaecation response

This would mean that the functional involvement of the extrinsic nervous control of the gastro-intestinal tract in general differs considerably in its various compartments thus including also the proximal and distal colonic parts The vagal relaxatory fibres to the stomach constitute an efferent link that causes prompt and apparently well graded reflex adjustments of the reservoir section of the organ to food intake (see e.g Jansson 1969) On the other hand the vagal excitatory influence in the stomach the small intestine and the proximal colon appears rather to be involved in facilitating mixing and churning of the contents mainly to subserve digestion and absorption rather than primarily executing propulsive activity which is normally fairly slow and continuous in these parts Once the contents have passed beyond these digestive-absorptive sections however a reflex pattern ensues which is of immediate effect and aimed at active emptying of the distal reservoir

The gastric and colonic reservoirs at the two ends of the gastrointestinal system have therefore much in common with respect to their extrinsic nervous control Apart from a vagal excitatory influence probably in both organs aimed at facilitating mixing of the contents for digestion and absorption there are specific fibre sets for adjusting the reservoir function of the two organs in appropriate directions. Thus active widening occurs in the stomach and active expulsion occurs from the colon both responses being conveyed by specific parasympathetic fibre sets. Further both exhibit neural vasodilator responses in the mucosa which may well be secondary to the neurogenic secretory responses because the blood flow increase seems to be mainly restricted to those mucosal sections where the increased secretion occurs

The sympathico-inhibitory fibres appear to exert their suppressing effect on gastrointestinal motility mainly or perhaps only at the intramural ganglionic level and this is evidently also the case in the colon. However their reflex involvement appears to differ somewhat in the proximal and distal colon. Thus spinal sympathetic reflex arcs ("intestino-gastric intestino-intestinal and intestino-colonic inhibitory reflexes respectively) which are exposed to tonic supraspinal inhibition and involve splanchnic afferent and efferents promptly inhibit cholinergic motility in the stomach (e.g Jansson and Martinsson 1966) and the entire small intestine (e.g Johansson and Langston 1964) but only the proximal part of the colon. The sympathetic lumbar colonic nerves affecting the entire muscularis of the cat colon via the intramural ganglia, appear to be little if at all involved in such spinal inhibitory reflexes. This sympathetic supply which overlaps in the proximal colonic parts with the splanchnic supply rather appears to mediate tonic activity of mainly supraspinal

origin. Thus, the parasympathetic pelvo-pelvic reflexes are if anything released from this type of sympathetic inhibitory influence by high spinal transection. This procedure normally enhances the intestino-intestinal type of sympathetic inhibitory reflex that is mediated by the splanchnic efferents

Associated with the pelvo-pelvic reflex increase of motility there occurs, as mentioned an increase secretion and a mucosal vasodilatation. This has also been observed in man (Grace *et al* 1951) Previously specific vasodilator fibres were supposed to be widely distributed in the gastrointestinal tract but recent investigations including the present one indicate that the parasympathetic vasodilator mechanisms are restricted to the salivary glands, the stomach and distal parts of the colon The mechanism behind this atropine-resistant gastro-intestinal vasodilatation has been extensively studied in recent years in salivary glands. Even though specific vasodilator fibres may also be present in this particular organ it appears that the main part of the neurogenic vasodilator response is *not* due to such fibres but rather due to the local formation of a potent vasodilator polypeptide kallidin to which the effect of unspecific "vasodilator metabolites may to some extent contribute

It is possible that similar indirect mechanisms may be responsible of the vasodilatation occurring not only in the gastric mucosa during vagal stimulation (see Martinson 1965) but also that occurring in distal colonic parts during pelvic nerve stimulation. Preliminary observations (Hultén and Jodal) suggest that the colonic secretion, like the saliva, contains a factor that on injection into the colonic circulation causes a profound vasodilatation. However much further study is needed before it can be settled whether this vasodilator principle is in any way related to the kinin-producing enzyme kallikrein present in the saliva In any case the situation in the colon resembles that of the salivary gland and also the gastric mucosa in the respect that the intense *initial* parasympathetic vasodilator response is largely unaffected by atropine, although the *recurring* blood flow increases probably associated with the secretory response is eliminated by this drug (see chapter IV)

From a circulatory point of view the colon can be looked upon as an abundantly perfused glandular tissue enclosed by a less well perfused smooth muscle coat One important function of this glandular tissue especially in the distal colonic parts, is to rapidly produce a mucoid secretion that presumably serves to lubricate inspissated faecal contents in association with defaecation. As the raw material for any secretion formation is ultimately blood plasma, an increased blood flow is necessary to cope with the suddenly increased fluid demands connected to this neurogenic mucoid production to which comes the increased glandular metabolism The pelvic nerves can in fact produce virtually maximal vasodilatation in the mucosa of the distal colon, where flow figures around 400 ml/min $\times$ 100 g of tissue appear to be reached in its superficial parts at intense pelvic nerve activation.

The gastro-intestinal vascular bed is as mentioned, also under the influence of vasoconstrictor fibres which in e.g the colon can produce fairly intense and well maintained flow reductions in the outer mucosa. Indeed vasoconstrictor fibres are

present to about an equal extent in all consecutive compartments of the gastrointestinal tract In contrast to the more restricted neurogenic vasodilator mechanisms. Thus antagonistic vasomotor mechanisms are only present in the stomach and the distal colon and this might be of importance in some pathophysiological situations For example strong emotions and psychic conflicts are known to cause considerable gastro-intestinal disturbances and it appears as if motility blood flow and secretion are especially affected in the stomach and the distal colon In fact the mucosal colour in the two organs may reflect emotions as expressively as the blanching and flushing of the face (Wolf and Wolff 1947)

In both these compartments the parasympathetic control of the mentioned functional parameters is pronounced and at the same time the sympathetic vasoconstrictor fibre influence is powerful not the least with respect to the outer mucosa This might constitute a potential source of especially pronounced disturbances of function and blood supply in the stomach and distal colon e g in situations of centrally elicited increases of autonomic discharge For example vasoconstrictor fibre activation increases flow resistance but the increased resistance in the postcapillary vascular section appears to be better maintained than that in the precapillary section In the resting gastrointestinal tissue this causes capillary hydrostatic pressure to remain largely unchanged during vasoconstrictor fibre activity while in e g skeletal muscle it is much lowered If however parasympathetic activation is superimposed upon an enhanced constrictor fibre tone in the stomach or and colon leading to increased secretion coupled to a preferential precapillary dilatation of mucosal vessels the vasoconstrictor fibre influence will be counteracted mainly on the precapillary side This will in turn result in a considerable increase of mean hydrostatic capillary pressure at the same time as most mucosal capillaries are probably opened up by the parasympathetic influence A mucosal edema may then ensue especially if capillary permeability is increased which seems to be the case when kinins are locally released from local sweat glands Often repeated bouts of marked mucosal edema would implicate large increases of the diffusion distance between blood and tissue Especially if this is combined with intermittent neurogenic reduction of mucosal blood flow this may in the long run have harmful effects on mucosal nutrition. This is, of course just a theoretically possible sequence of events even if mucosal control in the two organs is so organized that each single event is entirely plausible It remains to be shown that e g often repeated emotionally evoked autonomic activations produce the required interactions between parasympathetic and sympathetic influences However it might be more than a coincidence that gross mucosal disturbances and even ulcerative lesions are not only fairly common but almost exclusively confined to the stomach and the colon which are the only compartments of the gastrointestinal tract which exhibit the mentioned type of nervous control of mucosal secretion and blood flow

SUMMARY

The extent to which the parasympathetic nerves (the vagus and the pelvic nerves) and the sympathetic nerves (the splanchnic and the lumbar colonic nerves) influence motility and blood flow in various parts of the colon was studied in a series of experiments on cats. The results suggest the following general organization of the extrinsic nervous control

1 The parasympathetic excitatory and sympathetic inhibitory control of colonic motility is so organized as to form two different sets of partly overlapping antagonistic systems. These two fibre systems appear to be functionally differentiated in the sense that the vagal fibres, antagonized by both sets of sympathetic fibres facilitate mixing and churning in the *proximal* colon only here evidently favouring absorption processes rather than mere expulsion The pelvic nerves executing expulsion from virtually the *entire* colon, are in the distal colonic parts antagonized by the lumbar colonic nerves only In the proximal colon the expulsive influence of the pelvic nerves overlapping the mixing influence of the vagal fibres, is antagonized by the two sets of sympathetic fibres distributed to this part

2 The sympathetic supply appears to exert its inhibitory influence on colonic motility mainly perhaps only at the intramural ganglionic level. The splanchnic fibres are distributed only to the proximal colon, while the lumbar colonic fibres, affecting motility reach largely the entire colon.

3 The splanchnic nerve supply is strongly engaged in the inhibitory intestino-colonic reflexes as is the case also in the intestino-gastric and intestino-intestinal reflexes. The lumbar colonic supply on the other hand appears to be less involved in these types of spinal reflexes mediating instead a tonic centrogenic inhibitory influence that particularly affects the motility in distal *colonic parts*.

4 The pelvic nerves induce a largely maximal vasodilatation in superficial mucosal sections, but in the distal colon only Blood flow figures of 400 ml/min × 100 g mucosa may here be reached and it appears as if the vessels surrounding the secretory crypts are particularly involved. This powerful vasodilator response seems to be closely linked to the secretion of mucus In contrast the vagal nerves do not induce any vasodilator effects in proximal colonic parts, analogous to the situation in the small intestine

5 The splanchnic and lumbar colonic nerves carry vasoconstrictor fibres to proximal and distal colonic parts, respectively with only slight overlap in contrast to their overlapping inhibitory influence on colonic motility The colonic resistance vessels exhibit like those of the small intestine an "autoregulatory escape from the vasoconstrictor fibre influence, which seems to imply a redistribution of blood flow from superficial to deeper mucosal-submucosal parts.

6 The pelvic nerve effects on motility mucosal blood flow and secretion constitute a coupled response that may be mediated by one and the same group of preganglionic fibres. Only the secretory response is readily blocked by atropine. The vasodilator

response might at least in part be induced by locally formed specific vasodilator agents like kinin as a result of the glandular activation.

All the three pelvic nerve effects are readily induced by a sacrospinal "pelvo-pelvical reflex constituting the defaecation reflex in which no other autonomic excitatory fibres appear to be involved However the defaecation reflex which strongly contracts the entire colon, can be markedly damped by the lumbar colonic nerves by means of their inhibitory influence on the parasympathetic intramural synapses

7 There is no evidence that the vagal fibres to the proximal colon should be involved in either the so-called gastrocolic reflex or in the defaecation reflex These fibres, which are readily blocked by atropine are probably activated together with the vagal excitatory fibres to the stomach and small intestine in such reflex and central autonomic patterns that are involved in connection with digestive activity and feeding responses"

8 The combined results thus suggest that the colon is comprised of two functionally different parts, where each exhibits a differently organized control of motility blood flow and secretion but where some overlap exists at least with respect to the motility control.

The motility control of the proximal colon seems to have much in common with that of the small intestine and there is here like in the small intestine no sign of any direct parasympathetic influence on either blood flow or secretion.

The extrinsic nervous control of motility blood flow and secretion in the distal part of the colon is on the other hand in important respects arranged in a very similar way as in the stomach. Thus both organs have a reservoir function, where the nervous control of the stomach is specialized for rapid reception and storage and that of the colon for storage and prompt expulsion In each organ a specific set of parasympathetic fibres appears to cause an appropriate motility adjustment—relaxation (vagal relaxatory fibres) and powerful contraction (pelvic fibres) respectively—which is closely linked to secretion and enhanced mucosal blood supply

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Storage of Histamine in Mast Cells
Evidence for an Ionic Binding of Histamine to Protein Carboxyls in the Granule Heparin-Protein Complex

By

BÖRJE UVNÄS, CARL HUGO ÅBORG and ANDERS BERGENDORFF

STOCKHOLM 1970

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1969

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The basophil granules of rat peritoneal mast cells contain two main constituents, heparin and protein. In histamine-free granules these two constituents account for over 95 per cent of the granule dry weight other constituents such as ATP lipids, phospholipids etc. amounting to only a few per cent (Lagunoff *et al.* 1964) The mast cell granules are insoluble in water and in physiological saline but dissolve in 1—2 M KCl solution, and at pHs above 10—11 (Lagunoff 1965 Thon and Uvnäs 1966) Protein and heparin can be separated from the resulting solution by column chromatography When the protein and heparin fractions are recombined in water a precipitate is formed (unpublished observation)

The heparin of rat mast cells shows similar characteristics to commercial heparin preparations. Its strongly negative charge is due to the presence of ester sulphate groups In rat mast cell heparin 3 13 such groups are present per mole of hexosamine (Schiller and Dorfman 1959) The granule protein is composed of basic polypeptides with an I.P around 9 (Lagunoff *et al.* 1964 Bergqvist *et al* unpublished) To all appearances then the matrix of the mast cell granules consists of a water insoluble heparin-protein complex held together by the strong attractive forces between two oppositely charged macromolecules, the acid heparin and the basic protein.

As reported in previous papers, granules isolated from mast cells lysed in distilled water were able to retain histamine as long as they were suspended in water When the granules were suspended in physiological sodium chloride solution the histamine was immediately released (Uvnäs 1964 Uvnäs and Thon 1965) and in exchange, sodium was taken up Aborg, Novotny and Uvnäs 1967) The pH-dependence of the histamine storage mechanism, with its almost complete breakdown already around pH 4 indicated a binding of histamine to weakly acid groups (Thon and Uvnäs 1966 Aborg *et al.* 1967) It remained an open question, however whether these groups belonged to the heparin or to the protein part of the granule complex.

The granule protein is composed of basic polypeptides with a minimal molecular weight around 5 600 (Bergqvist Samuelsson and Uvnäs, to be published) Studies on the histamine binding properties of a water-insoluble complex formed *in vitro* by reaction of a basic polypeptide with a molecular weight around 4 000 (clupein—a protamine) and heparin have recently been reported (Aborg and Uvnäs 1968) Quantitative observations on the uptake by this complex of sodium, histamine and other biogenic amines, as well as titration studies, yielded data not only about the nature and number of the ionic binding sites but also about their location. The histamine taken up by the heparin-clupein complex at pH 7 was apparently exclusively linked to the free terminal COO^--groups of the protamine. The suggested gross structure of the heparin-clupein histamine complex at pH 7 is shown in Fig 1 a.

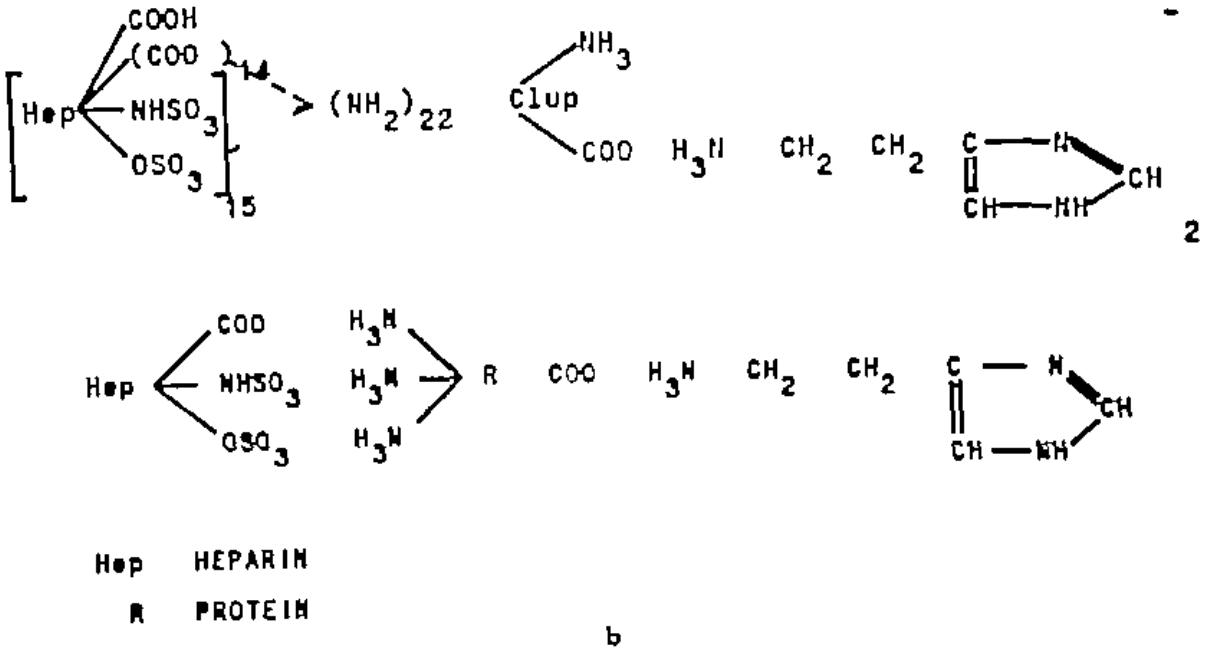


Fig. 1 Assumed gross-structure of
a. heparin-clupein-histamine complex formed *in vitro* at pH 7 (from Åborg and Uvnäs 1968)
b. heparin-protein-histamine complex of mast cell granules at pH

The similarities in physico-chemical properties of the heparin protein complex in the mast cell granules and the heparin-clupein complex formed *in vitro* suggested to us a possible gross structure of the granule heparin protein-histamine complex as shown in Fig. 1 b. According to our hypothesis, in the intact complex at pH 7 the acid groups of heparin should be linked to the amino (imino) groups of the basic protein, leaving only COO^--groups of the protein available for histamine binding.

In the present paper experimental data will be presented to support this hypothesis. In section A of the paper evidence will be given to support the assumption that histamine is stored in the granules in ionic linkage with COO^--groups. In section B data will then be presented to demonstrate the cation binding capacity of the granule ionic sites. Finally in section C titration experiments will show the protein carboxyls to be the most plausible binding sites.

Methods

Isolation of mast cells

Mast cells were isolated from the abdominal and thoracic cavities of male Sprague-Dawley rats weighing 300—400 g as described by Thon and Uvnäs (1967). The slight modification was made that after differential centrifugation the cells were not washed in isotonic sucrose solution.

Isolation of mast cell granules

Mast cells from several—usually 20—rats were pooled, washed in 0.9 % NaCl solution, 2 ml per rat, and centrifuged at 400 × *g* for 10 min. The precipitated cells were suspended in deionized water (2 ml per rat) and the pH was adjusted to 7.1 with 0.1 M NaOH. The suspension was gently shaken for 5 min and the resulting slightly acid medium again carefully adjusted to pH 7.1. The shaking and the adjustment to pH 7.1 were repeated a further twice and the suspension of lysed mast cells was then centrifuged at 400 × *g* for 10 min to precipitate coarse material. The granule-containing supernatant was centrifuged at 3000 × *g* for 20 min. All procedures were performed at room temperature. About mg of granules (dry weight) were obtained. Due to scarcity of granular material most of the uptake and release studies described below are based upon single point determinations.

Note. The suspension fluid was adjusted to pH 7.1 since previous experience had shown this pH to give an optimal yield of granules. These have a strong tendency to adhere to cell membranes, thereby forming aggregates which precipitate with coarser cell material. This tendency is minimized—although not abolished—at pH 7.1.

Preparation of histamine and sodium free granules

Histamine-containing granules obtained from mast cells lysed in deionized water as described above were suspended in 10 mM sodium phosphate buffer pH 7 (2 ml per rat) and gently shaken for 15 min. This procedure results in an almost total release of the granule histamine and an influx of sodium into the granules (Fig. 9). The sodium-charged granules were recovered by centrifugation at 3000 × *g* for 20 min and then suspended in deionized water (2 ml per rat). After gentle shaking they were recentrifuged and resuspended in deionized water. The washing procedures were repeated a further twice and—as checked in experiments with the admixture of ^{22}NaCl—the now sodium- and histamine-free granules (Fig. 5) were collected by a final centrifugation.

Depletion with deionized water of sodium or histamine recharged granules

Sodium

Granules were depleted of histamine and sodium as described above. About 2 mg of such granules suspended in 2 ml of deionized water were recharged with sodium by suspension in 10 ml of 30 mM sodium phosphate buffer pH 7 containing ^{22}NaCl. After incubation with gentle stirring for 15 min, 1 ml aliquots were taken and centrifuged at 3000 × *g* for 20 min. The supernatants were sucked off the granules dissolved in 0.1 M NaOH and the radioactivity of the resulting solution recorded in one of the samples. To each of the remaining samples 1 ml of deionized water was added. The suspensions were gently stirred for 5 min and, after centrifugation, the radioactivity of the precipitate was again measured in one of the samples. The washing procedure was repeated 3—4 times.

Out of the 0.1 M NaOH solution of the granules used for radioactivity measurements 0.4 ml from each sample was taken for the determination of granule protein (in pepsin units). The protein values were needed to follow the losses of granules during the resuspension procedures (about 10 per cent loss per procedure) and to allow reliable calculations of the sodium content of the remaining granules.

Histamine

The procedures were the same as described bov for sod um cept for a few details. The granules were first depleted of histamine by sodium exchange nd then of sodium by repeated washing in deionized water. Such histamine and sodium-depleted gr nules were incubated in 0.5 mM histamine solution pH 7 (a mixture of histamine base and histamine dihydrochloride) containing ^{3} C-histamine. The histamine refilled granules were the washed repeatedly as described abov for the sodium harged granules.

In other experiments, about 1 mg of histamine- and sodium-depleted granules were incubated i 15 ml of 1 mM histamine solution ntaining 75 μCi ^{3} C-histamine with gentle stirring for 15 ml at room temperature w th ontinuou adjustment of the pH to 7 with 1 mM Hi-base. After centrifugation and resuspen ion in 15 ml of deionized water the granules were spun down and resuspended in 0.5 ml of wh wat To 10 μl of the homogeneous suspension (in cut hematocrit tubes) was dded 10 30 0 150 μl of deionized water. After 10 min incubation followed by centrifugation t 3000 g 10 μl of the supernatant was added to 15 ml f scintillation fluid ontaining 0 d of 1 M NaOH. As controls tubes containing 10 μl of granule suspension 15 ml of sci t ll t fl id and 0.2 ml of 1 M N OH were used.

Depletion with deioni d u at f dium or histamine-charged Amberlite IRC 50

Amberlite IRC 50 in sodium form was converted i t hydrogen form with M HCl, washed in deionized water until consta t pH (bout 6.2) and dried t tac over P_2O_5. Before use the dried material was educed to a f ne powder order t facilitate the preparation of homogeneous suspensions.

Amberlit IRC 50 s stated t ha maximal content of COO -groups of about 10,000 mueq/mg (t pH 10—12). The mast cell granules hav cation binding pacity at pH 7 of about 1000 mueq/mg (p. 20 p 1). To facilitat comparison of the Amberl te and the granule studies 0.2 mg of the resin and mg of the granule material were used in each experiment.

0.2 mg of Amberlit was suspended ther in 1 ml of 30 mM sodium phosphat buffer pH 7 containing ^{22}N Cl r in 0.5 mM histamine solution (histamine base + histamine di hydrochloride t pH 7) containing ^{14}C-histamine. The suspensions were then gently stirred for 15 min, divided into 1 ml aliquots, centrifuged t 3000 × g for 20 min and the radioactivity in the supernatant of one of the samples was determined. The other samples were sequentially washed and rewashed as described above for gra ule material.

Influence of pH on the granule histamine store

Granules recharged with histamine in vitro

About 2.5 mg of granules (0.1 ml of suspension) depleted of histamine and sodium were recharged with histamine by incubation with gentle stirring for 10 min at room temperatu and pH 7 in 15 ml f 10 1.0 or 0.1 mM histamine solution, containing ^{14}C-histamine. In these concentrations of histamine the granule histamine stores will be filled t about 95 %, 75 % and 50 % respectively of the maximal ptake at pH 7 (around 1000 mueq/mg, see Fig. 7).

After one wash in 15 ml of deionized water the granules were resuspended in 30 ml of water and the suspension was continuously acidified d ring gentle stirring with 5 M HCl. The increase in volume was negligible. At desired pHs, 2 ml aliquots of the suspension were removed for centrifugation and 1 ml of the resulting supernatant + 0.1 ml 1 M N OH was used for recording of C-activity.

As a control 1 ml f solution containing 1 ml f granule suspension (out of the 30 ml mentioned above) 1 ml of 0.1 M NaOH (which dissolves the granules) and 0.1 ml of water were used.

Influence of pH on the uptake of histamine and sodium by granules

Histamine

A 0 1 M sol tion of histamine base was gradually acidified with conc H_3PO_4 (Agla micro syringe) At desired pHs, 2 ml samples were taken and 100 µl granule suspension (0.. mg granules) added. After Incubation with gentle shaking for 10 min t room temperature the granules were spun down to enable thei ^{14}C-activity to be recorded.

Sodium

A fresh buffer solution was made by dissolving 70 mg of boric acid and 15 mg of citric acid in 60 ml of 10 mM NaCl sol tion containing $^{22}NaCl$. The pH was adjusted t 9 with 10 mM N OH and then gradually acidified with 5 M HCl (Agla micro syringe) At desired pHs 2 ml samples of the buffer solution were pipetted into plastic tubes and 0.2 mg of granules (0.1 ml) added. After Incubation with gentle shaking for 10 min at room temperature the granules were precipitated by centrifugation and the ^{22}Na acti ity of the precipitate recorded.

Uptake studies

For each experiment about 2 mg of histamine and sodium-free granules (dry weight) was suspended in 2 ml of deionized water and gently shaken to secure a homogenous suspension. About half of the granule suspension was used for uptake and release studies, the other half for determining its granul content (dry weight) in order to allow uptake and release figures to be related to granule weight. An exactly measured vol me (between 500—1000 µl) was evaporated to dryness in *vacuo* over P_2O_5 and the residue weighed. In some experiments such dried granules were resuspended nd used for further experimentation, since dried granules were found to retain thei cation storing qualities unchanged (Fig. 12)

Sodium

A fresh sodium phosphat buffer was made by titrating 100 mM NaOH with conc. H_3PO_4 to pH 7 This stock solution was used for preparing 30 10 3 1 0.1 0 03 and 0.01 mM dil tions. To 9 ml samples of each of these dilutions was added 0 1 ml of ^{22}N Cl sol tion. 2 ml samples f the ^{22}N Cl-containing sodium phosphate buffer solutions were pipetted into weighed polypropylene tubes with plastic stoppers. T each tube was added 100 µl of granul suspension. After 10 minutes' incubation at room temperature the granules were sedimented by centrifugation t 5000 × *g* for 20 min. The supernatant was carefully sucked off except for 1—2 drops which were left in the tube. The pH of the supernatant was determined. The tube was reweighed to allow calculation of the volume of the remaining fluid and thereby calcula tion of th contaminating radioactivity The granul precipitate was dissolved in 0.5 ml of 1 M N OH. 200 µl of the granule solution was then added to 15 ml of scintillation fluid and the ^{22}Na activity recorded.

For each granule sample control sample was made up. 1—. drops of the corresponding $^{22}NaCl$-containing sodium phosphate buffer solution were pipetted into weighed polypropylene tube. After reweighing to determine the volume, 0.5 ml 1 M NaOH was added, the tube reweighed and 200 µl taken for recording ^{22}Na activity (in 15 ml of scintillation fluid) The values obtained fo the ^{22}Na activity per µl of the control samples were used for calculating the uptake of sodium in the granules (in nmoles per mg granules)

Histamine

For determining histamine uptake the granules were incubated in ^{1} C-histamine-containing media and the samples treated as described above for sodium uptake.

Release studies

Histamine- and sodium-free granules were recharged with histamine containing ^{14}C-histamine as described above. After precipitation at 3000 × *g* 2 ml of 10 mM sodium phosphate solution pH 7 was added to each sample and the granules spun down again. 1 ml of the supernatants were removed for recording the ^{14}C-activity and for calculating the sodium induced release of histamine. Similarly by using ^{22}NaCl instead of ^{14}C-histamine the uptake of sodium was calculated.

Incorporation of ^{14}C histamine into mast cell granules

Mast cells from 10 rats were incubated for 2 hours at 37° C in 15 ml of isotonic salt solution containing 10 % v/v Sörensen phosphate buffer pH 7.1 mg human serum albumin per ml and 10 µl of ^{14}C-histidine (Slorach and Uvnäs 1968).

Radioactivity measurements were made with a Packard Tri-Carb liquid scintillation spectrometer model 2002. The liquid scintillation fluid contained 0.4 % 2,5-diphenyl oxazole and 0.01 % 1.4-bis-2(4 methyl-5-phenyloxazol) benzene in a mixture of equal volumes of toluene and ethylene glycol monoethyl ether.

The amounts of radioactive material added in the uptake and release studies were ^{22}Na about 1 µCi per ml, ^{14}C-histamine about 0.1 µCi per ml.

Titration of mast cell granules

Mast cell granules (from 30 rats) depleted of histamine and sodium (see p. 5) were suspended in about 2 ml of deionized water. Using a Carlsberg pipette 1.00 ml was transferred to a weighing bottle dried over P_2O_5 in vacuo and weighed. 1.00 ml was transferred to a titration vessel and during gentle stirring exposed to a stream of nitrogen gas for 5—10 min until the pH was constant (pH 5). The acidity is probably due to the fact that in washed granules the cationic sites are occupied by hydrogen ions. The pH was adjusted to 3 with 0.1 M HCl. Titration from pH 3 to pH 10 was then performed with 0.1 mM KOH (or histamine base) adding 1 µl between each pH determination. For titration of dissolved granules 75 mg of KCl was added together with 1 ml 1 M KCl solution. The granule heparin went into solution and the pH shifted to 9.2. Titration to pH 3 was then performed with 0.1 M HCl.

For the blank titration 1.00 ml of deionized water was exposed to a stream of nitrogen gas until the pH was constant (pH 6). Titration was performed to pH 3 with 0.1 M HCl and then to pH 10 with 0.1 M KOH. The blank values were deducted from the titration values of the granule suspension (Kerschington 1960).

For all titrations and pH adjustments an Agla micrometer syringe was used.

Protein assay

Protein was assayed by the method of Lowry *et al.* (1951) and the results are expressed in terms of the crystalline pepsin standard used.

Materials

Histamine dihydrochloride Sp. act. 30.5 mCi/mM. The Radiochemical Centre Amersham, England.
^{22}NaCl. 1 mCi/mg. Phillips—Duphar Holland.
Na^{36}Cl. 565 µCi/g Cl. The Radiochemical Centre Amersham, England.
Ficoll AB Pharmacia, Uppsala, Sweden.
Human serum albumin (free from preservatives) AB Kabi, Stockholm, Sweden.

H paris (B.P 63) Activity 138. I U/mg, calculated as the dried base AB Vitrum, Stockholm Sweden.

Lysozyme from egg white grade I 3× crystallized, dialyzed nd lyophylized Sigma Chem. Co., St. Louis, U.S.A.

Isotonic salt solutio $1,5 \times 10^{-1}$ M N Cl 2.7 10^{-6} M KCl; 10^{-3} M $CaCl_2$.

For tri ations were as d

Titration assembly type TTA 31 Radiometer Copenhagen, Denmark.

Agla micrometer syringe Shaedlow micrometers Ltd. Sheffield, England.

Results

A. Qualitative observations indicating the binding of histamine to COO -groups in mast cell granules

Influence of pH on the granule histamine stores

If histamine was stored in the granules in ionic linkage with COO -groups the storage capacity of the granules should vary with the degree of dissociation of these groups. We therefore studied the influence of pH on the histamine content of granules.

Histamine and sodium depleted granules were recharged with histamine to about 95 % 75 % and 50 % of their maximal uptake capacity at pH 7 by incubation in 10 1 0 and 0 1 mM histamine solution respectively as described on p. 6. Irrespective of the degree of saturation the histamine stores showed the same pH dependence. The content steeply fell when the pH was reduced from 6 to 4 (Fig 2)

In order to meet the argument that granule histamine stores recharged with histamine *in vitro* should behave differently from granule histamine stores containing endogenous histamine the influence of pH was also studied on granules isolated from mast cells containing ^{4}C-histamine. This histamine had been endogenously formed during incubation of mast cells in a ^{14}C-histidine containing medium according to Slorach and Uvnäs (1968) and the granules were isolated by water lysis of such cells as described at p 5 The granules were then resuspended in 30 ml of water and the suspension was gradually acidified with HCl as described above for the *in vitro* recharged granules. The store of endogenously formed histamine showed the same pH dependence as observed for exogenous histamine (Fig 2)

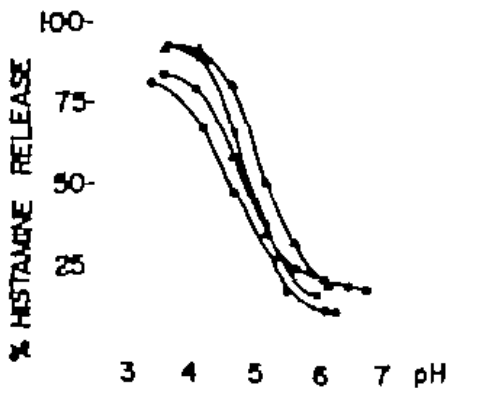


Fig. 2. Influence of pH on granule histamine storage. Granules recharged with histamine by incubating them in 0.1 mM (×—×) 1 mM (▲—▲) and 10 mM (●—●) histamine solutions. Granules from mast cells charged with C-histamine by incubation of the cells with C histidine (○—○).

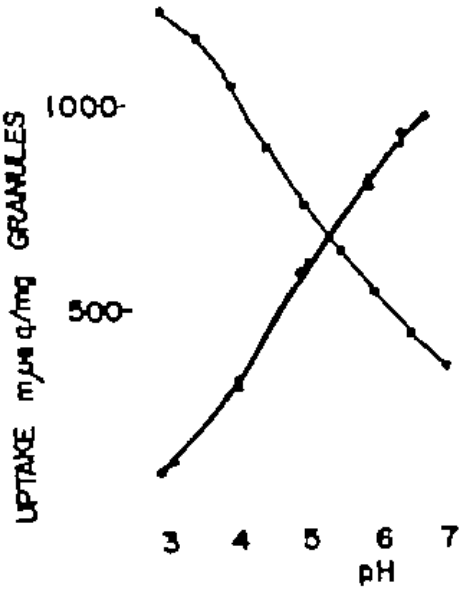


Fig. 3

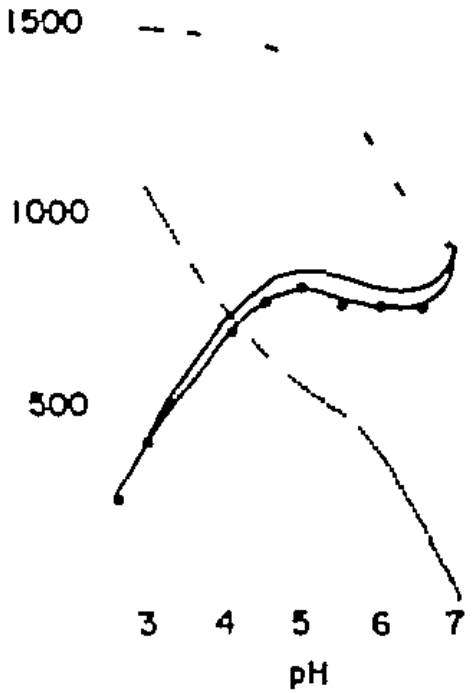


Fig. 4

Fig. 3 Influence of pH on uptake of Na ions and H ions in sodium and histamine depleted granules.
—●—●—● Uptake of Na ions
■—■—■— Uptake of H ions (obtained from the titration values in Fig. 10)
▲—▲—▲— Reversed curve for uptake of H ions. For discussion see p. 13

Fig. 4 Influence of pH on the uptake of histamine in sodium and histamine depleted granules. Histamine conc. 10 mM.
—○—○—○ Histamine uptake (mμeq/mg) curve observed.
———— Histamine uptake (mμeq/mg) curve constructed from
– – – – – Histamine dissociation curve (titration with HCl) and COO⁻-dissociation curve (titration with H-base)

Influence of pH on the uptake of histamine and sodium by granules in vitro

The ionization of histamine varies with pH ($pK_{a_1} = 5.9$ $pK_{a_2} = \sim 10$). At pH 7 histamine is monobasic but the ionization increases rapidly below neutrality and it becomes dibasic at pH 4—3. (See titration curve in Åborg *et al.* 1967 Fig. 6.) Consequently the affinity of histamine for cationic binding sites will increase with falling pH. On the other hand the ionization of the carboxyl groups in the granules will be reduced with increasing acidity thereby diminishing the histamine binding capacity of the granules. Consequently the histamine uptake by the granules should be expected to show a pH-dependence curve reflecting the influence of two oppositely changing dissociation curves — a falling dissociation of carboxyl groups and an increasing dissociation of amino groups.

On the other hand a monovalent cation *e.g.* sodium ion, should be expected to run an uncomplicated course, simply reflecting the dissociation of the carboxyl groups

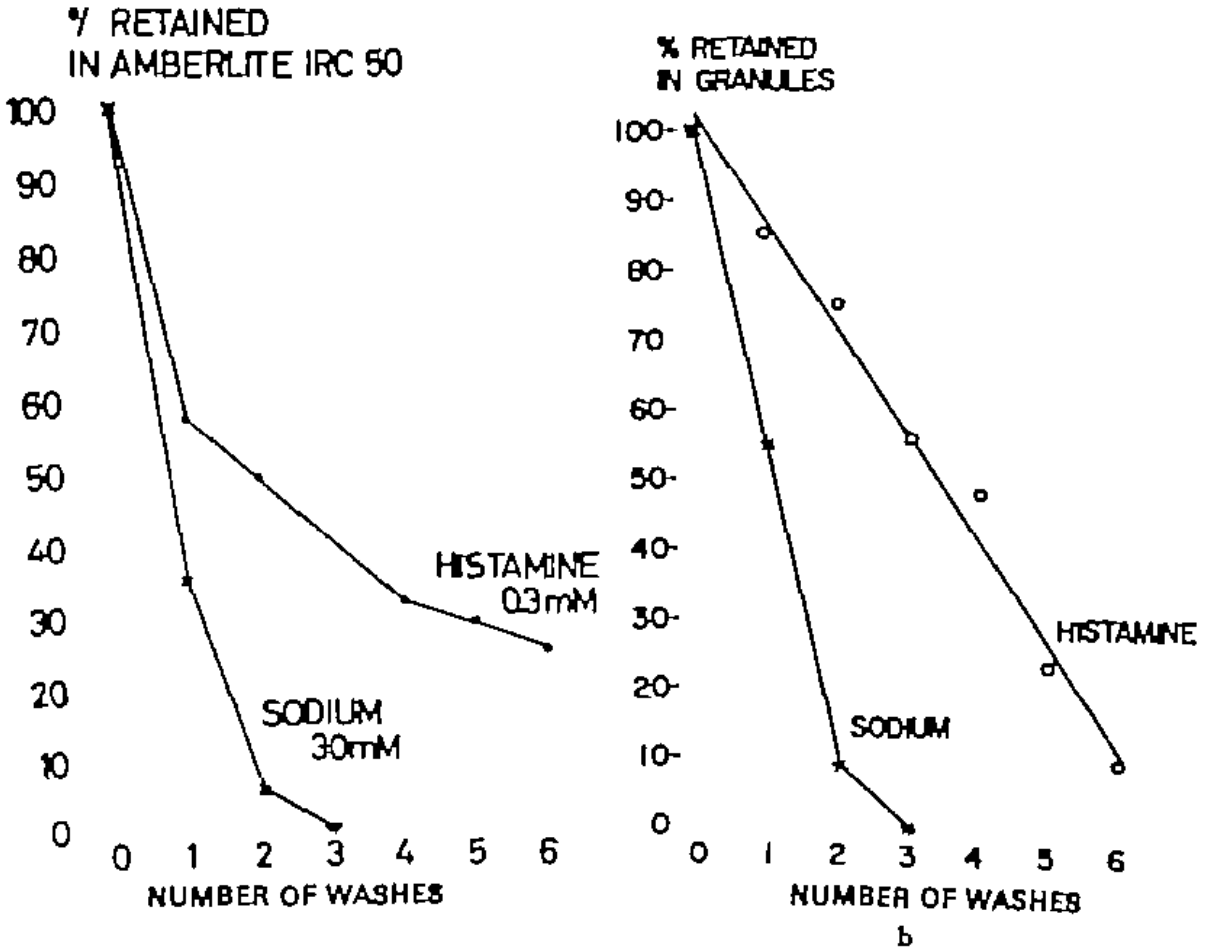


Fig. 5 Depletory action of washing) Amberlite IRC 50 and b) mast cell granules in deionized water on thei contents of
sodium (×—×—×) and
histamine (o—o—o)

The materials were charged by suspension f 30 mM sodium and 0.3 mM histamine solutions respectively

in the granules. It should form the mirror image of the carboxyl titration curve. The sodium uptake curve was in fact found to fulfill the criteria. It runs an uncomplicated course and when reversed the carboxyl titration curve followed the sodium uptake curve very closely (Fig. 3) In contrast, the histamine uptake showed a more complicated two phase course (Fig 4) In Fig 4 is also plotted the dissociation curve for the secondary amino group of histamine (the curve is constructed from the titration values given by Aborg and Uvnäs 1967 with its origin at pH 7) In the same figure are plotted the values obtained on titration of granules with histamine base By deducting the granule titration values from the histamine dissociation curve values a histamine uptake curve was constructed. This constructed curve followed fairly well the found uptake curve, supporting the assumption that the complexity of the pH dependence curve for the histamine uptake into granules is due to the increasing protonization below neutrality of the secondary amino group of histamine

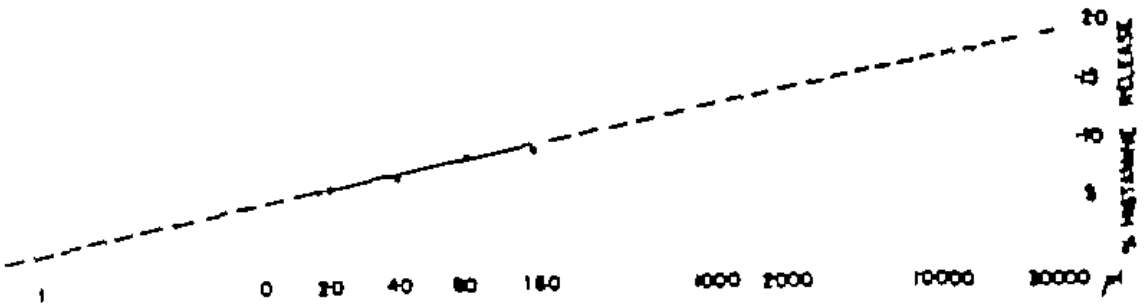


Fig. 6. Influence of suspension volume (deionized water) on the ratio between histamine retained in the granules and histamine released to the suspension medium (ratio "bound" histamine/"free" histamine) × indicates values obtained from the "wash experiment" plotted in Fig. 5 b.

the increasing affinity of histamine to ionic sites counteracting the effect of the diminishing ionization of carboxyl groups.

Depletory effect of deionized water on granules and on Amberlite IRC 50 charged with sodium or histamine

The assumption of the mast cell histamine being stored in ionic linkage with COO groups and being exchangeable against other cations e.g. sodium ions means that the mast cell granules should have the properties of a weak cation exchange material. Some experiments were therefore made to compare the cation storage abilities of mast cell granules and the carboxyl ion exchange resin Amberlite IRC 50.

At pH 7 the granules took up about 1000 the Amberlite about 2000 mμeq of anions/mg dry material (see Fig. 7 respectively Åborg *et al.* 1967 Fig. 5). When washed repeatedly in deionized water as described at p. 5—6 the sodium stores were completely emptied in both materials within 2—3 washes (Fig. 5). The histamine stores were more difficult to deplete. After 6 washes 10 % was still retained in the granules and about 25 % in the Amberlite.

An ionic binding of histamine to granule sites will be influenced by the histamine concentration in the granule suspension medium. With increasing suspension volume i.e. diminishing extragranular histamine concentration the amine should be released from the granules. Fig. 6 shows a linear correlation between the suspension volume (deionized water) and the percentage release of granule histamine.

Discussion

The influence of pH on the granule storage of exogenous as well as of endogenous histamine suggests a binding of the histamine to weakly acid groups, which become protonized and thus increasingly unavailable for binding with falling pH. The dissociation curve of protein carboxyls comprises the pH range 6.5—2 (Kenchington

1960) a fact which makes such groups the probable candidates for the binding sites. It is true that since at pH 3.5 10—20 per cent of the histamine has still not been released from the granules (Fig 2) the results do not exclude the possibility that below this pH part of the granule histamine is linked to more acid groups, *i.e* sulphate groups of the heparin. However it should be emphasized that COO groups cannot be expected to be completely undissociated until the pH approaches 2.

Furthermore, with the technique used granule histamine released with falling pH will accumulate in the incubation fluid and this histamine will compete with the hydrogen ions for granule ionic sites. For example when completely depleted of their histamine those granules which have been loaded by suspension in 10 mM histamine solution will have released into the suspension fluid at pH 3.5 an amount of histamine sufficient to give a concentration there approaching 0.1 mM (Fig 2) The accumulation of released histamine in the suspension medium therefore prevents the complete depletion of the granule histamine stores. The fact that the released histamine at pH 3.5 will be divalent with increased affinity for cationic binding sites further counteracts the depletory actions of the hydrogen ions.

The pH curve for the sodium uptake forming below pH 7 the mirror image of the carboxyl titration curve, is in complete agreement with an ionic binding of sodium ions to granule carboxyl groups, so is the pH curve for the uptake of histamine, due regard being taken to the increasing valency of histamine below neutrality

Our deionized water was slightly acid—around pH 6.0—since no precautions were taken to prevent CO_2 uptake from the air The depletion of the sodium and histamine stores of the granules on washing with this slightly acid water is well in accordance with the assumption of an ionic linkage of sodium and histamine to weakly acid groups in the granule heparin-protein complex. The more rapid depletion of sodium than of histamine from the granule stores indicate a weaker affinity of sodium than of histamine for the granule ionic sites.

The ratio between granule-bound histamine and supernatant histamine varied with the suspension volume as should be expected if histamine was ionically bound to weakly acid groups in the granules. The slope of the curve indicates that at infinitesimal volumes, as can be expected to occur intracellularly and especially intragranularly in the mast cell, free histamine is likely to exist in only minute amounts. This observation has a bearing on the question whether or not "free" histamine occurs in mast cells (see Discussion p 25)

The similarities in the behaviour of the granule and Amberlite sodium and histamine stores on exposure to deionized water are also striking

In conclusion The influence of pH on the storage of histamine in mast cell granules *in vitro* points to COO^--groups of the heparin-protein complex as the cationic binding sites.

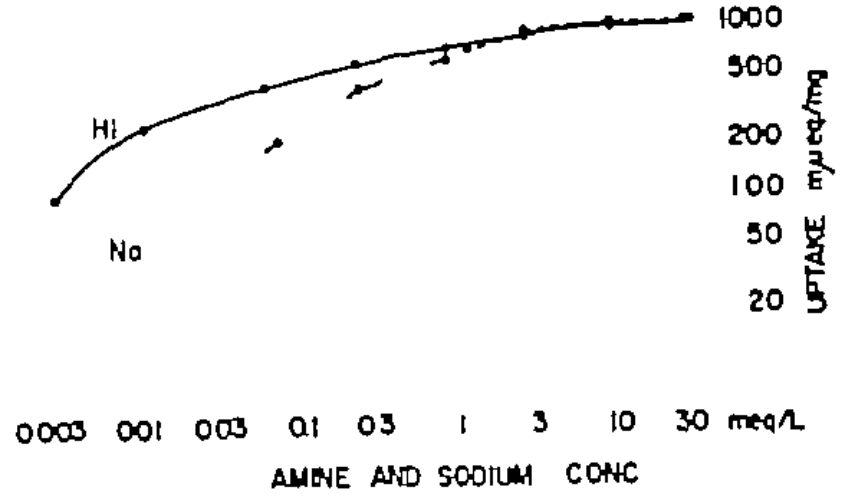


Fig. 7 Uptake of histamine and sodium in rat cell granules suspended in sodium and histamine solutions of various concentrations (pH and at room temperature. Corrections were made for concentration reductions due to granule uptake of histamine and sodium.

B Quantitative aspects of the uptake and release of cations by granules in vitro

Uptake at pH of histamine and sodium in granules (Fig 7)

Histamine and sodium-free granules were resuspended in histamine- or sodium-containing solutions with concentrations ranging from 0.03 to 30 mM. There was an appreciable uptake (100 mμeq/mg) already at the lowest histamine concentration used (0.003 mM) while the sodium uptake was still only around 30 mμeq/mg in 0.01 mM sodium concentration. Both uptake curves rose with increasing extragranular histamine and sodium concentrations to merge and level off at an uptake of 1000 mμeq/mg in 10—30 mM solutions. Uptake studies in histamine or sodium concentrations above 30 mM became unreliable since the granules began to dissolve at such concentrations.

Competitive uptake of histamine and sodium at pH 7

Histamine and sodium-free granules were incubated in media with a constant histamine concentration of 10 mM but with sodium concentrations ranging from 5—30 mM. To measure the uptake of sodium and histamine two parallel experimental series were performed one with the admixture of ^{22}NaCl, another with the admixture of ^{14}C-histamine.

The uptake of histamine fell and the uptake of sodium rose with increasing sodium concentration in the incubation medium (Fig. 8). Irrespective of the mutual concentrations of histamine and sodium the sum of the corresponding uptake values for histamine and sodium approached the same level as that observed with histamine

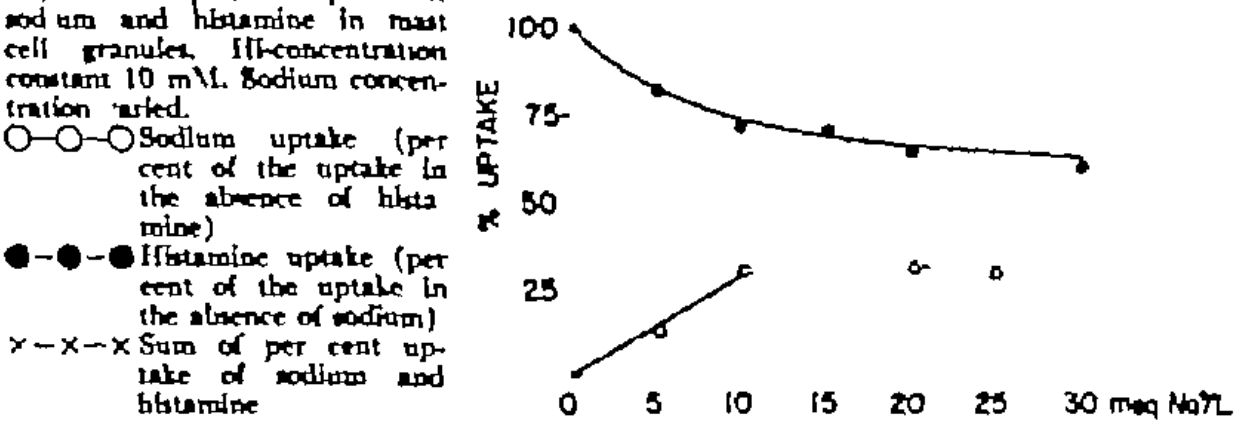


Fig. 8. Competitive uptake of sodium and histamine in mast cell granules. Hi-concentration constant 10 mM. Sodium concentration varied.
○–○–○ Sodium uptake (per cent of the uptake in the absence of histamine)
●–●–● Histamine uptake (per cent of the uptake in the absence of sodium)
×–×–× Sum of per cent uptake of sodium and histamine

alone in the incubation medium. Observations with sodium concentrations above 25—30 mM were not possible since the granules began to dissolve at such high concentrations.

Molar exchange ratio between Na uptake and Hi release at pH 7

As reported previously (Åborg *et al* 1967) and as illustrated in Fig. 9 a sodium induced release of histamine occurs with a molar ratio of sodium uptake/histamine release around 2 or higher

On the assumption that the sodium induced release of histamine was the result of a competition for identical ionic sites in the granules between sodium and histamine one might expect the molar exchange ratio Na /Hi to be 1 The higher exchange ratios found by Åborg *et al* therefore deserved further investigation.

The susceptibility of the granule histamine storage mechanism to washing in water as shown in Fig 3 suggested to us that the unexpectedly high molar exchange ratio was due to a partial desaturation of the granule histamine binding sites caused by the repeated washing of the granules during their isolation. Experiments were therefore designed to recharge granules with histamine to various degrees of saturation.

Two series of experiments were made In one series histamine and sodium-free granules were incubated in histamine solutions of concentrations ranging between 0 1—10 mM containing ^{14}C-histamine. These histamine recharged granules, designed to be 25—100 % saturated with histamine (see Fig. 9 a) were then suspended in 10 mM sodium chloride and the sodium induced release of histamine calculated from the ^{14}C-activity appearing in the supernatant as described under Methods p 8

In a parallel series granule samples were incubated in the same concentrations of histamine as above but no labelled histamine was added. Instead the histamine-containing granules were exposed to 10 mM sodium phosphate buffer containing ^{22}Na Cl After centrifugation the uptake of sodium was calculated from the ^{22}Na activity of the various precipitates.

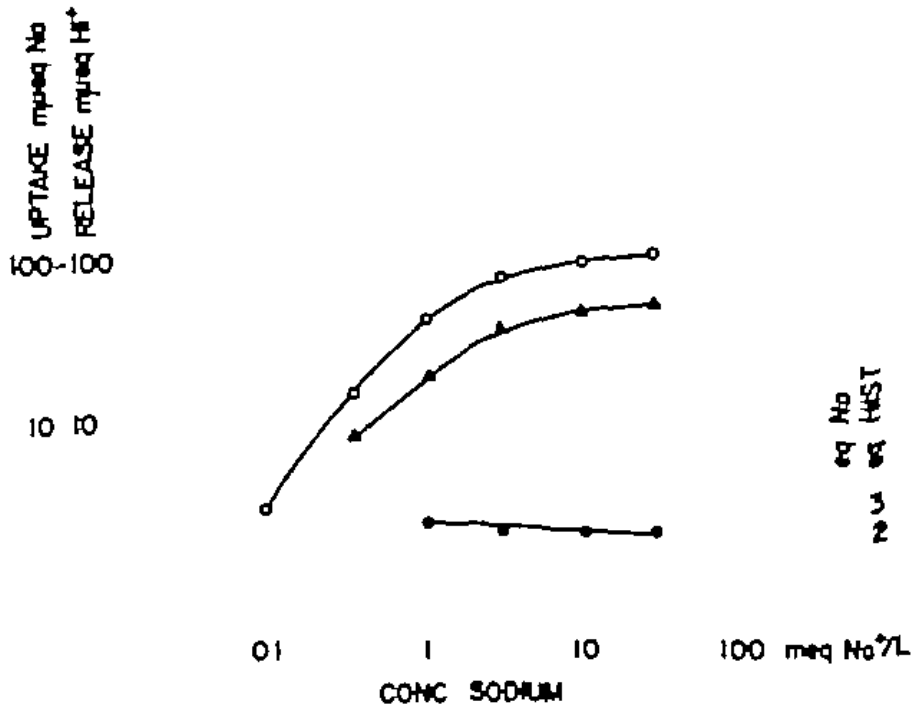


Fig 9 a) Uptake of sodium and release of histamine in granules isolated from water-lysed mast cells and suspended in sodium phosphate buffers of various concentrations. ○—○—○ uptake —○—○—○ H release —△—△—△ Not the ratio Na uptake / H release between 2—3 ●—●—●
(The figure taken from Åborg et al 1967)

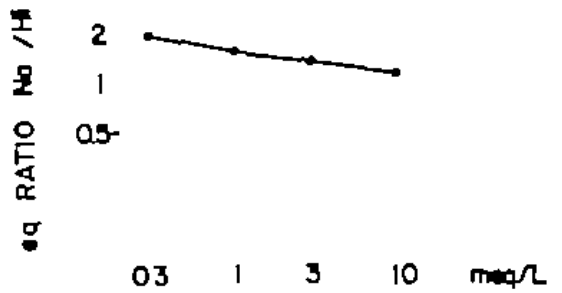


Fig 9 b. The influence of granule histamine content (degree of saturation of histamine binding ionic sites) on the ratio Na uptake/ H release The higher the saturation of histamine stores the closer is the Na /H ratio to 1

The results showed that when the granules were incubated in high concentrations of histamine (10 mM) the molar exchange ratio Na /H approached 1 With declining histamine concentrations in the incubation medium, *i.e.* with expected incomplete saturation of granule ionic sites with histamine the exchange ratio Na /H increased, approaching 2 at the lowest histamine concentrations used

Uptake of Cl ions

0 135 mg of histamine- and sodium-depleted granules were suspended in 2 ml of 0.9 4.5 9 and 20 mM NaCl solutions containing $Na^{36}Cl$ No significant uptake of ^{36}Cl was observed.

Discussion

Equal maximal uptake by the granules of two cations is to be expected if these are linked to identical ionic sites. The confluence of the histamine and sodium uptake curves at the same maximal level of 1000 m/eq/mg (Fig 7) therefore supports the assumption that histamine and sodium are located at identical granule storage sites.

The fact that when histamine and sodium are simultaneously present in the incubation medium the granule uptake of the two cations added together approaches the same level irrespective of the mutual concentrations of the two ions (vide Fig 8) is also consistent with their binding to identical sites.

The relative courses of the uptake curves in Fig 7 indicate a lower affinity of sodium than histamine for these sites. However quantitative evaluation of the relative affinities of the two ions—histamine and sodium—for the granule sites is complicated by the slight acidification of the granule suspensions that occurs at low concentrations of these ions. This acidification is due to the low buffering capacity of the histamine and sodium containing solutions which allows the acidity of the deionized water and the depleted granules to break through. As is evident from Fig 2 even such a slight acidification as to pH 6.5—6 will noticeably reduce the granule storage capacity for sodium and histamine *i.e.* the sodium and histamine ions have to compete with the hydrogen ions for the same granule ionic sites. This pH influence will increase with decreasing extragranular sodium and histamine concentrations and will be most evident for the sodium ion which has the weaker affinity to the granule sites.

The experiments illustrated in Fig 9 show that the Na /Hi ratio increases with decreasing histamine concentrations in the granule suspension medium and consequent increasing unsaturation of the granule histamine binding sites. This supports our assumption that the molar exchange ratios Na /Hi above 1 as seen in our experiments reflect the degree of depletion of the granule histamine stores caused by washing the granules in deionized water during the isolation procedure.

1 2 and 3 washes reduced the histamine content of "histamine refilled" granules to about 85 75 and 55 per cent respectively (Fig 5) of the starting level. Similar effects were observed on the endogenous histamine stores of granules isolated from water lysed mast cells (Thon and Uvnäs 1966) Since the granules used in our experiments were routinely washed at least 2—3 times in deionized water a considerable proportion of the histamine binding sites in our granules probably were depleted of histamine and the sites occupied by H ions. On suspension of such granules in a sodium containing medium Na ions would exchange with both Hi and H ions at the granule sites. The more often the granules were washed, the more of the histamine should be expected to be replaced by hydrogen ions, and consequently the higher should be the molar exchange ratio Na /H In Fig 5 it can be seen that three washes deplete the saturated granules of about 50 per cent of their histamine. A molar exchange ratio around 2 should be expected which is in fact the approximate value found in most of our experiments. A similar explanation may

be given for the increase of the Na /H1 exchange ratio in low sodium concentrations as seen in Fig 9 The insufficient capacity of the sodium phosphate buffer allows the pH of the suspension to approach 5.5—6. Histamine is released and the granule ionic sites become increasingly occupied by H ions. Consequently the exchange ratio Na /H1 increases.

The values of the exchange ratio above 1 therefore are considered to be experimental artefacts not invalidating our hypothesis of the histamine release from the granules as being the result of an equivalent ion exchange between sodium and histamine at identical ionic sites.

Conclusion

The mast cell granules have the properties of a weak cation exchange material with a maximal binding capacity at pH 7 of 1000 mueq/mg

C. Titration experiments on mast cell granules

Attempts were made to determine by titrations the capacity and the localization of the cation binding sites of the mast cell granules. For details of the titration procedures see Methods p 8

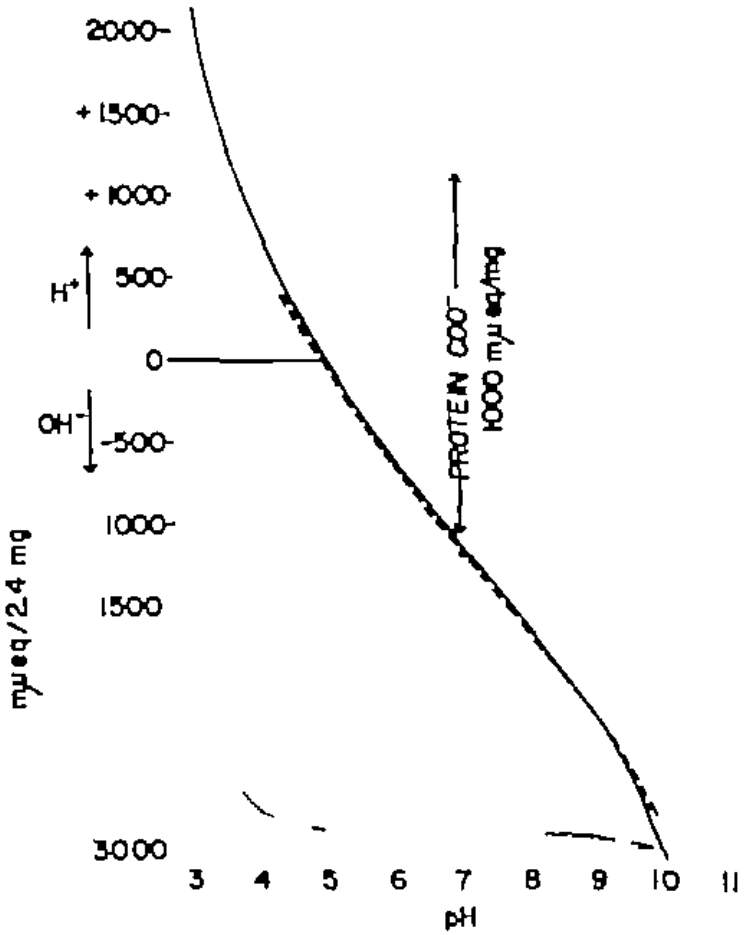


Fig 10 Titration of histamine and sodium-depleted granules suspended deionized water (2.4 mg 1 ml H_2O) with 0 1 M HCl and 0 1 M KOH

—— Total consumption of KOH between pH 3—10 (after adjusting the pH of the granule suspension from 5 t 3 with 0 1 M HCl)

Net consumption of KOH between pH 3—7

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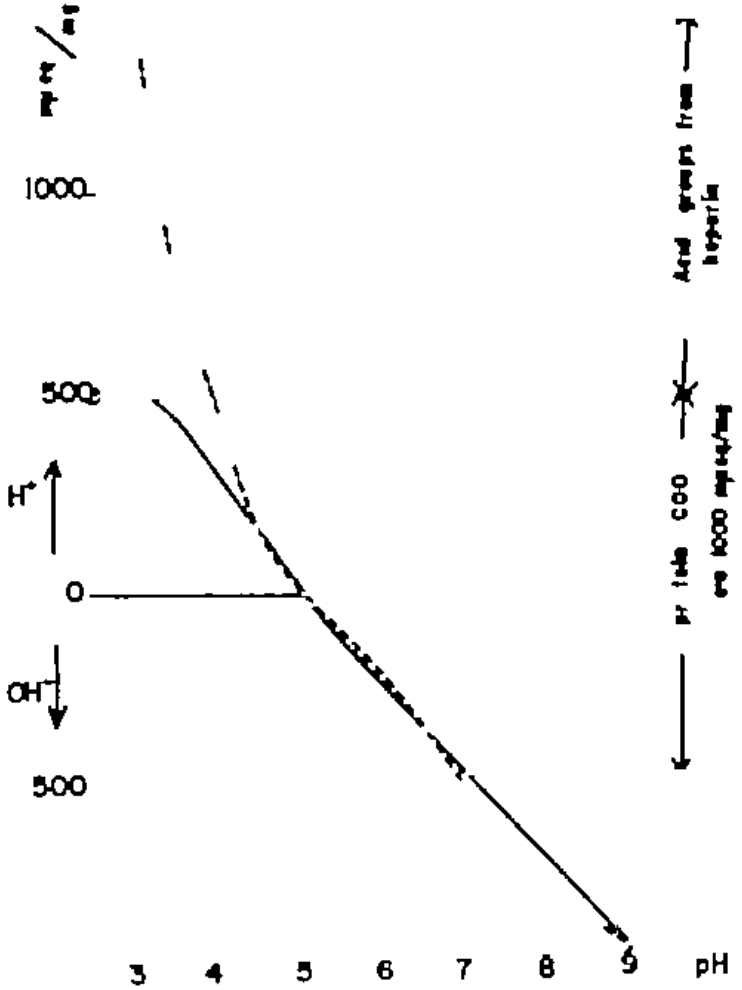


Fig. 11 Titration of histamine- and sodium-depleted granules (2.4 mg) with 0.1 M HCl and 0.1 M KOH.
——— Net consumption between pH 3—10 of KOH by granules suspended in deionized water (from Fig. 10)
Gross consumption of HCl between pH 9—3 of granules (from above) dissolved in 1 M KCl.

On titration with 0.1 M KOH a suspension of granules depleted of histamine and sodium was found to consume between pH 3—7 about 1000 mμeq/mg (Fig. 10). After dissolution of the granules in 1 M KCl the consumption of HCl on back titration to pH 3 with 0.1 M HCl was found to have increased considerably (Fig. 11). Drying the granules *in vacuo* over P_2O_5 did not change the titration curve (Fig. 12).

The granule heparin protein complex is composed of about 30 % heparin and 70 % protein wt/wt (Lagunoff *et al.* 1964, Bergendorff *et al.* unpublished data). On the assumption that the heparin of rat mast cell granules is similar in its composition to bovine heparin (Schiller and Dorfman 1959) titration of an equivalent amount of bovine heparin should give an approximate value for the number of acid groups present in rat mast cell heparin. Titrations were made on granules dissolved in 1 M KCl (curve a in Fig. 13). Provided that after dissolution of the granules in 1 M KCl titration with HCl records all the available free acid groups in both the protein and the heparin of the granule complex at pH 7 the deduction from the titration values of the dissolved granules of those of the calculated amount of bovine heparin should give an estimate of the cationic binding capacity of the granule protein at pH 7. This operation gave curve c in Fig. 13 with a calculated consump-

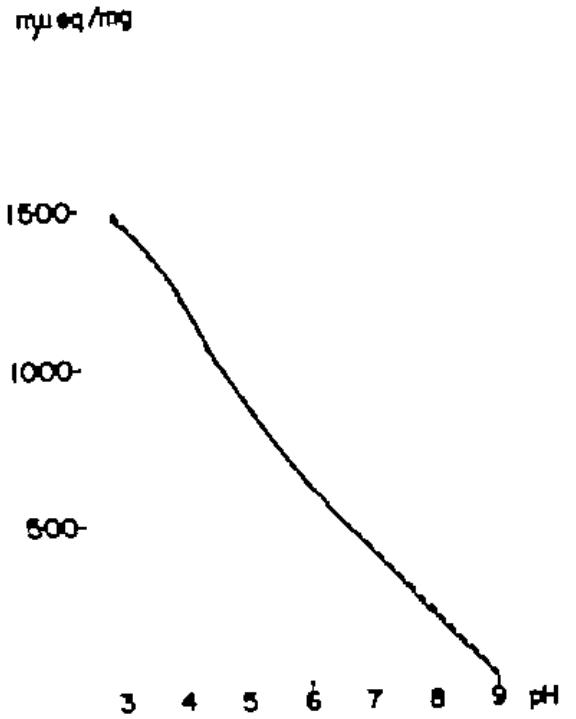


Fig 14. Titration with 0 1 M KOH of —— isolated, isolated and dried granules.

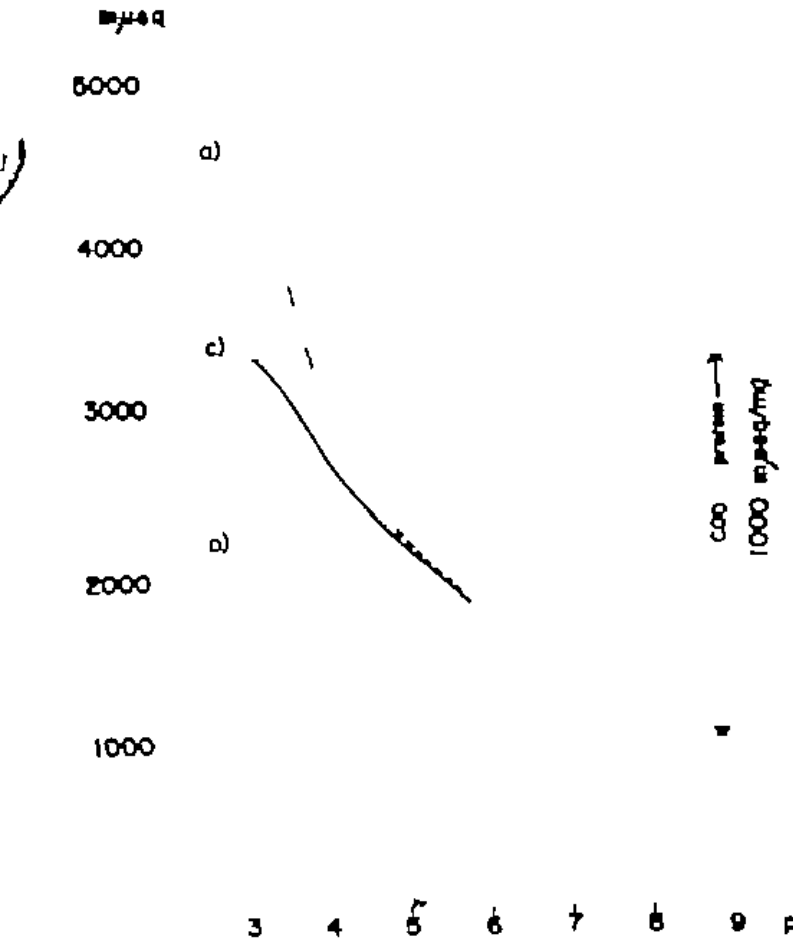


Fig. 15 Titration with 0 1 M HCl of mast cell granules (2 4 mg) dissolved in 1 M KCl (curve) and of sodium heparinate (bovine) (0 73 mg) in 1 M KCl (curve b) Constructed protei carboxyl curve (a—b)

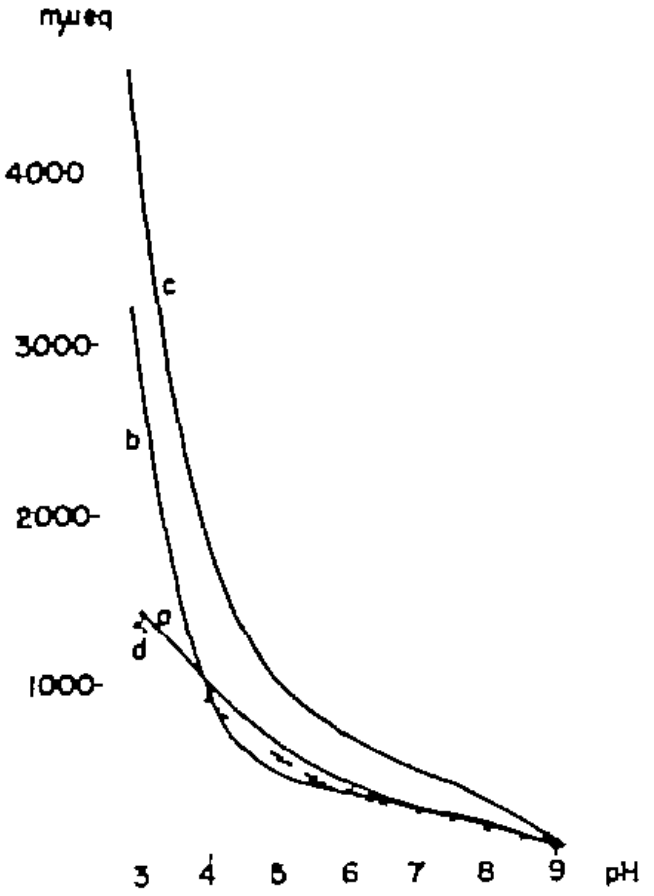


Fig. 14 Titration with 0 1 M KOH and 0 1 M HCl in M KCl of
a) 2 mg lysozyme
b) 2 mg sodium heparinate
) 2 mg lysozyme + mg sodium heparinate
d) constructed lysozyme titration curve (—b)
Curve a corrected according to the formula 1 — antilog (pH$_2$—pH$_1$) (Cohn and Edsall 1943)

tion of the protein between pH 3—7 of about 1000 mμeq/mg granules. This value corresponds well with the value obtained from the titrations on suspended granules (Fig 10)

Lysozyme is an arginine rich basic protein with an i p at 10.5—11 and a mol. wt of 14—17 000 When mixed with heparin in water an insoluble complex forms this complex dissolves in 1 M KCl

Fig. 14 shows titrations of solutions in 1 M KCl of lysozyme (a) sodium heparinate (b) and a mixture of lysozyme and sodium heparinate (c) with 0 1 M HCl. The calculated protein carboxyl curve (d) obtained by deducting the titration values of the heparinate from those of the heparin-lysozyme mixture agreed fairly well with the values obtained by direct titration of the lysozyme. Our calculations above concerning the cationic binding capacity of the heparin-protein complex and its components based on the titrations (Fig 12) therefore to all appearances seem to be valid.

Discussion

The titration studies show that at pH 7 the mast cell granules have a cation binding capacity corresponding to about 1000 mμeq/mg and agree with the assumption that the cation binding sites are located to the protein part of the heparin-protein

complex. Heparin probably does not contribute to any appreciable extent to the cationic binding capacity of the granules at pH 7 since the acid groups of heparin at this pH are occupied with the basic groups of the protein (Fig 1 b)

General discussion

The present observations lend further support to previous proposals from our laboratory that histamine is stored in the mast cell granules in ionic linkage to weakly acid sites in the granule heparin protein complex.

The present and previous experiments are in accordance with the hypothesis that histamine and sodium are linked to identical ionic sites. The uptake capacity at pH 7 of the two ions is the same (1000 mueq/mg granules) and the molar exchange ratio between Na and Hi in histamine-saturated granule stores approaches 1 When present simultaneously these cations compete for ionic sites and the sum of their uptakes is constant irrespective of their relative proportions in the suspension medium (Fig 8) The observed variations in the equivalent exchange ratio Na /Hi is discussed and explained on p 17

An ionic binding of the mast cell histamine in the granules has been proposed earlier by several authors (for references see Thon and Uvnäs 1966) The main new principle of our postulate is the binding of histamine to the protein part of the granule heparin-protein complex—not to heparin as previously assumed—in such a weak linkage that histamine is immediately released when the mast cell granules on mast cell degranulation become exposed to the extracellular ionic milieu.

Previous suggestions as to an ionic binding of histamine to the heparin of the mast cell granules were based on the fact that in *vitro* heparin and histamine form a rather stable complex. In this complex the amino groups of histamine are attached to the acid groups of heparin. Due to the strength of the attractive forces of the sulphated groups of heparin the heparin-histamine complex is stable down to pH 2—3 In contrast as demonstrated in the present and previous studies from our laboratory the granule histamine stores are far more sensitive to acidification. The sensitivity to pH and the resulting incapacity of the granules to store histamine below pH 3 eliminate the sulphated groups of heparin as the main histamine binding sites in the granules In fact as indicated by our titrations, in the granules these groups of the heparin are masked by electrostatic binding to basic groups of the granule protein

The protein of the granule heparin-protein complex consists of basic polypeptides with a minimal molecular weight around 5600 Among the 16 amino acids identified are three basic amino acids—arginine lysine and histidine—and 2 dicarboxylic amino acids—aspartic and glutamic acid (Bergqvist, Samuelsson and Uvnäs to be published)

The granule complex is insoluble in water but dissociates in the presence of cations, *e.g* in 1 M NaCl solution. The two components heparin and protein can be

separated by electrophoresis or by column chromatography On mixing they again form a water insoluble complex (Bergqvist, Samuelsson and Uvnäs to be published)

Åborg and Uvnäs (1968) have recently studied the properties of another water insoluble complex betwen heparin and a basic polypeptide. The complex was able to bind histamine as well as other biogenic amines, and inorganic cations. The histamine was released in the presence of sodium chloride, the release being due to an equivalent ionic exchange between histamine and sodium In fact qualitatively the *in vitro* formed heparin-clupein complex showed the same storage properties as did the mast cell granules.

Titration of the heparin-clupein complex as well as quantitative studies of its sodium and histamine binding capacity showed that the only ionic sites available for cationic binding in this complex at pH 7 were the terminal carboxyls of the clupein. The acid groups of the heparin—the sulphated groups as well as the carboxyls—were all occupied by the basic groups of the clupein. In other words, in the heparin-clupein complex the cationic binding sites were exclusively localized to the carboxyls of the protein part of the heparin-protein complex (Fig 1 a)

If we make the assumption that the histamine binding sites in the granules are carboxyl groups the question remains where these are located. Both the heparin and the protein part of the heparin-protein complex contain such groups. However our lack of knowledge about the exact structures of the heparin and protein composing the heparin-protein complex makes it impossible to decide directly to which carboxyl groups histamine is located. However indirect evidence suggests that they are localized to the granule protein.

Titration of the acid groups of the undissolved granules with HCl showed a consumption of 1000 mμeq/mg granules (dry weight) between pH 7—3 This figure agrees well with the maximal capacity of the granules to bind histamine and sodium at pH 7 Both uptakes were found to approach 1000 mμeq/mg granules.

Protein and heparin carboxyls are completely dissociated at pH 6.5—7 (Kenching ton and Ward 1954 Åborg and Uvnäs 1969) The consumption of KOH between pH 3—7 by a granule suspension (1000 mμeq/mg) is therefore a measure of the main part of the COO^--groups available for cationic binding in intact granules at pH 7 The increased titration values after dissolution of the granules in 1 M KCl (Fig 11) to all appearances reflect mainly the unmasking of acid heparin groups as the result of the breakdown of the heparin-protein complex.

On deducting from the titration values of dissolved granules the titration val es of the amount of heparinate calculated to be present also about 1000 mμeq/mg granules could be attributed to the granule protein. The agreement between this indirectly obtained value of the protein carboxyls available for ionic binding with the values found by direct titration is good.

When dissolved in 1 M KCl the granule heparin occurs as potassium heparinate Fig 15 shows that titration of sodium heparinate and of a corresponding amount of heparinic acid (both in 1 M KCl) gave identical values. Taken together the titration results therefore agree with the assumption that in the intact granule the acid

1000 mueq per mg granules for both lots. Titrations of dissolved granules, heparinate lysozyme etc. supported the view that the granule cation binding sites are located in the protein part of the heparin-protein complex.

The storage of histamine ionically linked to protein COO^--groups in the granule heparin-protein complex is consistent with the observation that histamine is instantaneously released when mast cell granules are discharged from the cells and exposed to the cation-containing extracellular fluid. Free histamine in mast cells suggested by some others to exist in mast cells is considered to occur mainly as an experimental artifact.

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EFFECTS OF FIBRINOLYTIC INHIBITORS AND HEPARIN ON INDUCED INTRAVASCULAR COAGULATION

BY

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IV Nordström, S and E. Zetterqvist, Effects of epsilon aminocaproic acid (EACA) and Trasylol on thromboplastin induced intravascular coagulation studied in dogs with iodine-labelled fibrinogen. *Acta physiol scand* 1969 *76* 93—105

V Nordström, S and E. Zetterqvist, Effects of thrombin infusions upon ^{131}I labelled fibrinogen in dogs. *Acta physiol scand* 1968 *72*. 85—99

VI. Egberg, N and S Nordström, Effects of Reptilase-induced intravascular coagulation in dogs. *Acta physiol scand* Accepted for publication.

References to these papers will be made in the text with the Roman numerals I—VI

CONTENTS

INTRODUCTION 7

THROMBOPLASTIN AND THROMBIN INDUCED COAGULATION 9
Previous investigations 9
Present investigation 11
Experimental conditions 11
Early effects of thromboplastin and thrombin 12
Late effects of thromboplastin 13
Effects of thromboplastin and thrombin in heparinized dogs 15
Summary 15

REPTILASE INDUCED COAGULATION 17
Previous investigations 17
Present investigation 18
Experimental conditions 18
Effects of Reptilase 18
Effects of Reptilase in heparinized dogs 19
Summary 19

EFFECTS OF FIBRINOLYTIC INHIBITORS ON INDUCED COAGULATION 21
Previous investigations 21
Present investigation 22
Experimental conditions 22
Effect of Trasylol *in vitro* 23
Influence of epsilon aminocaproic acid (EACA) and Trasylol on the early effects of procoagulants 24
Influence of Trasylol on the late effects of thromboplastin 25
Influence of Trasylol on the whole blood clotting time 26
Influence of Trasylol on thromboplastin-induced changes in blood pressure 27
Summary 28

GENERAL SUMMARY 29
ACKNOWLEDGEMENTS 30
REFERENCES 31

INTRODUCTION

Major operations and clinical conditions, in which injury or necrosis of tissue occurs, are followed by an increased coagulability of the blood, sometimes leading to disseminated intravascular coagulation with concomitant thrombus formation or severe bleeding disorder

Tissue thromboplastin, released to the circulating blood from injured cells, is considered the most important trigger of intravascular coagulation (Hjort 1967) Plasma coagulation factors will be involved in a stepwise process leading to thrombin formation. Thrombin is a proteolytic enzyme, which converts fibrinogen to fibrin by release of the fibrinopeptides A and B from the fibrinogen molecule. The deposition of fibrin in intravascular coagulation may be compensated by a secondary induced fibrinolytic process and by the ability of the reticuloendothelial system to engulf fibrin particles. The dissolution of fibrin clots occurs through the action of fibrinolytic enzymes, with the concomitant formation of fibrin degradation products In states of disseminated intravascular coagulation, the blood concentration of fibrinogen and other coagulation factors may decrease considerably which together with the anticoagulant action of fibrinolytic degradation products renders the blood incoagulable and may give rise to bleeding. (For review on intravascular coagulation, see Penick and Roberts 1964 McKay 1965 Selye 1966, McKay and Müller Berghaus 1967)

In treatment of bleeding disorders secondary to intravascular coagulation, both anticoagulant and antifibrinolytic drugs have been used Treatment with anticoagulants seems most adequate, since these drugs prevent clotting and, consequently indirectly inhibit the secondary induced fibrinolytic process. The use of only fibrinolytic inhibitors seems not appropriate. However there are rather few reports dealing with deleterious effects of antifibrinolytic drugs in bleeding disorders.

Local thrombosis has since many years been treated by administration of anticoagulants or operative procedures. Recently reports on a therapeutic effect of an enzyme preparation purified from the venom of a Malayan snake have appeared (Sharp et al. 1968 Bell, Pitney and Goodwin 1968) The preparation has a thrombin-like activity *in vivo* but causes no bleeding tendency

The present investigation aims to a better understanding of the processes involved in intravascular coagulation It deals with experimental

in dogs, in which the coagulation system has been activated by intravenous administration of different procoagulants — thromboplastin thrombin and a thrombin like enzyme preparation (Reptilase) isolated from the venom of the snake Bothrops atrox. All these procoagulants induce a secondary fibrinolytic process The influence of heparin and fibrinolytic inhibitors on the induced intravascular coagulation has been investigated The *in vitro* effect of heparin epsilon aminocaproic acid and Trasylol on the clotting activity of thromboplastin preparations has been studied, in addition.

THROMBOPLASTIN AND THROMBIN INDUCED COAGULATION

Previous investigations

Buchanan observed 1845 that *tissue extracts possessed clotting-accelerating effects* when added to blood *in vitro* Since then a large number of investigations have been made on the nature of the coagulation promoting activity in tissue extracts with respect to its chemical and physical properties and its mechanism of action on blood coagulation *in vitro* and *in vivo*

Wooldridge (1886) regarded the *active principle* as a compoud of phospholipids and protein. Later Deutsch, Irrgler and Lomoschitz (1964) showed that tissue thromboplastin prepared from human brain is a lipoprotein. By separation they found the lipid part to contain the procoagulant activity and the protein part to be inactive in blood coagulation. Clarke and O'Meara (1966) investigated subcellular components of human chorion cells and found that the greatest coagulative activity was associated with the microsomal fraction.

A large number of hypotheses on the mechanism of *action of tissue thromboplastin* in blood coagulation have appeared. Morawitz (1904 1905) suggested that the tissue substance initiated and hastened the formation of thrombin from prothrombin in the presence of calcium in plasma. Biggs, Douglas and McFarlane (1953) found that brain extracts, factor V and factor VII took part in a reaction producing a prothrombin converting principle. According to Hjort (1957) a reaction product is formed between factor VII tissue thromboplastin and calcium. Nemerson (1966) prostulated that a rapid reaction between factor VII and tissue thromboplastin formed a product, which could activate factor X.

The methods for comparing the concentrations of the active compound in different thromboplastin extracts have been based on the *linear relation between log coagulation time and log thromboplastin concentration* as found by Mills (1921) In most investigations, the clotting time has been determined as the recalcification time of plasma thromboplastin mixtures (Astrup 1965 Clarke 1966)

In experiments *in vivo* Naunyn (1868) showed that intravenous injec-

tion of lysed red cells resulted in *intravascular coagulation* Wooldridge (1886) revealed that the intravascular coagulation, which occurred in animals receiving tissue extracts intravenously was proportional to the amount of substance injected and that in surviving dogs a period of hypocoagulability followed. Since then a large number of investigations have been made on *intravascular coagulation induced by thromboplastin prepared* from different organs and by thrombin (for reviews on induced intravascular coagulation, see Penick and Roberts 1964 McKay 1965 Hardaway 1966)

Placental extracts were infused into animals by Fulton and Page (1948) and Schneider (1950) They could reproduce the *defibrination syndrom* found in delivery and suggested that tissue thromboplastin from placenta may escape into the maternal circulation, giving rise to fibrinogen consumption, fibrin formation and concomitant incoagulable blood. Ratnoff and Conley (1951) infused homologous brain thromboplastin into dogs, and found that a thrombin inhibitory activity appeared in the plasma, but only after an appre iable fall in the concentration of plasma fibrinogen had occurred Lewis and Szeto (1962) identified an anticoagulant in blood after infusion of brain thromboplastin and found it identical to the antithrombin produced by fibrinolytic agents as described by Niewiarowsky and Kowalski (1958)

Basinger and Allen (1951) reported that the *fibrinolytic properties* of plasma increased following intravenous injections of thromboplastin and thrombin into dogs. Later investigations lend support to the opinion that the fibrinolytic process is induced secondarily to the coagulation process (Merskey et al. 1964 Kowalski et al. 1965 Girolami, Cliffton and Agostino 1966 Phillips, Margaretten and McKay 1968) In similar experiments Leandoer and Nilehn (1969) and Zetterqvist (1969) have demonstrated appearance of a variety of fibrinolytic degradation products.

Soluble complexes between fibrinogen and fibrin monomer or between these and fibrinogen degradation products are also involved in the pathophysiological phenomena observed during intravascular coagulation and fibrinolysis (Shainoff and Page 1962, Copley and Luchini 1964). Kopec et al. (1968) observed that the appearance of complexes between fibrin monomer and fibrinogen degradation products was accompanied by a considerable decrease of platelet adhesion and aggregation. This results in decreased disruption of platelets on thrombin action and, consequently decreased release of platelet factor 4 (PF4), which by Farbiszewski et al. (1968) was found to be a potent and specific trigger for platelet aggregation and blood clotting.

Evidence that the liver reduces the effects of artificially induced intravascular coagulation was reported by Spaet et el. (1961) They found in studies on rats that blood thromboplastin was selectively taken up by *reticuloendothelial cells* following intravenous injection and also demonstrated that blockade of the reticuloendothelial system (RES) resulted in enhancement of defibrination produced by blood thromboplastin. Later Gans and Lowman (1967) studied the uptake of Se^{75} labelled rat fibrin and of fibrin degradation products by an isolated perfused rat liver the loss of activity from the blood closely paralleled the increased activity recorded in the liver Clearance of the fibrin degradation products occurred at a much slower rate than that of fibrin Their findings confirmed the observation by Lee (1962) that cells of the reticuloendothelial system are involved in the elimination of free circulating fibrin and its metabolic products. In recent studies in heparinized dogs Gans, Stern and Tan (1969) have demonstrated that intravenously injected thrombin is rapidly removed by clearance predominantly in the liver

Present investigation

Homologus brain thromboplastin was injected or infused intravenously in dogs in order to study the early (IV) and late (II) effects on various coagulation factors. Thrombin was infused for studies of the early effects only (V)

Thromboplastin was prepared from dog brain essentially according to the method of Owren (1949) In order to determine the doses of thromboplastin given to dogs comparison between the different thromboplastin preparations in terms of concentrations of the active compound had to be performed (I) The thromboplastin was assayed *in vitro* by the one-stage prothrombin time method, essentially according to Quick (1935) The coagulation times of different thromboplastin dilutions were plotted for each batch on a double logarithmic graph as used by Astrup (1965) The concentrations of 10 thromboplastin preparations, expressed in arbitrary units, were found to range from 4 to 33 arbitrary units per ml of the preparation. One arbitrary unit was defined as the amount of thromboplastin producing a clotting time of 18.5 sec with dog plasma in the assay system used.

Experimental conditions

Dogs subjected to studies of the *early effects* of thromboplastin (IV)

and thrombin (V) were injected with iodine-labelled fibrinogen. When equilibration of the isotop with the extravascular space had occurred, experiments with procoagulants were made. After i.v anesthesia, dog brain thromboplastin was injected i.v for 10—20 sec, whereas bovine thrombin was infused for 1 hr In the studies of the *late effects* (II) thromboplastin was administered in different ways — by rapid or slow i.v injections of undiluted thromboplastin by infusion of thromboplastin in saline solution or by subcutaneous injection of undiluted thromboplastin. Only dogs given infusions were anesthetized Blood samples were collected for up to 9 days.

Hematocrit, platelet count, fibrinogen concentration fibrinolytic activity frequency of thromboemboli and occasionally heparin co-factor activity of plasma were determined. In the dogs used for studies of the early effects of thromboplastin and thrombin radioactivity in plasma and in fibrinogen clottable with thrombin was also determined

In *control* animals the basic half life time of radioactivity in plasma and fibrinogen the effect of iodinated fibrinogen without isotop and the influence of anesthesia and indwelling catheter were studied (V) In the studies of the late effects of thromboplastin dogs, injected or infused with heated thromboplastin and saline respectively were used as controls (II)

Early effects of thromboplastin and thrombin (IV V)

The immediate reactions of the dogs injected with thromboplastin within 10—20 sec were severe with respiratory distress, tachycardia and diarrhoea. Of 8 dogs given thromboplastin, 6 died within 20 hrs. Five of these 6 dogs showed occluding thromboemboli in the right side of the heart and pulmonary arteries. The dogs given thrombin infusions showed only slight reactions of the type described, only 1 died during the experiment and in this occluding thromboemboli were demonstrated at autopsy

In general, the effects after thromboplastin injection and thrombin infusion were of the same type, but occurred earlier and more dramatically after the rapid thromboplastin injections. There was very soon an increased disappearance rate of both plasma and fibrinogen radioactivity After 15—30 min the two radioactivity curves started to separate. The plasma radioactivity then increased to a maximum at 30 min to 2 hrs while that of the thrombin-coagulable fibrinogen remained low The fibrinogen concentration decreased in parallel with the fibrinogen radioactivity The platelet count was also rapidly lowered.

The fibrinolytic activity *in vitro* determined in 2 dogs of the thrombin group showed in 1 dog a slight increase immediately and at 1 hr after the start of the infusion. In the thromboplastin group the determination in 3 dogs showed an increased fibrinolytic activity at 15 min but normalization within 2 hrs.

Based on the pre-infusion specific activity of the fibrinogen radioactivity the secondary plasma radioactivity increase — representing degraded non-clottable fibrinogen fibrin (NCFP) — could be calculated as equivalent amounts of fibrinogen. At the time of the maximal plasma radioactivity increase the degradation products amounted to between 40 and 100 pr cent of the fibrinogen decrease up to that point. With increasing doses of thromboplastin and thrombin, the fibrinogen disappeared faster and proportionally larger amounts of non-clottable labelled fibrinogen products appeared in the circulation. This indicates an intimate relation between the intensity of the coagulation process and the mechanism for fibrinogen degradation.

Although in the experiments with thrombin no significantly increased fibrinolytic activity could be demonstrated in the circulating blood there were indications that the subsequent plasma radioactivity increase could be caused by a fibrinolytic process, probably induced secondarily to intravascular coagulation. Evidence for this has been given by the demonstration of fibrinogen degradation products in studies on separation of plasma by gel filtration and the immunological behaviour of serum (Zetterqvist 1969) as well as by the effects of simultaneously administered fibrinolytic inhibitors (IV V) This is also in accordance with the suggestions by Kowalski et al. (1965) In contrast to the findings in the thrombin experiments, an elevated fibrinolytic activity was recorded in the circulating blood after thromboplastin injections. Because of the shorter injection time of thromboplastin the coagulation trauma was probably more intense. This might explain why a fibrinolytic process could be more intensively evoked, and therefore demonstrable in the circulating blood.

Late effects of thromboplastin (II)

Four of ten dogs injected with thromboplastin for less than 20 sec died. The mortality was not correlated to the thromboplastin dose. All four dogs, which died during the experiment showed thromboemboli, three of which occluded the outflow of the right ventricle or the pulmonary artery In 5 of the 6 surviving dogs, killed 9 days later small to massive thromboemboli had occured.

The platelet count decreased even after small doses of thromboplastin however only slightly if thromboplastin was given slowly After rapid injections of large thromboplastin doses the platelet count was depressed for at least 7 days. The decrease in fibrinogen concentration during the first days was correlated to the dose injection rate of thromboplastin Slow rate injections of thromboplastin caused slight or no decrease in fibrinogen concentration In contrast to the fibrinogen reaction after thromboplastin injections at high dose injection rate, hyperfibrinogenemia developed already from the first day in anesthetized dogs infused with thromboplastin

Increased fibrinolytic activity occurred only in one of the dogs given rapid or slow injections of thromboplastin In the 2 anesthetized dogs, however infusion of thromboplastin was followed by moderately increased activity during 9 days After infusion of saline alone in 3 anesthetized dogs, slightly increased fibrinolytic activity was recorded

The heparin co-factor activity of plasma decreased only by 10—20 per cent after administration of thromboplastin.

Intravenous injection of heated thromboplastin and subcutaneous injection of thromboplastin did not cause any significant changes

The more pronounced and prolonged decrease in platelet count and fibrinogen concentration after rapid than after slow injections of thromboplastin is in conformity with the result of previous animal studies (Ratnoff and Conley 1951 Adelson Rheingold and Parker 1960 Lewis and Szeto 1962) Relatively small total amounts of thromboplastin, if given rapidly could be sufficient for consumption of platelets and fibrinogen and thrombus formation while slow injections of large amounts of thromboplastin were less harmfull The critical thromboplastin concentration seemed to vary in the individual dogs, probably due to differences in blood flow rate, anticoagulant activity of plasma, blockade of the reticuloendothelial system and general condition of the animal These differences might explain the poor correlation between thromboplastin dose on one side and the rate of thromboemboli and mortality on the other

The nature of the different reactions in fibrinogen concentration after rapid and slow dose injection rate of thromboplastin is unclear The slow thromboplastin infusions in anesthetized dogs may simulate conditions during operations, after wich supernormal fibrinogen values are a regular finding (Godal 1962) Thromboplastin in high concentration might interfere with metabolic and/or catabolic processes or with the synthesis of fibrinogen.

With one exception, hyperfibrinolysis occurred only in anesthetized

dogs infused with thromboplastin or saline alone This indicates that anesthesia, or fluid infusion, or a combination of both is responsible for the increased fibrinolytic activity

Effects of thromboplastin and thrombin in heparinized dogs (II V)

In dogs injected intravenously with heparin (1 000 IU/kg b w) before the trombin infusion, the early changes induced by thrombin were almost completely absent.

In heparinized dogs, thromboplastin produced no thromboembolic phenomena and no marked changes in platelet count and fibrinogen concentration.

Injections of heparin alone resulted in a slight initial decrease in platelet count followed on the third day by an increase. The fibrinogen concentration increased. As the increased fibrinogen level remained for more than 1 week this phenomenon can hardly be explained by an inhibition of a permanent coagulation process occurring normally in the circulating blood.

The heparin co-factor decreased by 35 per cent after intravenous injection of 2,500 IU heparin This effect of heparin was observed by Olsson (1963) and explained as being caused by a complex formation between heparin and its co-factor When thromboplastin was injected after heparin, the co-factor decreased, however only to a small degree, indicating a competition for heparin between the co-factor and thromboplastin.

Summary

The *early* effects of intravenous administration of dog brain thromboplastin and bovine thrombin were studied in dogs injected with iodine-labelled fibrinogen. In general, thromboplastin and thrombin provoked similar effects There occurred, however earlier and more dramatically after the rapid thromboplastin injections than after the slow thrombin infusions. There was a decrease in platelet count, fibrinogen concentration and radioactivity of plasma and coagulable fibrinogen. A following secondary increase of the plasma radioactivity was assumed to reflect the appearance of degradation products of fibrinogen or fibrin, obtained through a secondary activated fibrinolytic process. An increased fibrinolytic activity *in vitro* could be demonstrated only after the more rapid coagulation traumata. The effects were almost completely prevented when the dogs were heparinized before administration of procoagulants.

Present investigation

The aim of this study (VI) was to investigate whether the thrombin like enzyme Reptilase, like other procoagulants (IV V) would give rise to a secondary activation of fibrinolytic processes. The influence of Reptilase on platelets and the effect of daily injections of Reptilase for several weeks were also studied.

Experimental conditions

Dogs were injected with ^{125}I fibrinogen and at steady state catabolism, anesthetized and infused intravenously with Reptilase for 1 hr Two dogs were used for long term treatment with daily injections of Reptilase for 10 and 16 days, respectively Blood samples were collected for determinations of platelet count, fibrinogen concentration and radioactivity in plasma, fibrinogen and serum. Immunoelectrophoresis and immunodiffusion were performed on serum against rabbit anti-dog fibrinogen serum

The dose response and effects on general circulation and on some coagulation factors were studied in preliminary experiments.

Effects of Reptilase (VI)

In preliminary experiments, no influence on arterial blood pressure pulse rate or rectal temperature was observed There seemed to be at most a moderate decrease of prothrombin. Factor V as determined in a one-stage system decreased by 75 per cent for the first 8 hrs.

Reptilase caused an immediate decrease in fibrinogen concentration. By increasing the dose of Reptilase complete defibrination could be obtained within 2 hrs. Even after the highest doses of Reptilase the platelets never fell below 40 per cent of the pre-infusion value. The radioactivity curves were, in principle of the same type as after thromboplastin and thrombin administration. One hr after the start of the Reptilase infusion the serum radioactivity increased. At the same time the immunoelectrophoretic investigations showed precipitation arcs which were localized in the regions where the lytic degradation products of fibrinogen and fibrin are expected to be found (Nussenzweig, Seligmann and Grabar 1961) In the 24 hr sample, the precipitation bands had almost completely disappeared. The immunodiffusion investigation gave the same results. It is obvious that the non-clottable degradation products, containing the antigenic determinants,

were brought back to the circulation secondary to the defibrination pr cess. All dogs survived the experiment and no thromboemboli were foun at autopsy

Daily intravenous injections of Reptilase kept the fibrinogen concen tion on a rather constant level between 0 4 and 0 9 mg/ml of plasma t 16 days without adverse effects. After cessation of Reptilase treatmer the fibrinogen concentration rose to the initial level for 4—5 days The platelet count did not change markedly No significant bleeding tenden was observed.

Reptilase, like thromboplastin and thrombin, was capable of inducing intravascular coagulation The coagulation promoting activity remaine for at least 24 hrs, probably due to the absence of plasma inhibitors to Reptilase combined with a slow excretion rate of Reptilase. The coagula tion induced by Reptilase was followed by a fibrinolytic process with return of degraded fibrin into the circulation as indicated by radioactivity measurements and immunological investigations. The main cause of the maintained hemostasis must be that the platelet function is essentially undisturbed by Reptilase. The failure to demonstrate any thrombi at autopsy shows that a successive and complete removal of fibrin took place. As the action of Reptilase seems to be very similar to that of Arvin, Reptilase might be useful in clinical treatment of thrombot manifestations.

Effects of Reptilase in heparinized dogs

Heparin did not inhibit the Reptilase induced coagulation and the secondary fibrinolytic process Thus, the decrease in fibrinogen concentra tion, the radioactivity curves and the results of the immunological investigations were mainly in agreement with those obtained in unheparinized dogs. However the platelet count was essentially unaffected, while in the non-heparinized animals there was a moderate decrease. This may be due to traces of a thromboplastin-like activity in the Reptilase prepara tion.

Summary

The effect of the thrombin like enzyme preparation Reptilase, purified from the venom of Bothrops atrox was studied in dogs injected with ^{125}I labelled fibrinogen. Reptilase, like thromboplastin and thrombin was capable of inducing intravascular coagulation with secondary

of fibrinolytic processes This was evidenced by the results of radioactivity measurements and immunological methods By daily injections of Reptilase it was possible to keep the fibrinogen concentration on low levels for several weeks without adverse effects. No bleeding tendency was observ ed, probably because the platelet count was essentially unchanged. No thromboemboli occurred. On account of the demonstrated properties Reptilase may be useful in treatment of clinical thrombotic disorders.

EFFECTS OF FIBRINOLYTIC INHIBITORS ON INDUCED COAGULATION

Previous investigations

Concerning the states of pathological fibrinolysis, there have been conflicting views as to the underlying mechanisms, diagnosis and treatment (for review see Fletcher Alkjaersig and Sherry 1962) Important for understanding of the pathogenesis of fibrinolysis was the finding, that lysed fibrinogen, besides losing its clottability with thrombin, aquires anticoagulant properties (Niewiarowski and Kowalski 1958 Stormorken 1957 Triantaphyllopoulos 1958) Furthermore, fibrinogen degradation products inhibit polymerization of fibrin (Godal and Helle 1963) and decrease platelet aggregation, adhesiveness and viscous metamorphosis (Kowalski, Kopec and Wegrzynowicz 1964)

The connection between coagulation and fibrinolysis was emphasized by Schneider (1951) and Müllertz (1957), who found that when tissues are damaged by trauma or anoxia both processes are activated or accelerated by the release of tissue thromboplastin and/or fibrinolytic tissue activator Similar effects were seen after trauma (Bergentz and Nilsson 1961) and after administration of thromboplastin, thrombin (Basinger and Allen 1951) and certain snake venoms (Regoeczi, Gergely and Mc Farlane 1966) In investigations on the effect of thrombin in dogs, Kowalski et al. (1965) and Girolami, Cliffton and Agostino (1966) suggested that the increased fibrinolytic activity was secondary to the induced coagulation process. Phillips, Margaretten and Mc Kay (1968) showed in similar experiments that plasminogen decreased gradually and reached low levels after 24 hrs, when the fibrinogen had returned to normal.

Based on assumptions of a secondary compensatory fibrinolysis after coagulation trauma, studies have been made on the influence of the fibrinolytic inhibitors, epsilon aminocaproic acid (EACA) and Trasylol, administered simultaneously with procoagulants. *EACA* inhibits the activation of plasminogen to plasmin, but its antiplasmin activity is weak (Alkjaersig, Fletcher and Sherry 1959) Besides its antifibrinolytic activity EACA has been reported to retard the endogenic thromboplastin generation and to inhibit the prothrombin activation slightly (Rezakovic, Nikulin and Stern 1966, Blombäck, Blombäck and Olsson 1967) *Trasylol*

a polypeptide found in several organs, inhibits the proteolytic action of kallikrein (Frey and Kraut 1928) trypsin (Werle, Maier and Ringelmann 1952) chymotrypsin (Kraut and Körbel Enckhardt 1958) and plasmin (Marx et al 1959) Trasylol also inhibits the activation of plasminogen to plasmin (Berghoff and Glatzel 1963) Furthermore, in investigations *in vitro* Trasylol inhibits thromboplastin but not thrombin (Amris 1964 Godal and Theodor 1965 Blombäck, Blomback and Olsson 1966 Andersson Nilsson and Hedner 1967 Dubber et al 1968)

In animal experiments EACA given together with thrombin promotes the development of the generalized Shwartzman phenomenon (Lee 1962). Margaretten Zunker and McKay (1964) demonstrated persistence of fibrin in the glomeruli of the kidney as long as the infusion of EACA was continued. Gans (1966) found that EACA given simultaneous with thrombin into dogs, increased the mortality and frequency of thromboembolu Beller Mitchell and Gorstein (1967) tested the effect of different proteolysis inhibitors together with thrombin in rabbits and found that all of the inhibitors resulted in fibrin deposition in the glomeruli of rabbit kidney Mammen and Poucho (1966) reported that Trasylol prevented formation of thromboemboli in dogs injected with thromboplastin. In dogs given EACA before intravenous injection of extracts from snake venoms, Chan and Reid (1964) did not observe any adverse effects. Thus, in states of intravascular coagulation the use of fibrinolytic inhibitors has given varying results.

Present investigations

The problems of these studies were:

1 Do substances with antifibrinolytic activity influence upon the early and late changes obtained after intravenous administration of procoagulants?

2 Can an anticoagulant effect of Trasylol be demonstrated in different *in vitro* tests and also *in vivo* after injection of procoagulants?

3 How does the whole blood clotting time of dog blood mixed with Trasylol *in vivo* compare to that of dog blood mixed with Trasylol *in vitro*?

4 How does Trasylol affect the changes in blood pressure following rapid injections of thromboplastin?

Experimental conditions

In vitro the effect of Trasylol on the recalcification time of different

plasma—thromboplastin mixtures was compared to that of EACA and heparin (I) The effect of Trasylol on a one-stage thrombin titration test used for the determination of heparin co-factor activity of plasma (Blombäck et al 1963) was also examined (I)

The *in vivo* investigations were performed as described earlier with the modification that antifibrinolytic drugs were administered before or together with procoagulants. Dogs subjected to studies of the *early* effects were infused with EACA or Trasylol simultaneously with injections or infusions of thromboplastin (IV) thrombin (V) or Reptilase (VI), respectively In the studies of the *late effects* (II) dogs were treated intravenously with various doses of Trasylol prior to or in event of infusion, simultaneously with thromboplastin Dogs injected or infused with Trasylol and saline, as well as those given thromboplastin alone as described in a previous chapter served as controls. Only dogs given infusions were anesthetized.

In order to study the influence of Trasylol on the *whole blood clotting time* (I) blood was collected from dogs into siliconized glass tubes containing saline or different concentrations of Trasylol Thereafter the dogs were infused with saline or Trasylol in amounts calculated to give the same concentration *in vivo* as those used *in vitro* Samples were taken frequently for 1 hr The times of beginning and of complete coagulation at 37 C were noted.

Finally in another series of dogs (III) polyethylen catheters were introduced into the aorta and the right ventricle or the pulmonary artery in order to follow changes in *blood pressure* after rapid injections of thromboplastin with or without pretreatment with Trasylol.

Effect of Trasylol in vitro (I)

It was found that the recalcification time of plasma—thromboplastin mixtures (one-stage prothrombin time) was successively prolonged with increasing concentrations of Trasylol. However this was only applicable to concentrations exceeding those corresponding to 1 000 KIU per arb unit of thromboplastin The inhibition by Trasylol seemed to approach its maximum at a concentration of about 2 000 KIU per arb unit of thromboplastin. It appeared that Trasylol, even in very high concentrations, could inactivate only part of the thromboplastin activity This differed from the effect of heparin, indicating that heparin acts in a different way Judging from these *in vitro* investigations, 10 000 KIU of Trasylol will, in the range of comparison, inhibit the same amount of

thromboplastin as 4—5 IU of heparin EACA was without effect on the one-stage prothrombin time In the test system used for determination of the heparin co-factor activity Trasylol in high concentrations showed slight antithrombin activity The effect of Trasylol was not related to the concentration of plasma This is also in contrast to the effect of heparin which, to a high degree, is dependent on the plasma content of heparin co-factor (Olsson 1963)

Influence of EACA and Trasylol on the early effects of procoagulants (IV V VI)

The divergence between the curves for plasma and fibrinogen radioactivity obtained after thromboplastin, thrombin and Reptilase, was abolished or delayed with large doses of EACA and Trasylol and the amount of calculated degradation products was reduced. However although a fibrinolytic activity seemed to be inhibited significant amounts of degradation products were still calculated and in Reptilase-infused dogs, there was immunological evidence for the appearance of degradation products. This is in accordance with the findings of Barnhart (1966) who identified and quantitated by means of immunological methods degradation products after combined administration of EACA and thrombin in dogs. She concluded that a dose of EACA, large enough to inhibit effectively a general plasma fibrinolytic activity could not block a local fibrinolysis proceeding in microthrombi in the circulation

Trasylol in large doses, has been shown to inhibit *in vitro* the activation phase of blood coagulation without affecting thrombin activity (Amris 1964 Blombäck Blombäck and Olsson 1966) In the present study no effect on the disappearance of fibrinogen in dogs given thrombin was found (V) which is in conformity with the results of the *in vitro* studies. After thromboplastin injections (IV), the initial decrease in fibrinogen radioactivity and platelet count was somewhat smaller if the dogs received EACA or Trasylol. This might be due to a slight retardation of the thromboplastin effect. The Reptilase-induced fibrinogen decrease was not influenced by EACA and Trasylol (VI) The moderate decrease in platelet count, however was prevented if the dogs were pretreated with EACA or Trasylol. It is assumed that some thromboplastin like activity contained in the Reptilase preparations and responsible for the platelet decrease was inhibited by EACA and Trasylol.

Among the dogs given thrombin as procoagulant, the mortality rate was highest in the Trasylol thrombin group With thromboplastin the

highest mortality was obtained in the group given only procoagulant. In both series EACA treated dogs showed the lowest mortality Thus, Trasylol seemed to protect against thromboplastin but not against thrombin while EACA seemed to exert some protection against both procoagulants. The higher mortality rate in Trasylol than in EACA treated dogs might be due to the fact that Trasylol, but not EACA, inhibits plasmin directly An inhibiting effect by EACA upon fibrin polymerization (Godal and Helle 1963 Godal and Theodor 1965) might also have contributed to the protective action by this substance. Concerning the effect of Trasylol, Mammen and Poucho (1966) suggested that the protective action of Trasylol in decreasing thrombus formation after thromboplastin injections was rather by interference with the cross linking of fibrin monomers than by its antithromboplastin activity When Reptilase was infused simultaneously with EACA or Trasylol all dogs survived

Influence of Trasylol on the late effects of thromboplastin (II)

Three of eight dogs given Trasylol before a rapid thromboplastin injection died. These 3 dogs had received large amounts of thromboplastin at a high injection rate. In groups with comparable thromboplastin doses, thromboemboli occurred in 9 out of 10 dogs given thromboplastin alone and in 2 out of 8 dogs injected with Trasylol before the thromboplastin injection. This "thrombus protective effect of Trasylol was also found by Mammen and Poucho (1966)

The decrease in platelet count seemed to be smaller after pretreatment with Trasylol, but this was not statistically verified. Small and large doses of Trasylol inhibited to about the same degree the fall in fibrinogen concentration, which is in conformity with the *in vitro* findings that Trasylol inactivates only part of the clotting activity of thromboplastin (I) In dogs given comparable doses of thromboplastin the decrease in fibrinogen concentration during the first 1—3 days was significantly less ($p<0.01$) after pretreatment with Trasylol. Intravenous injections of Trasylol alone in doses up to 100 000 KIU per kg b w had only slight or no influence on platelet count and fibrinogen concentration.

The less marked decrease and earlier return of platelets and fibrinogen, together with the lower incidence of thromboembolic complications in the Trasylol treated animals, suggest that Trasylol has an *in vivo* anticoagulant effect. *In vitro* Trasylol was found to inhibit the initial phase of coagulation (Amris 1964 Blombäck, Blombäck and Olsson 1966) Mammen and Poucho (1966), however postulated that *in vivo* the action of

Trasylol is directed against the final phase of coagulation causing an impaired fibrin clot formation. Trasylol, which inhibits a variety of proteases, may interfere with several reactions in the coagulation process. Trasylol is a weak anticoagulant compared to heparin

Trasylol, which is a potent antifibrinolytic substance (Marx et al 1959 Godal and Theodor 1965) had no influence on the slightly increased fibrinolysis, which appeared in anesthetized dogs after slow infusion of thromboplastin and of saline. Anesthesia, or fluid infusion or a combination of both might thus give rise to a type of hyperfibrinolysis which is uninfluenced by Trasylol or appears after the period during which Trasylol is effective

The small decrease of heparin co-factor following intravenous administration of thromboplastin seemed to be prevented by Trasylol pretreatment. Trasylol alone caused a small increase of the co-factor activity This is in conformity with the finding from the *in vivo* administration of Trasylol during surgical interventions in man (Olsson and Nordström 1964) The results of the present *in vivo* experiments are in contrast to the effect of Trasylol *in vitro* where no influence on the antithrombin system of plasma was observed (I) This suggests that the effect of Trasylol on the antithrombin activity is indirect

Influence of Trasylol on the whole blood clotting time (I)

The clotting time was determined in dog blood after addition of Trasylol *in vitro* and *in vivo* The coagulation times were prolonged with increasing Trasylol concentraton added *in vitro* The time of starting as well as of complete coagulation was doubled at a Trasylol concentration of 400 KIU per ml blood. The differences were highly significant ($p<0.001$) The maximal effect of intravenously injected Trasylol was recorded immediately after the injection At that time, however the effect of 1 000 KIU Trasylol added per ml blood *in vivo* was smaller than that of 400 KIU Trasylol added per ml blood *in vitro* Injection of 40 000 KIU Trasylol per kg b w., giving a calculated initial concentration of 400 KIU per ml blood, caused a slight prolongation of the clotting time for at least 1 hr The time of complete coagulation was more influenced by Trasylol than that of beginning coagulation

The difference in whole blood clotting time between blood mixed with Trasylol *in vitro* and *in vivo* is indicative of a fast distribution of Trasylol to the extravascular compartment. The fact that Trasylol prolonged the time for complete coagulation to a greater degree may support the

suggestion of Mammen and Poucho (1966) that the action of Trasylol is directed against the final phase of coagulation.

Influence of Trasylol on thromboplastin-induced changes in blood pressure (III)

Hemodynamic changes are an important manifestation of intravascular coagulation (McKay 1965 Hardaway 1966) Delin, Olsson and Teger Nilsson (1967) have shown that thrombin and thromboplastin cause peripheral vasodilatation followed by intravascular coagulation. By dialysis of tissue thromboplastin, they succeeded in separating a vasodilating activity from the procoagulant activity Consequently several mechanisms may contribute to the hypotension in thromboplastin-induced intravascular coagulation. Trasylol which inhibits the release of vasoactive kinins (Back 1966) and acts as a weak antithromboplastin (Amris 1964 *Blombäck, Blombäck and Olsson 1966) might, therefore, interfere with* the thromboplastin-induced hypotension.

The rapid intravenous injections of thromboplastin caused a decrease in platelet count and fibrinogen concentration, which is indicative of intravascular coagulation. As in previous investigations (II) pretreatment with Trasylol partly inhibited the decrease in fibrinogen concentration indicating an anticoagulant activity of Trasylol. The initial fall in aortic blood pressure to a lowest value after 1 min was less influenced by Trasylol. The return of systemic blood pressure was, however significantly faster ($p<0.01$) in Trasylol-treated than in untreated dogs. Ten min after the thromboplastin injection, the blood pressure had returned to 80 per cent of the pre-injection value in 6 out of 7 dogs given Trasylol, but in none of 6 dogs without pretreatment. In both groups of dogs, the pulmonary arterial pressure started to increase when the aortic pressure had reached its minimum value. The pulmonary arterial pressure returned to pre-injection levels within 5 min

The hemodynamic changes obtained in the present investigation resemble those after thrombin infusion as described by Olsson, Rådegran and Taylor (1969) They found the cardiac output unchanged at the time when the systemic hypotension was most pronounced, and suggested that the early effects are due to pulmonary vasoconstriction and systemic vasodilatation rather than to formed fibrin clots. On the other hand later hemodynamic events may depend on clot formation. From the results obtained in the present study the conclusion is drawn that the effect of Trasylol on the thromboplastin-induced hemodynamic changes might

depend on interference with coagulation processes as well as with release of vasoactive substances.

Summary

In vitro Trasylol was shown to prolong the one-stage prothrombin time, to exert some slight antithrombin activity in an assay of heparin co-factor activity and to prolong the time of beginning and complete whole blood coagulation although only at high concentrations. *In vivo* Trasylol inhibited partly the decrease in platelet count and fibrinogen concentration and reduced the frequency of thromboemboli after intravenous thromboplastin administration Trasylol also seemed to exert some slight influence on the antithrombin level in the circulating blood and to prolong the whole blood coagulation times It is believed that the anticoagulant effect of Trasylol is the result of partial inhibition of more than one reaction in the coagulation process Trasylol is a weak anticoagulant compared to heparin. Due to the weak effect and to the fast extravascular distribution of infused Trasylol large doses on repeated occasions would be needed for anticoagulant effect in humans.

Epsilon aminocaproic acid (EACA) seemed also to give some protection against thromboplastin induced intravascular coagulation. The coagulation process induced by thrombin and Reptilase was not appreciably influenced by EACA and Trasylol

The fibrinolytic process, activated secondary to the induced coagulation process by thromboplastin thrombin and Reptilase could be delayed but not completely abolished by EACA and Trasylol.

Trasylol accelerated the normalization of the decreased arterial blood pressure after thromboplastin injections. This effect of Trasylol is assumed to depend on interference with coagulation processes as well as with release of vasoactive substances.

GENERAL SUMMARY

The effect of the fibrinolytic inhibitors epsilon aminocaproic acid (EACA) and Trasylol was investigated *in vitro* and *in vivo* in dogs. Intravenous administration of homologous brain thromboplastin, bovine thrombin and the thrombin like enzyme preparation Reptilase resulted in a consumption coagulopathi accompanied by a secondary fibrinolytic process. This fibrinolysis was delayed and reduced, but not completely blocked, by simultaneous treatment with large doses of EACA and Trasylol. Pretreatment with heparin prevented the coagulation process induced by thromboplastin and thrombin but not that induced by Reptilase

Besides its antifibrinolytic effect, Trasylol was shown to exert some anticoagulant activity and to influence the hemodynamic changes following coagulation trauma. The anticoagulant effect of Trasylol is probably the result of partial inhibition of more than one reaction in the coagulation process Compaired to heparin, Trasylol is a weak anticoagulant. The antifibrinolytic activity of Trasylol is much stronger than the anticoagulant one, and at least as effective as that of EACA.

In long term treatment with Reptilase the fibrinogen concentration was kept at low levels without adverse effects. No bleeding tendency was observed, probably because the platelet count was essentially unchanged. On account of the demonstrated properties Reptilase may be useful in treatment of clinical thrombotic disorders.

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Metabolism of L(+)-Lactate in Human Skeletal Muscle during Exercise

BY

LENNART JORFELDT

STOCKHOLM 1970

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CONTENTS

I	Introduction	5
II	Determination of ^{1} C-L(+) lactate in blood	8
	Materials	8
	Reagents	9
	Procedure	9
	Experimental, results and comments	10
	Discussion	20
III	Metabolic studies	
III.A.	General Procedures and Methods	21
	Procedures	21
	Blood Analyses	24
	Definitions	26
III B.	Blood Flow Determination	27
	Procedure and Experimental	27
	Calculations	28
	Results.	29
	Discussion	31
III C.	Uptake, Release and Metabolism of Lactate at Normal Arterial Lactate Concentration	32
	Subjects	32
	Procedure	32
	Results	34
	Discussion	41
III.D	Uptake, Release and Metabolism of Lactate at Elevated Arterial Lactate Concentration	45
	Subjects	45
	Procedure	45
	Control studies	46
	Results	48
	Discussion	54
IV	General Discussion.	56
V	Summary	60
	Acknowledgements	62
	References	63

CHAPTER I

Introduction

In his paper "On milk and its acid" Scheele (1780–1786) described the isolation of lactic acid from sour milk. It was soon evident, however, that lactic acid could be isolated also from other material. Thus Berzelius (1812) demonstrated the presence of lactic acid in skeletal muscle, but it was not until about 100 years later that definite evidence of the relationship between muscle activity and production of lactic acid in the muscle could be provided (Fletcher and Hopkins 1907). That this lactic acid production constituted a final product in the glycolysis was shown first by Meyerhof and Hill (see Meyerhof 1930). Until the 1930's, when Lundsgaard (1930 and 1931) and Lohman (1935) demonstrated the significance of creatine phosphate and adenosine triphosphate, respectively, as source of energy in muscle contraction, lactic acid formation was assumed to be necessary in conjunction with the conversion of the chemically bound energy to mechanical energy in muscle contraction.

It has now been established that adenosine triphosphate (ATP) is the immediate source of energy for muscle contraction (Cain and Davies 1962). The store of ATP in the muscle is limited, however, so that a continuous resynthesis of ATP must take place. This resynthesis of ATP occurs mostly intramitochondrially in the oxidative phosphorylation, i.e. when NADH, NADPH and FADH formed in the breakdown of fat and carbohydrate are oxidized with oxygen as final electron acceptor. A small quantity of ATP is also regenerated in the cytoplasm in the breakdown of carbohydrate. If the pyruvate and NADH formed in this process cannot be oxidized intramitochondrially, the ATP synthesis can nevertheless be sustained since pyruvate, under formation of lactate, functions as electron acceptor for NADH. As this glycolytic resynthesis of ATP takes place anaerobically, production of lactate from working muscle may be caused by a deficiency or absence of oxygen, as for example during very heavy exercise (Gollnick and King 1969), during exercise at altitude (Hermansen and Saltin 1966) and in clinical conditions with impairment of the arterial inflow to the exercising muscles (Carlson and Pernow 1959). In a wider sense the lactate production may be considered to be caused by the fact that the oxidative resynthesis of ATP does not keep step with the

This term is used in the sequel since the pK_a value for lactic acid is more than 3 pH units lower than physiological pH.

consumption or that there is an unbalance between the glycolysis and the oxidative capacity in the muscle (Keul Doll and Keppler 1969) The limitation of the oxidative capacity would seem mainly to relate to the pyruvate oxidation (Holloszy 1967) and/or the electron transport through the mitochondrial membrane (Boxer and Devlin 1961 Alpert 1965) which is necessary for intramitochondrial oxidation of extramitochondrial NADH

The conversion of pyruvate to lactate is catalysed by the enzyme lactic dehydrogenase (LDH) The molecules of this enzyme are made up of four subunits of which there are two types M and H The proportion between these subunits in the molecule affects the activity of the enzyme (Dawson, Goodfriend and Kaplan 1964) LDH made up predominantly of M type subunits has a high turnover number for pyruvate It thus has a high pyruvate to-lactate conversion activity In tissues with high capacity for aerobic metabolism, and in tissues where lactate is normally converted to pyruvate LDH consists chiefly of H type subunit

The skeletal muscle is made up of white and red muscle fibres The white fibres are functionally adapted to resynthesize a large part of their ATP through glycolysis, while the red fibres are adapted for a chiefly oxidative resynthesis of ATP with the metabolism probably based largely on blood-borne substrate.

A comprehensive survey of the muscle metabolism has recently been presented by Keul Doll and Keppler (1969)

Production and to some extent uptake of lactic acid in human and animal skeletal muscle have been the subject of a large number of studies, both at rest and during varying types of exercise In these studies the above factors have been assessed both by analysis of arterio-venous concentration differences of lactate over individual muscles or groups of muscles and by observing variations in the arterial concentration of lactate Recently the lactate formation in muscle during exercise has been studied also by analysis of the lactate content in muscle tissue collected by needle biopsy technique immediately after exercise (Diamant Karlsson and Saltin 1968)

A release of lactate from skeletal muscle takes place already at rest (Kramer Quensel and Schafer 1939 Andres, Cader and Zierler 1956) This lactate production becomes greatly elevated in conjunction with muscular exercise The veno-arterial concentration difference, production and arterial concentration of lactate, all rise approximately exponentially with increasing intensity of work (Bang 1936 Pernow Wahren and Zetterquist 1965 Wahren 1966 Hermansen and Saltin 1966)

A significant rise of the lactate concentration in the venous blood from the forearm muscle is observable only a few seconds after the start of muscular exercise (Pernow and Wahren 1962) On continued exercise of long duration

at submaximal level the lactate production falls after an initial rise (Bang 1936, Bergström *et al* 1967 Wahren and Hagenfeldt 1968) which is not the case at high relative work intensities (Bang 1936 and others)

The skeletal muscle has the capacity not only to produce lactate but also to extract it. Thus Margaria, Edwards and Dill (1933) and Carlson and Pernow (1959) found that during muscular exercise an uptake of lactate took place in muscle not participating in the exercise. At high arterial concentrations of lactate there may be an uptake of lactate also in exercising muscle (Stainsby and Welch 1966, Freyschuss and Strandell 1967) Quantitatively this lactate uptake seems to be even greater when the muscle performs light exercise than when it is at rest (Rimmal and Ström 1949 Freyschuss and Strandell 1967) In the myocardium, where an uptake of lactate normally takes place even at normal arterial lactate concentration, the uptake of lactate increases with rising arterial concentration (Goodale, Olson and Hackel 1959) Whether a similar relationship exists in skeletal muscle is not known. Nor is it fully understood to what extent the lactate taken up in a skeletal muscle under these conditions is oxidized to CO_2 or metabolized by other means.

Our knowledge of the mutual relation between uptake and release of lactate in skeletal muscle, and of the metabolism of the lactate taken up, is incomplete. Studies of this kind would appear to be possible only with labelled lactate. In animal experiments with labelled lactate it has been shown that lactate taken up in the muscle is partially oxidized and gives rise to release of labelled CO_2 (Drury and Wick 1956) In a study of isolated perfused canine gastrocnemius Omachi and Lifson (1956) found that the fraction of the released carbon dioxide deriving from the added isotope-labelled lactate was larger in exercising than in resting muscle. A significant but quantitatively slight labelling of ^{14}C in muscle glycogen from administered ^{14}C-lactate has been observed (Drury and Wick 1956, Omachi and Lifson 1956, Koeppe *et al* 1964)

The objects of the present study were

1 to investigate whether simultaneous uptake and release of lactate occur in exercising human skeletal muscle,

2. to illustrate the mutual quantitative relations of these two—propably simultaneously occurring—processes both at different points of time during prolonged exercise and at varying arterial concentration of lactate,

3 to determine the extent to which lactate—if taken up—is oxidized to CO_2 or otherwise metabolized.

These questions appear to be best illustrated in studies with ^{14}C-labelled L(+) lactate. A method has therefore been devised for determination of ^{14}C-L(+) lactate in low specific activities in blood suited for analysis of large series of samples.

CHAPTER II

Determination of ^{14}C-L(+) lactate in blood

For determination of the specific activity of ^{14}C-lactate in blood urine and tissue, mainly two principles have been used 1 oxidation of lactic acid and measurement of the radioactivity in the products formed (Brin and Olson 1952 Friedman *et al* 1960 Pomerantz 1961) 2 separation by means of column (Forsander and Räihä 1960) or paper chromatography (Beaconsfield and Ginsburg 1966) Recently a gas radiochromatographic method has been described for determination of specific activities of individual organic acids (Hagenfeldt 196 Since the different oxidation methods described are not entirely specific (ery and Hastings 1931 Friedman *et al* 1960) a chromatographic metho metimes included in these procedures (Reichard *et al* 1961) Alth ic for lactate the chromatographic methods are not able to separat tereo-isomers of lactate of which only the L(+) form occurs n mammals. Furthermore the methods described are too ti t be suitable for analysis on a large scale.

L tat is today commonly determined by an enzymatic method (L t ndholm, Mohme Lundholm and Vamos 1963) When lactic deliv LDH) is used this method is specific for L(+) lactate (Lund h Lundholm and Svednyr 1963) It was the aim of the present i esti pply such an enzymatic method for the determination also of ^{14}C-L( ) L ate in blood.

The at in one sample may be oxidized to pyruvate by LDH and the pyruvate f ned precipitated as phenylhydrazone after addition of phenyl hydrazine hydrochloride and carrier The procedure is then repeated on another sample but without addition of LDH. The ^{14}C-radioactivity of the precipitates is then recorded and the difference between the two may be taken to reflect ^{14}C-L(+) lactate radioactivity

Materials

Sodium-DL-lactate 1 ^{14}C, sodium-L-ketoglutarate 1 ^{14}C and glucose-U ^{14}C were obtained from The Radiochemical Centre England and L(+)-lactate-1 ^{14}C from Volk Radiochemical Co USA. The radioactive substrates were

dissolved in sterile water (pyruvate in 0 6 M perchloric acid) dispensed into sterile ampoules and kept at −18° C.

The radiochemical purity of the DL-lactate tested by gas radiochromatography (Hagenfeldt 1968) and paper chromatography was found to be ⩾ 96 % when the crystalline substance had been freshly dissolved. No significant decomposition was found 3 months later In the calculations this substance was considered to be racemic. The radiochemical purity of the L(+) lactate supplied in water solution, was found to be more than 98 % To test its stereospecificity the labelled lactate was mixed with unlabelled lactate and converted to pyruvate by a modification of the enzymatic method for analysis of unlabelled lactate, with methoxyamine hydrochloride substituted for the hydrazine sulphate as pyruvate trap The reaction was found to be spectrophotometrically complete when compared with a standard solution. The residue was analysed by gas radiochromatography and found to contain more than 96 % of the activity as pyruvate and no significant radioactivity at the lactate position The purity of the sodium pyruvate was found to be about 95 % by gas radiochromatography when the crystalline substance was freshly dissolved. To avoid decrease in purity the pyruvate was stored in 0 6 M perchloric acid (Beatty *et al.* 1966)

Reagents

1 Glycine buffer 2 M glycine buffer containing 0.8 M hydrazine sulphate and 1.0 g EDTA per 100 ml adjusted to pH 10 0 with NaOH.

2. LDH (lactic dehydrogenase, from rabbit muscle) A crystalline suspension of 10 mg enzyme protein in 1 ml 2.2 M ammonium sulphate solution was used (Boehringer)

3 NAD (nicotinamide adenine dinucleotide) A 0 08% M NAD solution in water was used (Boehringer)

4 Phenylhydrazine hydrochloride, 4 g per 100 ml in water

5 Pyruvate carrier 1 M sodium pyruvate solution

6. Rinsing liquid. 0.2 M KCl HCl-buffer with pH 2.8

7 Scintillation liquid. Toluene containing 5 g per litre of 2,5-diphenyl oxazole (PPO) and 0.45 g per litre of 1 4-bis-2 (5-phenyloxazolyl)-benzene (POPOP)

Commercially available reagent grade chemicals were used The water was glass-distilled.

Procedure

5 ml blood was precipitated with an equal volume of ice-cold 0 6 M perchloric acid (PCA) within a few seconds. The blood and PCA mixture were carefully

stirred and then centrifuged for 5 min at 4500 rpm. The supernatant was centrifuged once again and 5 ml was taken off 1 ml 0 40 M K_3PO_4 was added and after cooling in ice for 10 minutes, the precipitated potassium perchlorate was eliminated by centrifugation. The blood extract prepared in this way was kept up to 6 days at 4° C until analysed.

In a test tube 2.5 ml blood extract were mixed with 3.5 ml glycine buffer 0 1 ml LDH solution and 0.5 ml NAD solution and incubated at 36° C

After 90 minutes the pH was adjusted to 2.8 by addition of 0 6—0.8 ml concentrated HCl. 0 6 ml phenylhydrazine hydrochloride solution and 0 1 ml pyruvate carrier were added and the phenylhydrazone was allowed to form for 20 min at room temperature. The precipitation was facilitated by rocking the test tubes.

The samples were cooled in ice for 20 min, whereafter the precipitate was filtered off and washed twice with 2 ml of the rinsing liquid The filter paper together with the precipitate was dried at room temperature for about 12 hours. It was then transferred into a glass vial for liquid scintillation counting 2 ml methanol and 12 ml scintillation liquid were added and the vial was shaken for 20 min. The radioactivity was determined in a liquid scintillation counter (Packard Tri Carb equipped with a Model 3003 spectrometer) Quenching was estimated by adding an internal standard

A blank was analysed in an identical manner but with the NAD and LDH solutions substituted by water and 2.2 M ammonium sulphate respectively

To determine the specific activity of lactate the total lactate concentration was analysed in 0 ml of the same blood extract by an enzymatic method based on the techniques described by Hohorst (1962) and Lundholm Mohme-Lundholm and Vamos (1963) as modified by Wahren (1966)

Statistical methods Most of the statistical calculations were analyses of variance as described by Dixon and Massey (1957) using a statistical model with one or two variables of classification and repeated measurements. The tests of individual means were performed according to Duncan as described in Documenta Geigy (1962) The following probability (p) levels of significance were used $p<0.001$ $p<0.01$ and $p<0.05$ Unless otherwise stated the results given in the text in this chapter are means ± standard error of estimate (M ± SE)

Experimental, results and comments

The recoveries reported are mostly expressed as per cent of the total radioactivity in the sample. The latter was calculated by counting 3 to 5 portions (0.5 ml) of the blood extract in a dioxane naphthalene scintillation liquid. Blanks were subtracted only when stated.

1 *Blood extract preparation*

A 5 ml and a 2 ml blood portion from 23 samples were deproteinized with equal volumes of ice-cold 0 6 M PCA. After stirring, cooling, centrifugation and perchlorate precipitation the lactate content was determined according to the method used for the determination of total lactate. No differences were found in lactate content or in blank extinctions when 5 or 2 ml blood was used.

Comments In order to be able to use small doses of the isotope it is desirable to extract the lactate for the determination of labelled lactate from fairly large blood volumes. It is impracticable, however to collect large volumes of blood each time since as a consequence of the rapid metabolism of lactate and pyruvate in blood cells, it is necessary to precipitate the blood proteins shortly after obtaining the blood sample. However the results from this experiment show that it is possible to increase the volumes of the blood samples to 5 ml without affecting the analyses of the concentration of total lactate in the blood.

2. *pH optimum for the enzymatic reaction*

0.5 ml blood extract containing labelled DL-lactate and about 2 mmoles/l L(+)-lactate was added to 10 different 1 M glycine buffers containing 0 4 M or 0 8 M hydrazine sulphate and 1.0 g per 100 ml EDTA and with pH adjusted to 7 0 8.0 9 0 10 0 and 11 0 Despite larger total volumes, the volume ratios, reagents and procedure were identical with the analytical procedure for total lactate The pH was determined after the addition of the PCA-extract and the enzyme solutions. The results are presented in Fig 1

The recovery increased markedly when the pH was raised above 7 In the pH interval 8.8—10 4 however the pH had no significant effect. No effect of the hydrazine sulphate concentration on the recovery was found.

3 *The eff ct on pH of type of buffer blood extract and reagents*

Four buffers with pH adjusted to 10.0 were used. They were 1 M or 2 M with respect to glycine and 0 4 or 0.8 M with respect to hydrazine sulphate and contained 1 0 g per cent EDTA. To 3.5 ml of the buffer were added 2.5 ml blood extract, 0.5 ml NAD solution (60 mg/ml) 0 15 ml 2.2 M ammonium sulphate and 0 02 ml 1 N HCl. The pH was determined after each addition.

With these experimental conditions the pH never fell below 9 6 when the weak buffer was used ($n = 10$) or 9 7 when the strong buffer was used ($n = 10$) The fall in pH was exclusively produced by the addition of the blood extract and the ammonium sulphate solution.

Comments From these studies it can be concluded that, using a 2 M glycine buffer it is possible to add 2.5 ml blood extract to 3.5 ml buffer using a 0.8 M

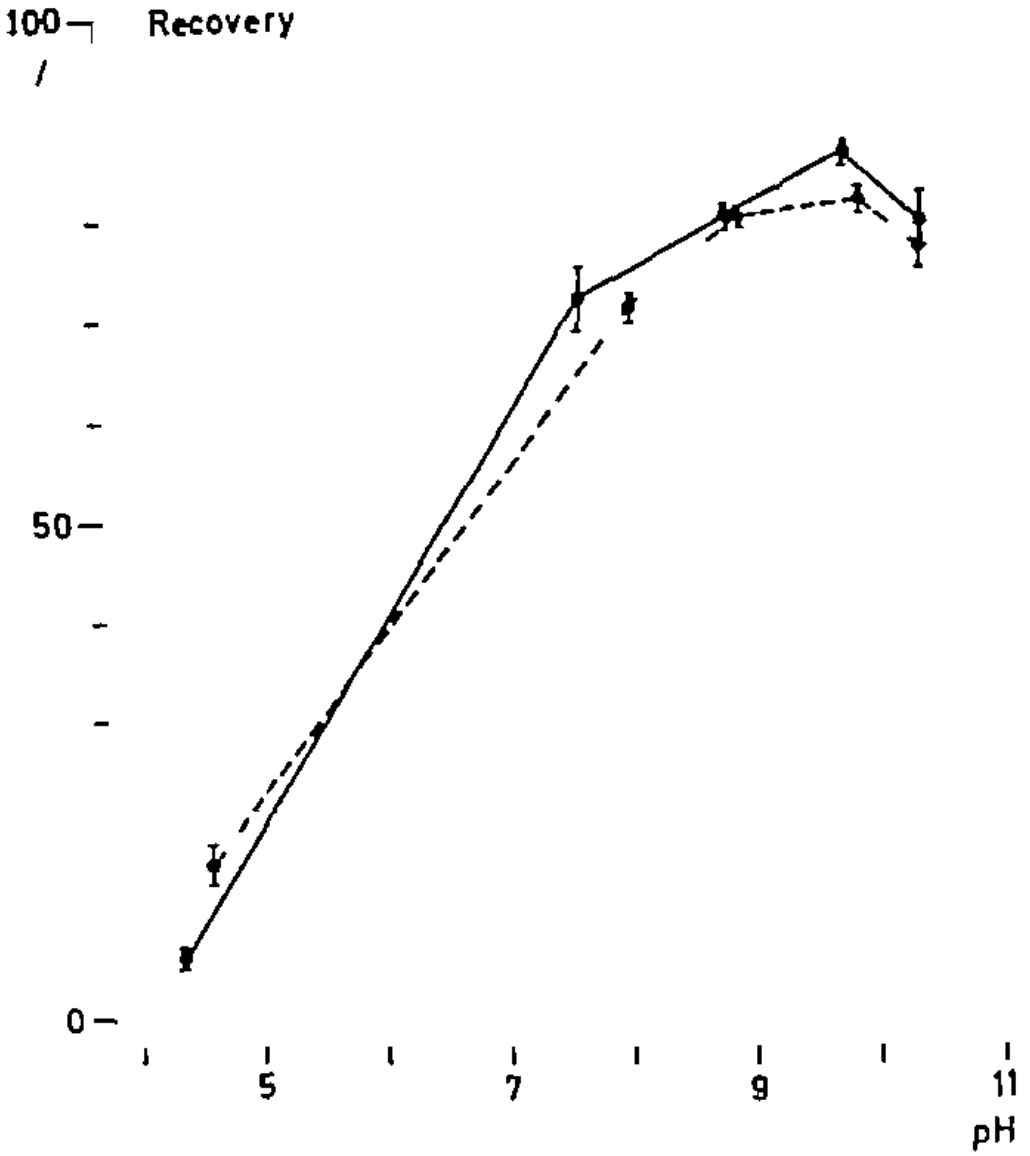


Fig. 1 Relationship between recoveries expressed as per cent of added L-L(+)-lactate and the pH in the enzymatic reaction with two buffers containing 0.4 M (●—●) and 0.8 M (● --●) hydrazin sulphate. For further explanation see experiment 2. Mean values and SE of 10 experiments of each series are given.

hydrazine sulphate concentration and 0.15 ml LDH solution and still keep the pH within the optimal range found for the enzymatic reaction. As shown in a later part of the experiments it is desirable to use the higher hydrazine sulphate concentration to improve the trapping of pyruvate.

Lundholm Mohme Lundholm and Vamos (1963) reported that the addition of the NAD and LDH solutions somewhat lowered the pH, and so did the free hydrogen ions formed at the reduction of lactate to pyruvate. The acidi-

fying effect of the LDH solution was found to be due to the 2.2 M ammonium sulphate solution in which the enzyme was suspended. The HCl was added in order to simulate the free hydrogen ions formed at the reduction of lactate. The fact that the addition of the HCl did not affect the pH significantly is of importance since the lactate concentration in the blood is not constant.

4 *The effect of the blood extract/buffer volume ratio on the recovery of ^{14}C-L(+)-lactate*

In this experiment the 2 M glycine buffer with 0.8 M hydrazine sulphate and 1.0 g per 100 ml EDTA was used. In three series increasing volumes of blood extract (0.5 2.5 and 3 0 ml) were added to 5.5 3.5 and 3 0 ml respectively of the buffer Labelled lactate was added to the blood extract in such a way that all samples contained about the same amount of radioactivity Also unlabelled lactate was added to the blood extract, corresponding to a blood concentration of about 10 mmoles/l. 0 15 ml LDH solution with 10 mg enzyme protein per ml was used The rest of the procedure was identical with that in the general description above. Three series of blanks were also analysed

The following recoveries were obtained 89.0 ± 1.8 86.8 ± 2.1 and 86.4 ± 1.3 % (n = 10) respectively The recovery decreased slightly with increasing volume ratios but the difference between the groups was not significant. No differences were found between the blanks.

Comments The lowest blood extract/buffer volume ratio used in this experiment is the same as in the method for determination of total lactate. In an additional experiment this method was spectrophotometrically found to give a complete recovery also at the lactate concentrations used in this experiment. The fact that no significant differences in recovery were found between the three series tested indicates that also in 3.0 ml blood extract, with a concentration of lactate corresponding to 10 mmoles/l blood, all the lactate was oxidized to pyruvate with the present procedure.

5 *Effect of the hydrazine sulphate and NAD concentrations on the recovery of ^{14}C-L(+)-lactat*

A. Three buffers containing 0 2 0 4 and 0 8 M hydrazine sulphate respectively were used in this experiment. Unlabelled lactate, corresponding to a blood concentration of about 10 mmoles/l, was added to the blood extract. Otherwise the procedure was identical with that in the general description. The recoveries were 83 1 ± 1 1 82.6 ± 0.9 and 86 0 ± 1.2 % (n = 10) respectively Although the recovery was highest in the series with the highest hydrazine sulphate concentration, no statistically significant difference was found between them

B Four buffers, one of them containing no hydrazine sulphate and the others 0 4 0.8 and 1 6 M hydrazine sulphate respectively were used in this experiment. Both unlabelled pyruvate corresponding to a blood concentration of about 10 mmoles/l and labelled pyruvate were added to the blood extract. The rest of the procedure was identical with that for the blanks in the general description.

The recoveries were 78.5 ± 0.9 78.6 ± 1 2, 75.8 ± 1 1 and 78.0 ± 1.3 % (n = 10) respectively No significant difference was found between the four series.

C. 0.5 ml of four NAD solutions (0 027 M 0 054 M 0 082 M and 0.108 M) were used. The rest of the procedure was identical with the general one.

The recoveries were 72.4 ± 2.3 82.9 ± 1 7 85.3 ± 1.8 and 84 2 ± 1 1 % (n = 10) respectively The means of the three groups with the highest concentrations of NAD were not significantly different.

Comments Lactate is oxidized enzymatically according to the reaction

$$\text{lactate} + \text{NAD} \xrightleftharpoons{\text{LDH}} \text{pyruvate} + \text{NADH} + \text{H}$$

The final position of equilibrium for this reaction can be kept to the extreme right by an excess of NAD and by trapping of the pyruvate as hydrazone in the presence of hydrazine sulphate. To compensate for high concentrations of lactate a high concentration of the hydrazine sulphate was used. This could be done since the results of these experiments indicated that the increased concentration of hydrazine sulphate did not exert a negative effect on the precipitation of the pyruvate as phenylhydrazone The results also suggest a slightly positive though not significant effect of an increased concentration of hydrazine sulphate on the recovery An improved recovery was found when the addition of NAD was increased up to 0.5 ml 0 054 M solution.

6. *Effect of the LDH concentration incubation time and temperature on the recovery of ^{1}C L(+) lactate*

A. The experiment included 12 series with 10 samples in each. In six series 0 15 ml LDH solution (10 mg/ml) was used and in the others 0 06 ml of the same solution plus 0.09 ml 2.2 M ammonium sulphate. Three incubation times (30, 60 and 120 min) and two incubation temperatures (25 C and 36° C) were used for each LDH concentration. Otherwise all steps were identical with the general procedure

The results are presented in Fig 2. Statistical analyses of the results showed that at 36° C maximal recovery was achieved after 60 min also when the lower concentration of LDH was used

B. 0.03 0 06 0 10 and 0 15 ml of the LDH solution (10 mg/ml) were used in this experiment 2 2 M ammonium sulphate was added in order to obtain

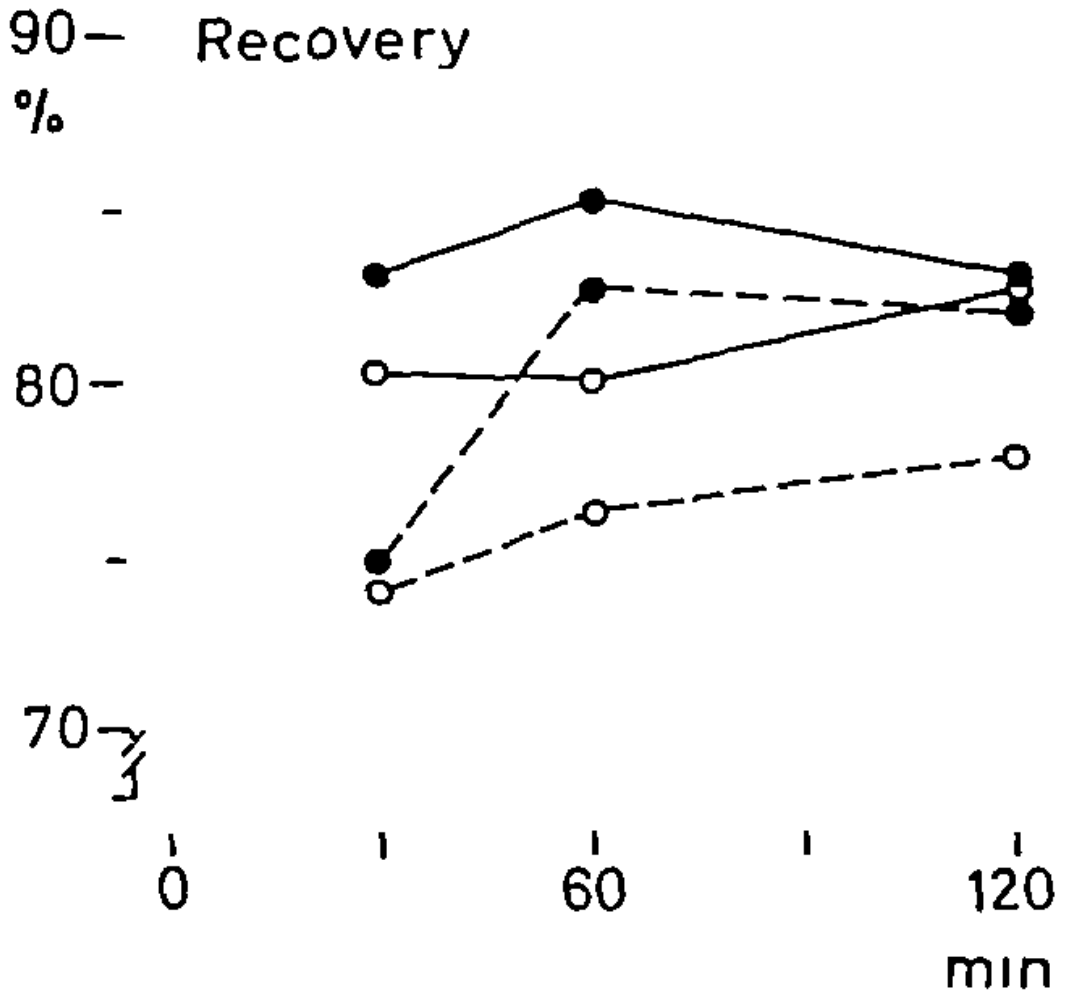


Fig 2. Relationship between recovery expressed as per cent of added ^{14}C-L(+)-lactate and incubation time t 25 C (○) and 36° C (●) and using 0.06 ml (— — —) and 0 15 ml LDH solution (———) Mean values for 10 experiments of each series are given. For further explanation see experiment 6 A.

identical volumes in the samples. As in the general procedure the incubation time was 90 min and the temperature 36° C. The recoveries were 79.9 ± 2.3 81 2 ± 1 4 84.9 ± 0.8 and 85.2 ± 1 4 % (n = 10) respectively The means of the three series with the highest LDH concentrations were not significantly dif ferent.

Comments As a consequence of the large amounts of lactate to be oxidized to pyruvate it is of importance that the reaction is allowed to occur at optimal temperature and that the LDH concentration is so high that it does not limit the reaction velocity As long as the enzyme is not inactivated, the reaction velocity can be expected to increase with temperature. In experiment A it was found that the optimal recovery was reached within 60 min at a reaction temperature of 36 C at both of the LDH concentrations used. At a reaction temperature of 25 C, however the optimal recovery was not obtained until

TABLE 1 The effect of pH on the precipitation of the phenylhydrazone of pyruvic acid expressed as fractional recovery For further explanation see the text t experiment 8 A. The probability (p) that the differences between the means were caused by random factors are indicated

pH	2.1	2.5	2 8	3 4	4 8—8.6
recovery % (Mean ± SE)	75.2 ± 0 7	77 0 ± 0.7	79.9 ± 1 1	79 0 ± 1 3	3 7 ± 1 1
n	10	10	10	10	10
analysis of variance		$p < 0.01$	—— $p > 0.05$ ——		

after 120 min incubation at the higher LDH concentration while the reaction was even slower at the lower enzyme concentration. These findings are not in agreement with the statement made by Horn and Burns (1956) that the LDH would be quickly destroyed at temperatures above 30° C. It was found that 0 1 ml of LDH solution containing 10 mg/ml of enzyme protein is enough for an optimal recovery if the reaction is allowed to occur for 90 min at 36° C.

7 *Effect of concentration of lactate*

Four portions of blood extract with different lactate concentrations were prepared but with the same concentration of labelled DL-lactate Samples from each of them were analysed for labelled lactate according to the general procedure. The total lactate concentration in the blood extract was also analysed and found to correspond to 2.7 5 1 7 9 and 11.8 mmoles lactate per litre blood. The recoveries were 87.3 ± 0 7 87 1 ± 1.3 86.7 ± 0.9 and 86.3 ± 0.9 % (n = 10) respectively No significant difference was found between the four means.

Comments In studies of lactate metabolism it is necessary that the analytical method permits a lactate concentration in blood of up to 10 mmoles/l. It is found in this experiment that the recovery is constant within the desired range.

8 *The precipitation of pyruvic acid as phenylhydrazone*

A series of experiments were performed to illustrate the different factors in the precipitation procedure. In all these experiments except in experiment D the oxidation procedure described was simulated by using labelled and unlabelled pyruvate. With the exception of the variable studied in each experiment the procedure was identical with the general procedure for the blanks.

A. Five volumes of concentrated HCl were added giving five series with different pH at the precipitation of the phenylhydrazone. The recoveries within the different series are given in Table 1

TABLE 2 The effect of the mount of added pyruvate carrier and phenylhydrazine hydrochloride on the precipitation of the phenylhydrazone of pyruvic acid expressed as fractional and absolute recovery (M±SE, n = 10) For further explanation see the text to experiment 8 B. The probability (p) that the differences between the means were caused by random factors are indicated

pyruvate carrier mmoles	0 06	0.08	0.10
phenylhydrazine hydrochloride mmoles	0 10	0.13	0 17
fractional recovery %	71 6±1 4	80.9±1.2	86.8±0.9
analysis of variance		p<0 01	———
absolute recovery cpm	10,950±250	11,580±150	11,330±100
analysis of variance		p<0.05	———
		——— p> 0.05 ———	

B. Increasing amounts of pyruvate carrier (0 06, 0.08 and 0 1 mmoles) and phenylhydrazine hydrochloride (0.10 0 13 and 0 17 mmoles respectively) were used the total volumes being kept constant. The recoveries within the different series are given in Table 2.

C. The filter paper with the precipitate was washed with either water or a 0 2 M KCl HCl buffer with pH 2.8 in both series with 2×1 ml, 1×3 ml or 2×2 ml The recoveries of total pyruvate were with water 83 1±0 9 80 0± 0 6 and 79 1±1 1 % (n=7) and with the buffer 84 6±0.8 84 1±1 9 and 84.3±2.8 % (n=7) respectively The difference in recovery between the washing liquids was significant (p<0 01) but not the effect of volumes.

D Labelled and unlabelled L(+)-lactate was added to the blood extract. The rest of the procedure was identical with the foregoing one, i.e. no LDH or NAD was added The remaining activity in the filter papers in per cent of the added activity was for water 2.07±0.05 1 74±0 04 and 1 05±0 06 % (n=8) and for the buffer 1.85±0 06 1 57±0.04 and 1.04±0.05 % (n=8) respec tively A significant interaction existed between type of liquid and volumes.

Comments The isolation of pyruvate by precipitation as phenylhydrazone was found to be superior to the generally used 2,4-dinitrophenylhydrazone (Sacks 1956 1961) since the yellow colour of the precipitate was so weak that liquid scintillation could be performed without preceding combustion.

As found in experiment A, it is necessary to adjust the pH to about 3 in order to precipitate the pyruvate as phenylhydrazone and the most complete precipitation was found at about pH 2.8. This agrees with the observation by Friedemann and Haugen (1943) that the reaction of nitrophenylhydrazines with carbonyl compounds only proceeds in acid aqueous solutions, a fact which

also explains why nitrophenylhydrazines or phenylhydrazine cannot be substituted for hydrazine as a pyruvate trap in the enzymatic reaction.

The fact that the labelled lactate is not completely recovered as phenylhydrazone is probably due mainly to an incomplete precipitation of the phenyl hydrazone. Considering the recovery as a fraction of added radioactivity it would consequently be favourable to add large amounts of pyruvate carrier and phenylhydrazone. Since the error at the liquid scintillation decreases with increasing cpm, it is better to adjust the procedure so as to recover as much as possible of the added radioactivity as cpm. As found in experiment B the fractional recovery increased with added pyruvate carrier and phenylhydrazine as expected. However the radioactivity recovered as cpm rather decreased when the highest concentrations of these reagents were used.

If the phenylhydrazone formation was allowed to occur for 20 min at room temperature an optimal recovery was reached. Under these conditions no phenylosazone formation with glucose was found

It is necessary to wash the precipitate and the filter paper to remove radioactivity not precipitated as phenylhydrazone. However since the phenylhydrazone of the pyruvic acid is to some extent soluble in water it is desirable to wash with small volumes in order to avoid a decreased recovery As found in experiments C and D a good washing effect is achieved with 2×2 ml of the buffer without a significant decrease in recovery

9 *Determination of the radioactivity*

Since the total weight of material on the filter paper to be counted is rather great (about 40 mg) it is necessary that the material is dissolved in the scintillation liquid in order to avoid "layering" (Blair and Segal 1962) In experiments performed it was found most favourable to add 2 ml methanol both with respect to fractional recovery and recovered cpm

Because of the weak yellow colour of the phenylhydrazone to be counted, a test was made to see whether a somewhat higher concentration of the scintillators than ordinarily used would increase the counting efficiency This was found to be the case to some degree for the POPOP but an increase of the concentration of PPO as well did not improve the efficiency The efficiency was found to be 65.1 ± 0.5 %

The internal standard technique is valid only if the quenching is the same for the activity in the sample as for the added standard This was found to be the case when tested by the channel quotient technique ($n = 30$)

The geometry of the filter paper in the vials in relation to the photomultipliers was found to have no effect on the counting efficiency Blair and Segal (1960) also conclude that the orientation of the paper is of minor importance.

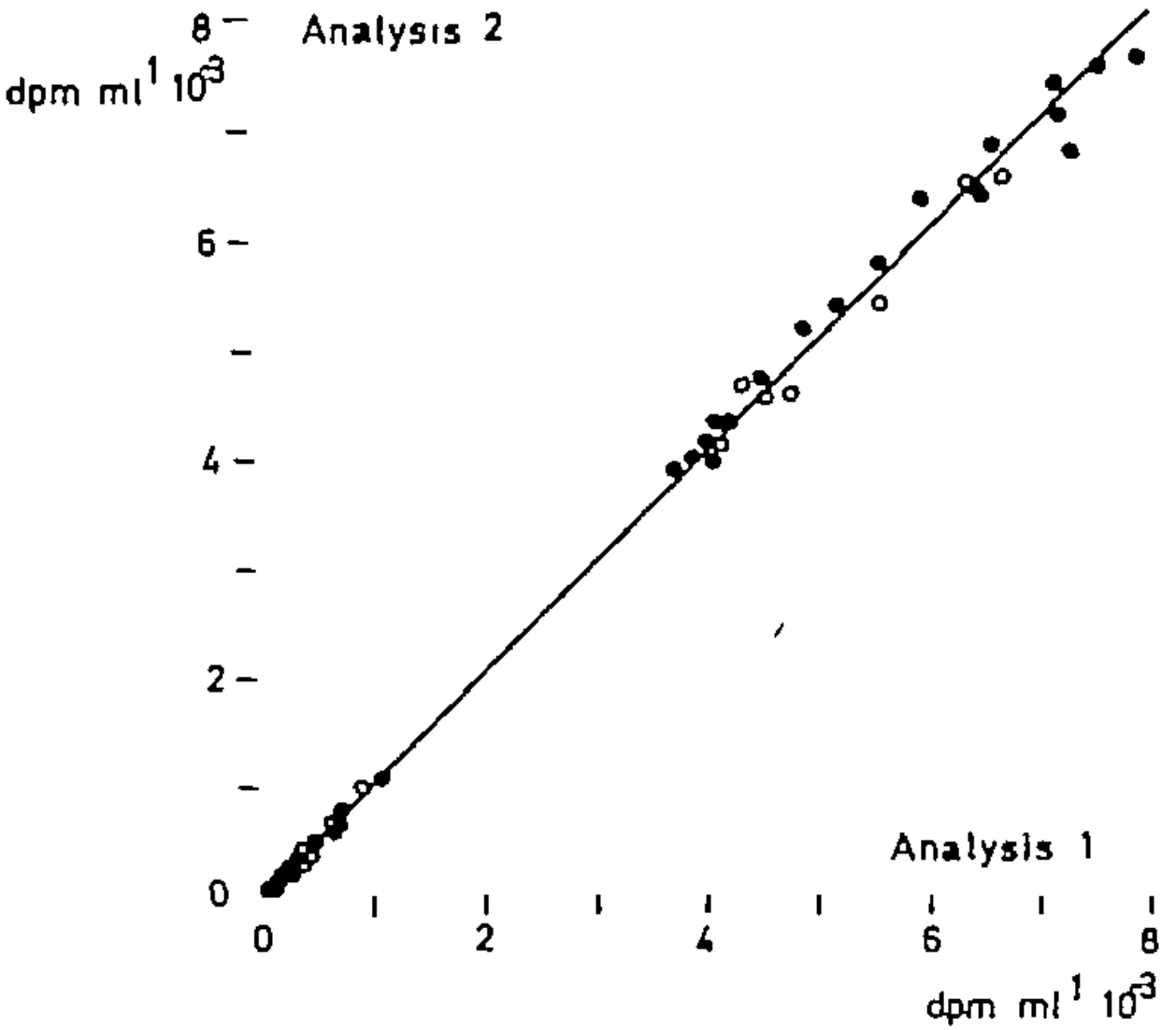


Fig 3 Comparison of duplicate analyses. Analysis 1 was performed after one day and analysis 2 after 2 (○) and 6 (●) days respectively The regression equations and correlation coefficients are

analysis 2 after 2 days $y = 1.01 + 4.6$ $= 0.99$
nalysis 2 after 6 days $y = 1.00x - 9.9$ $= 0.99^{*}$
otal $y = 1.00x - 7.4$; $= 0.99$

The line of identity is shown.

10 *Recovery and reproducibility*

A. Ten samples containing unlabelled L(+)-lactate corresponding to about 10 mmoles/l blood and ^{14}C-L(+) lactate were analysed The fractional recovery was 86.2 ± 4.0 % (mean ± SD) and the blank was 1.04 ± 0.14 % (mean ± SD) The total recovery was 85.2 ± 4.0 % (mean ± SD)

B. Duplicate analyses were performed on 64 blood samples from human studies. One determination was made after one day and the other after 2 and 6 days respectively In all cases the blood extract was prepared immediately

and then kept at +4° C. The results are presented in Fig 3 No statistical difference was found between analyses 1 and 2 in the two groups. The error expressed as the standard deviation (SD) for a single determination was found to be 156 dpm/ml and the corresponding coefficient of variation 2.8 % in samples containing more than 1000 dpm/ml and 72 dpm/ml and 7 2 % in samples containing less than 1000 dpm/ml.

Discussion

It is assumed that the enzymatic oxidation of lactate to pyruvate is complete. This is supported by the following facts

1) when the concentrations of LDH and NAD were increased, a constant level was reached for the recovery which could not be surpassed either by a further rise in enzyme concentration or by reducing the lactate concentration

2) when the analysis was repeated once again on the filtrate, no difference was found between the sample and its blank.

The loss in recovery is probably due to the incomplete precipitation of the phenylhydrazone. No other carbonyl reagent has been found, however which forms a colourless and more completely precipitable compound with pyruvic acid.

The specificity of the method is not entirely dependent on the specificity of the LDH used, since a product formed by interaction of impurities affects the results only if it is precipitated This is probably the case only for pyruvate. Of the substances tested by Lundholm, Mohme Lundholm and Svedmyr (1963) DL-glyceric acid was the only one which to an appreciable degree simulated lactate using LDH for enzymatic lactate determination. In this procedure the DL-glyceric acid would seem to be of importance only in case of a pathological metabolism of this substance (Williams and Smith 1968) In biological experiments, when labelled L(+)-lactate is infused, the blank values depend mainly of course on the labelled lactate converted to pyruvate In practical applications of the method the blanks have never exceeded 15 per cent of the "sample"

CHAPTER III A

General Procedures and Methods

Procedures

Catheterization The procedures for the catheterization of the forearm vessels agree with those described earlier (Wahren 1966) and are therefore only briefly outlined below After the hand and forearm had been immersed in hot water for 10 min a teflon catheter was inserted percutaneously into a cubital vein and manipulated into a deep forearm vein so far that its tip was no longer palpable. It had then been introduced approximately 3—9 cm. The radial artery was punctured at the level of the radial styloid process and the brachial artery at the intercondular line. Teflon catheters were inserted 2—5 cm in the proximal direction at both places. The right arm was used except in one subject who was left handed For administration of hypertonic sodium lactate solution (Chapter III.D) a teflon catheter was introd ced percu taneously into a cubital vein in the other arm and its tip was placed in the superior vena cava about 5 cm from the right atrium.

Exercise Rhythmic forearm exercise was performed on an adjustable hand ergometer according to the procedure described by Wahren (1966) The subjects were in the supine position and with the forearm horizontal at an angle of approximately 45 to the trunk. The elbow was extended and the volar side of the hand upwards. The resting position of the ergometer handles was adjusted until the middle phalanges of the second, third and fourth fingers were approximately vertical In all experiments the path of contraction was 30 mm and the exercise was performed with 60 contractions per min in time with a metronome. The work intensity was set to 10 kpm/min by using an average spring tension during contraction of 5 6 kp. The exercise performed was close to isotonic, the increase in tension during the contraction being 8 per cent of the mean tension. The movements of the ergometer handle were registered on an ink jet recorder (Mingograf 4? B Elema-Schönander) via a linear motion potentiometer connected to the handle and a direct current circuit.

Experimental design The experimental design of the studies in Chapter III-C and D is illustrated in Fig. 4 When the catheters had been inserted the subjects were allowed to rest for about 30 min before they started the exercise period, which lasted approximately 60 min. On two occasions during the

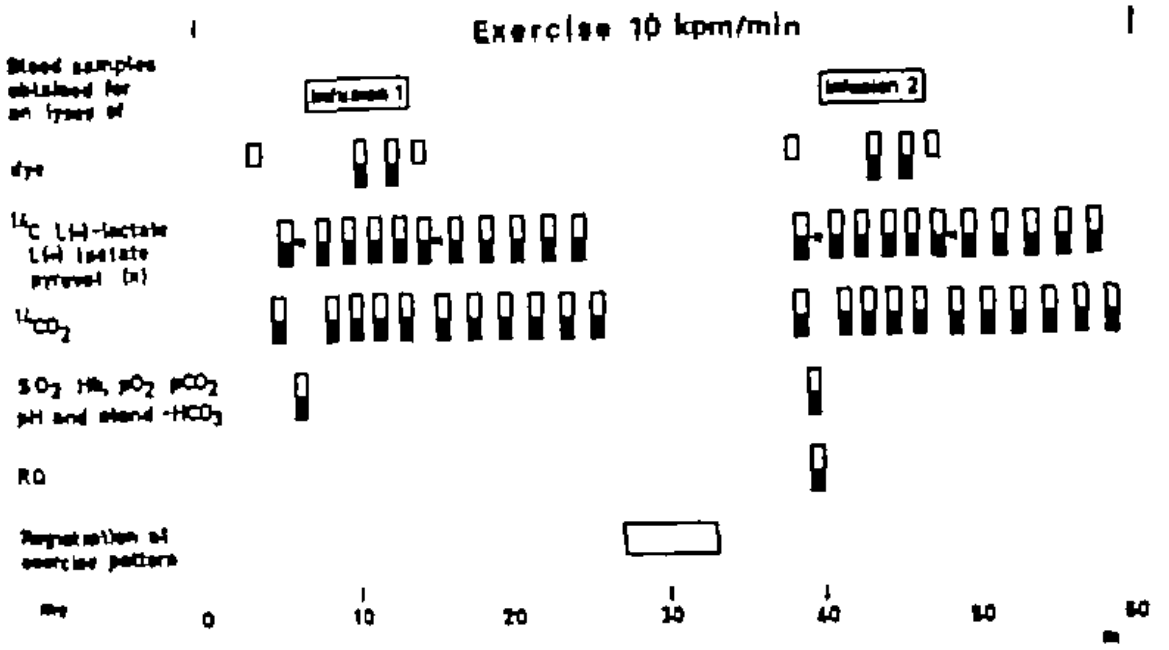


Fig 4 Th experimental design of th studies in Chapter III The pproximate occasions for blood sampling from th radial artery (□) and deep ein (■) are indicated. An asterisk denotes blood samples in which also the pyruvat concentration was analysed. For further explanation see text.

exercise period at about 7 and 40 min from the start (range 7—11 and 39—42 min respectively) infusions of a dye solution containing L(+) lactate-1 ^{14}C into the brachial artery for determination of blood flow and lactate turnover were started The infusions lasted 6—7 min. Background concentrations of dye ^{1} C-L(+)-lactate and $^{14}CO_2$ were determined before the infusion periods. The dye concentration of arterial and deep venous blood was analysed twice during the infusions and furthermore in arterial blood about 20 sec after the end of infusions Arterial and venous concentrations of ^{14}C-L(+)-lactate, total lactate and $^{14}CO_2$ were determined repeatedly during and up to 12 min after the infusions. The samples obtained just before and immediately after the infusions were also analysed for concentration of pyruvate. The total lactate and $^{4}CO_2$ values obtained during the infusions were corrected for dilution by the infused solution. Oxygen saturation hemoglobin concentration pO_2, pCO_2, pH and standard bicarbonate were determined in arterial and venous blood immediately before the infusions. The local respiratory quotient (RQ) was determined before the last infusion. The arterial and venous samples were obtained simultaneously and were thus not fully comparable. No correction was made for that fact.

The movements of the ergometer handle were registered in the interval be-

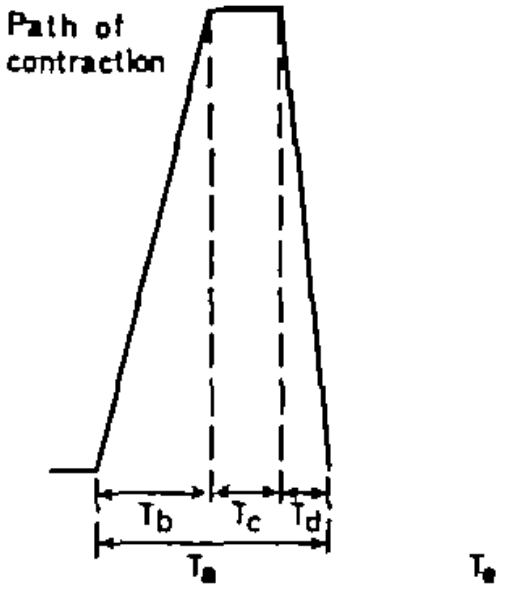


Fig 5. The symbols used for denoting the duration of the different phases of contraction and relaxation during exercise. The square root of the sum of the squares of the coefficients of variation for T_a, T_b, T_c, T and the contraction distance was calculated as an expression for the variability in exercise pattern.

tween the infusion periods. The symbols used for denoting the duration of the different phases of contraction and relaxation appear in Fig 5

Labelled lactate Sodium L(+)-lactate-1 ^{14}C, which was the only type of labelled lactate infused, was obtained from Volk Radiochemical Co., USA, and New England Nuclear Corp. USA. According to the suppliers the radiochemical purity of the substance was 98 %. When the labelled lactate was decarboxylated to acetaldehyde and CO_2 according to Brin and Olson (1952) the radioactivity in the aldehyde-dimedon complex obtained was less than 0.1 per cent of the radioactivity in the CO_2. This verifies that the lactate was labelled only in the carboxyl carbon. In all studies the radiochemical and optical purity of the substance used was tested by the analytical method described in Chapter II. Within the error of the method all the radioactivity was recovered as ^{14}C-L(+) lactate if it was taken into consideration that the recovery of the analytical method was 85.2 %

Unlabelled lactate The hyperosmotic sodium lactate solution which was used in the study described in Chapter III.D was obtained from Pharmacia, Sweden. When analysed enzymatically 90 % of the lactate was found to consist of the L(+) isomer

Forearm blood flow at rest ($\dot{Q}$ ml/min) was calculated from observed values of total forearm volume (TV ml) wrist circumference (W cm) and forearm skinfold thickness (S mm) using the following equation (Wahren and Hagenfeldt 1968) $\dot{Q} = 0.053\ TV + 9.20\ S - 7.45\ W + 70.3$ *The volume of the forearm* was measured by water displacement. The circumference of the wrist was measured with a tape just proximal to the radial styloid process. *Skinfold thickness* was measured with a skinfold caliper (Harpenden)

Forearm blood flow during exercise was determined as described by Wahren (1966) with the exception that a modification was introduced for background calculation during prolonged infusions (see Chapter III.B) *Muscle blood flow during exercise* ($\dot{Q}_m$) was calculated as the brachial artery blood flow determined by indicator dilution technique, minus half the resting forearm blood flow (Wahren 1966)

Maximal force of finger flexion was tested according to Tornvall (1963) using a strain gauge dynamometer and a measuring bridge (Philips PT 1200/01) This equipment was calibrated against known forces.

Statistical methods Most of the statistical calculations were performed according to Snedecor (1956) The following probability (p) levels of significance were used $p<0.001^{***}$ $p<0.01^{**}$ and $p<0.05^{*}$ Unless otherwise stated, the results given in the text in this Chapter are means $\pm$ one standard deviation ($M \pm SD$)

Blood analyses

Sampling Blood samples were drawn into disposable plastic syringes for the analyses performed on a perchloric acid blood extract and for the indocyanine green analyses. All other blood samples were collected in 10 or 20 ml siliconed glass syringes. The dead space in each syringe was filled with heparin solution (5000 I U /ml, Vitrum, Sweden) The hemoglobin concentration values were corrected for dilution by the heparin.

The *errors of the methods* are presented in Table 3.

^{14}C L(+)-lactate and total lactate concentration see Chapter II It was found that the presence of dextran (about 10 g/l) and indocyanine green (about 10 mg/l) did not influence ^{14}C-L(+) lactate or total lactate concentration analyses

Pyruvate concentrations were analysed enzymatically mainly according to Segal Blair and Wyngaarden (1956) and Bücher *et al* (1962)

$^{14}CO_2$ in blood was determined as described by Hagenfeldt (1967) It was found that the recovery in this method depended on the volume of the blood sample used. The recovery was complete up to 3 ? ml blood but then decreased linearly by 9 0 % per ml blood. On this account every sample was corrected for the incomplete recovery The blood samples used in the present studies were smaller than 5.5 ml. Lactic acid was used to liberate the CO_2 from blood in order to avoid oxidation of the ^{14}C-lactate present in the blood.

Oxygen saturation was determined spectrophotometrically (Holmgren and Pernow 1959)

pO_2 was measured with an oxygen electrode (Beckman Spinco Division) in

TABLE 3. The error for a single determination in various blood analyses, expressed as th standard deviation (SD) for single determination and the corresponding coefficient of variation. Th calculations are based on duplicate analyses on the same blood samples.

	Range	SD	Coefficient of variation	n
Oxygen sat. %	20—50	0.4	0.9	42
Oxygen sat., %	90—100	0.3	0.4	41
pO_2, mm Hg	15—30	0.8	5.4	15
pO_2, mm Hg	80—140	1 4	1.5	15
pCO_2, mm Hg	30—50	1.2	3 1	15
pH	7.30—7 60	0.01		15
Stand. bicarbonate meq/l	20—30	0.6	2 6	15
Blood CO_2 content, ml/100 ml	40—70	0.2	0.4	15
Blood O_2 content, ml/100 ml	3—20	0 15	1.3	15
Hemoglobin, g/100 ml	11—17	0.07	0.5	76
Hematocrit, %	34—47	0.2	0.5	87
Indocyanin green, mg/l	3—8	0 17	3 4	40
Lactate, mmoles/l	<1.0	0 05	6.8	33
Lactate, mmoles/l	1 0—5.0	0 06	3.2	36
Pyruvate, mmoles/l	<0.3	0.010	8.0	22
^{14}C-L(+)-lactate, dpm/ml	0—1000	22	7.2	38
^{14}C-L(+) lactate, dpm/ml	1000—10000	156	2.8	26
$^{14}CO_2$, dpm/ml	1500—3000	80	3.9	60

combination with a Beckman model 160 physiological gas analyzer The electrode was mounted in an open cuvette described by Holmgren (1965)

pH pCO and standard bicarbonate were measured with the microtechnique described by Siggaard Andersen (1963)

Carbon dioxide content of blood and for RQ determinations also the oxygen content, was determined according to Peters and van Slyke (1932)

Hemoglobin concentration was analyzed by the cyanmethemoglobin technique (Drabkin and Austin 1935) using a reagent and standard from Hycel Inc. USA.

Hematocrit was determined and corrected for trapped plasma (1.3 %) according to Garby and Vuille (1961) using a high speed, micro-capillary hematocrit centrifuge (Wifug Automatic 102—11)

Indocyanine green concentrations were measured spectrophotometrically in serum obtained after clotting and centrifugation of 2 ml blood samples. The analyses were made at 805 mμ using a Beckman B spectrophotometer A calibration curve was made for each subject with readings at 4 and 8 mg/l

Definitions

a = *arterial concentration* v = *deep venous concentration*

Fractional uptake of ^{14}C *L(+)-lactate* (F_{pl}) was calculated as the (a—v) difference of labelled lactate at relative steady state as a fraction of the arterial concentration of labelled lactate. Values given are means of at least 2 observations.

Uptake of lactate (synonym, extraction of lactate) was computed as the product of F_{pl} and the total concentration of lactate in arterial blood.

Rate of lactate uptake was calculated as the product of lactate uptake and muscle blood flow ($\dot{Q}_m$)

Net uptake and *net release of lactate* are defined as the magnitude of respectively a positive and a negative (a—v) difference of lactate.

Rate of net uptake and *rate of net release of lactate* were calculated as the product of net uptake and $\dot{Q}_m$ and of net release and $\dot{Q}_m$ respectively

Fractional oxidation of ^{14}C*-L(+)-lactate* was calculated as the (v—a) difference of $^{14}CO_2$ at steady state during the infusions as a fraction of the (a—v) difference of ^{14}C-L(+) lactate

CHAPTER III B

Blood Flow Determination

The procedure described by Hagenfeldt and Wahren (1968) for the study of human muscle metabolism during exercise has been used in the present study It is based on blood flow determination during forearm exercise by means of an indicator dilution technique with continuous infusion of the dye (indocyanine green) using an infusion rate of the dye solution of approximately 34 ml/min (Wahren 1966) the relatively high infusion rate being necessary for the achievement of complete mixing of blood and dye solution. Some modifications of the above procedure were required for the present study For determination of the blood flow it is necessary to calculate the concentration of recirculating dye. For infusion periods up to 2 min the concentration of recirculating dye may be considered with acceptable approximation to rise linearly with time (Wahren 1966) In metabolic studies, however it is often necessary to use longer infusion periods in order to obtain a constant (a—v) difference of the studied substance. A more exact model is then required for calculation of the concentration of recirculating dye.

This study was carried out in order to investigate how the concentration of recirculating dye can best be calculated for long infusion periods (6—7 min) and whether a somewhat lower rate of infusion than 34 ml/min could be used without affecting the mixing between the arterial blood and the infused solution.

Procedure and Experimental

A Recirculation

The investigation was made on two subjects who carried out a rhythmic forearm exercise with both arms at an intensity of 10 kpm/min. Through a catheter in the right brachial artery the dye solution (see Wahren 1966) was infused at a rate of 30 ml/min, corresponding to roughly 0.9 mg of dye per minute for 6—7 min. Blood samples were collected from the left brachial artery at 15-second intervals during and up to 1 min after the infusion for determination of the concentration of recirculating dye. Two infusions were made on each subject with an interval of about 40 min.

B Mixing of dye and blood

During the infusion of dye duplicate samples were drawn from the radial artery and deep forearm vein of 7 subjects on 15 occasions for determination of the concentration of dye. The samples from the artery and vein were drawn simultaneously

Calculations

The dye used, indocyanine green is eliminated from plasma at a rate proportional to the plasma concentration (Caesar *et al* 1961) As the rate of administration of dye is constant, the following approximate model can be set up for calculation of the concentration of recirculating dye The following symbols are used

t = time (sec)
a = quantity of dye infused per unit of time
Q = the plasma volume over which the dye is distributed
Y = quantity of dye in Q
C = the concentration of dye in plasma (mg/l) (C_1 C_2 and C_∞ are the concentrations of dye in plasma at times t_1 t_2 and infinity respectively)
k_1 k_2, k_3 and K = arbitrary constants

$$dY/dt = a - k \quad C \tag{1}$$

Under the assumption that $C = Y/Q$ the equation can be written

$$dY/dt + Y \quad k/Q = a \tag{2}$$

The general solution of this equation is

$$Y - a \quad Q/k = K \quad e^{-\quad k/Q} \tag{3}$$

which may also be written

$$C = a/k \quad + (K/Q) \quad e^{-\quad k/Q} \tag{4}$$

This expression yields

$$C_1 = C_2 (1 - k_3 \quad e^{-k_3 \quad t_1}) / (1 - \quad k_3 \quad e^{\quad k_3 \quad t_2}) \tag{5}$$

This relation can be used for calculation of the recirculating dye (C_1) at time t_1 when the corresponding concentration (C_2) is known at another time (t_2) The equation may also be written

$$C = C_\infty \quad (1 - k_3 \quad e^{-k_3 \quad t}) \tag{6}$$

or

$$1 - C/C_\infty = k_3 \quad e^{\quad k} \tag{7}$$

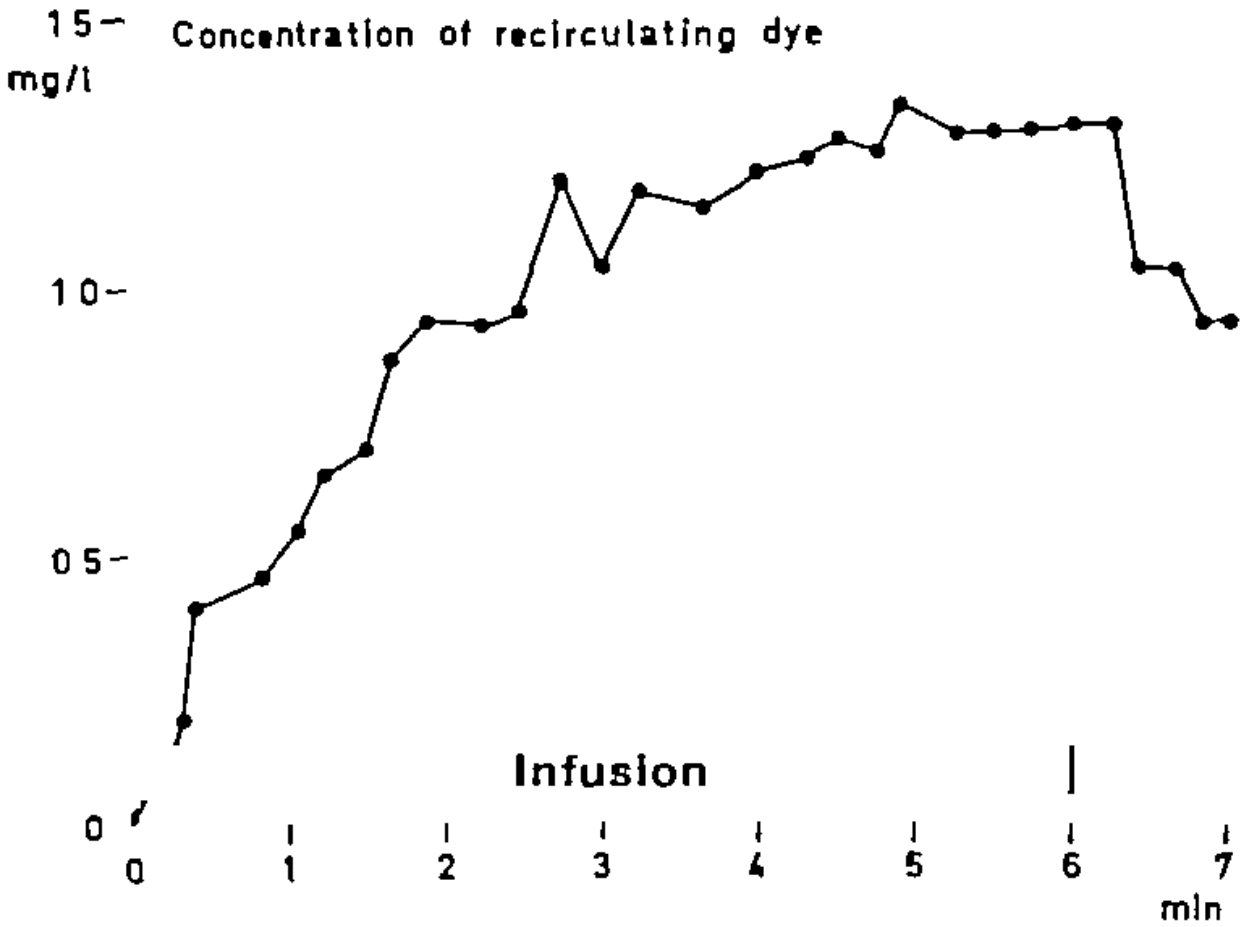


Fig 6 Concentration of recirculating dye during and after infusion of 0.9 mg/min of dye in one subject. Dye concentration determined in blood samples from the contralateral brachial artery during forearm exercise (10 kpm/min)

which makes it possible to determine empirically the arbitrary constants k_2 and k_3. For this purpose the concentrations of recirculating dye in the brachial artery was plotted for each of the four infusion periods, as function of the time, and C_∞ was estimated. For each infusion period the coefficient of correlation was calculated for the regression $^{e}\log (1-C/C_\infty) = {}^{e}\log k_2 - k_3\ t$ with $^{e}\log (1-C/C_\infty)$ as dependent variable both for the estimated value of C_∞ and for some slightly larger and smaller values. The values which corresponded to the found four regressions with optimal coefficient of correlation were lumped together and the constants $^{e}\log k_2$ and k_3 were calculated (Fig 7)

Results

A. (Fig 6 and 7) A typical course of the increase of concentration of recirculating dye is illustrated in Fig 6. The mean concentration of recirculating dye at the end of the infusions was 1.50 mg/l (range 1.35—1 65 mg/l) The mean value for C_∞ at optimal correlation was 1 65 mg/l (range 1 42—1.80

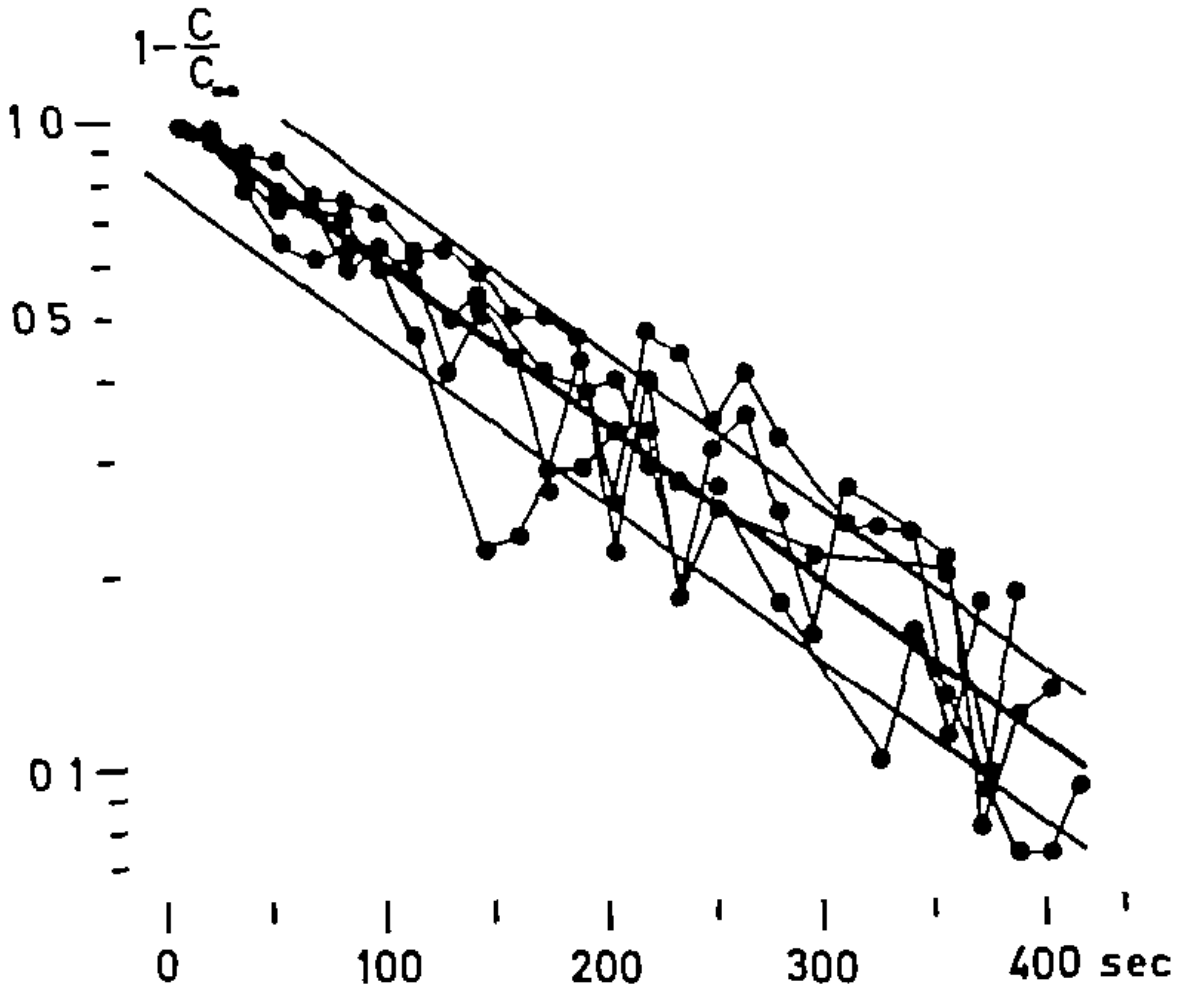


Fig 7 1—C/C$_\infty$ determined from 4 experiments, plotted semilogarithmically against time. For further explanation see text. The regression ($1 - C/C_\infty = 1\,09$ $^{-0.006}$ t $r = 0\,92$) ± 1 SD is shown. This figure can be used for calculation of the concentration of recirculating dye (C_1) at time t_1 if the concentration is known (C_0) at time t_0 $C_1 = ((1-y)/(1-y_0))$ C_0 where y is the ordinate ($1 - C/C_\infty$)

mg/l) The calculated mean values of the constants were $k_2 = 1\,09$ and $k_3 = 0\,0058$ sec The coefficient of correlation for the linear regression was $-0\,97^{***}$ The concentration of recirculating dye during infusion can be calculated by means of the expression

$$C_1 = C_0\,(3\,37 - e^{1.3\ \ 0.0058}\ )\,/\,(3\,37 - e^{1.3\,-\,0.0058\ t_1})$$

which is obtained from equation (5) after extension. Fig 7 can be used for the same purpose

B. The mean infusion rate was 30 ml/min (n = 15) with a lowest value of 29 6 ml/min. The coefficient of variation for a single determination of dye concentration was 3.9 per cent. F the ratio between the total intraindividual

variance for the 15 sets of 4 dye concentration values obtained on each occasion and the variance for a single determination was 1 27 ($p>0.05$) indicating uniform mixing of dye and blood.

Discussion

In the approximate mathematical model that has been set up it is assumed that the concentration of dye at a given moment is Y/Q, it being presumed that the dye is immediately distributed uniformly in the volume Q This approximation should be of subordinate importance apart from the first minute of the infusion period since the time taken for distribution of the dye in the plasma volume is short in relation to the time for the rise of concentration of the recirculating dye. Furthermore the arbitrary constants in the mathematical model have been determined empirically so that the formula set up for calculation of the concentration of the recirculating dye has been adapted to the prevailing experimental conditions.

The *mean value for the shortest recirculation time calculated from the relation* $\log (1-C/C_{\infty}) = 0.085 - 0\,0058\ t$ is about 15 sec. This agrees fairly well with earlier findings that the dye starts to appear in measurable quantities in the contralateral brachial artery 20—40 sec after the start of the infusions (Wahren 1966)

The concentration of recirculating dye constitutes only a small part of the total dye concentration in the forearm even after long periods of infusion. Small errors in the calculation of recirculating dye will thus have only a minimal influence on the blood flow calculation.

In the present experimental arrangement with long infusion periods the infusion rate was 30 ml/min. This rate is somewhat lower than the 34 ml/min recommended by Wahren (1966) The results show however that the infusion rate used produced an adequate mixture of infusion solution and blood.

CHAPTER III C

Uptake, Release and Metabolism of Lactate at Normal Arterial Lactate Concentration

Animal experimental studies (Omachi and Lifson 1956, Stainsby and Welch 1966) and to some extent studies on humans (Freyschuss and Strandell 1967) have given evidence that working skeletal muscle not only produces lactate but under certain conditions may take up lactate. One of the objects of the present study was by means of isotope technique, to investigate whether an uptake simultaneously with a net release of lactate takes place in human working skeletal muscle. It was also intended to quantitate the uptake if any and to determine the extent to which the lactate uptake was oxidized to CO_2. As the rate of net release of lactate from the forearm muscle varies during prolonged exercise, it was considered of interest to make these measurements both after 10 min of exercise when the rate is maximal, and after 40 min, when it has reached a lower and practically constant level (Wahren and Hagenfeldt 1968)

Subjects

The results presented in this chapter are based on data from 16 male volunteers aged 21—26 years. Five were students the others were firemen. All subjects had had regular physical training and health controls and were in good health at the time of the investigation. Arm dimensions of the subjects are given in Table 4

Procedure

The experiments were performed in the morning The subjects were fasting for at least 12 hours prior to the study

The study was arranged in two different series.

TABLE 4 Arm dimensions of the subjects in series 1 and 2

	n	Mean ± SD
Forearm volume, ml	15	1230 ± 190
Skinfold thickness, mm	15	3 9 ± 0.5
Wrist circumference, cm	15	17 6 ± 0 6
Isometric strength, kp	9	59.9 ± 7 5

Series 1 (n=10)

The experimental design is described in Chapter III.A and Fig. 4

Series 2 (n=5)

A discrepancy was found in series 1 between the amount of radioactivity which at steady state was taken up as ^{14}C-L(+) lactate and the radioactivity recovered as $^{14}CO_2$ and other ^{14}C-labelled metabolites. This discrepancy was tentatively assumed to be due to an incorporation of radioactivity into metabolites with a slow turnover rate. The studies in series 2 were undertaken to investigate whether this assumption was supported by the appearance of a slow phase in the release of $^{14}CO_2$ from the forearm muscles after infusion of ^{14}C-L(+) lactate.

For the right arm the same catheterization procedure was used as in series 1. In four of the experiments a catheter was also inserted into a deep forearm vein of the left arm. The subjects performed identical exercise with both arms using *two hand ergometers. Only one infusion was given starting after about 7* min of exercise (range 7—9 min). Blood samples were drawn from the deep vein of the contralateral arm. With this exception the procedure was identical with that in series 1 up to 12 min after the end of the infusion. For the rest of the exercise period arterial and venous blood samples were drawn every 5—10 min for analyses of lactate, ^{14}C-L(+)-lactate and $^{14}CO_2$ concentrations. No recording of the movements of the ergometer handle was performed in this series.

Special calculations in series 2

The $^{14}CO_2$ released after infusion of labelled lactate could be assumed to originate not only from oxidation of C-L(+)-lactate taken up during the infusions but possibly also from oxidation in the forearm muscles of recirculating ^{4}C-labelled substrates taken up during and after the infusions.

The latter component was probably of minor significance during the first period after the infusions, but it could possibly be of significance when the $^{14}CO_2$ release had reached a low level. The $^{14}CO_2$ release from oxidation of recirculating ^{14}C-labelled metabolites (U) was assumed to be a function of time ($U=f_U(t)$) and identical in both arms since the volunteers were performing exercise of the same intensity with both arms. The release of $^{1}CO_2$ originating from oxidation of ^{14}C-L(+)-lactate taken up during the infusion (Y) was also assumed to be a function of time ($Y=f_Y(t)$) and equal in both arms in proportion to the (a—v) difference of ^{14}C-L(+)-lactate at steady state during the infusion.

$$Y_l = Y_r \ ((a-v)_l{}^{14}\text{C-La}) / ((a-v)\ {}^{14}\text{C-La}) \quad (1)$$

r = right, l = left, La = L(+) lactate

The measured $^{14}CO_2$ release from either of the arms (Z) was a function of time ($Z = f_z\ (t)$) and was assumed to be the sum of $^{14}CO_2$ originating from oxidation of ^{14}C-L(+) lactate taken up during the infusions and recirculating ^{14}C-labelled substrates taken up after the infusions.

$$Z_r = U + Y \quad (2)$$

$$Z_l = U + Y_l \quad (3)$$

The following expression for Y can be calculated after combination and substitution in equations 1—3

$$Y = (Z_r - Z_l)\ ((a-v)\ {}^{1}\text{C-La}) / [((a-v)_r{}^{1}\text{C-La}) - ((a-v)_l\ \text{C-La})]$$

The arterial concentration of ^{14}C-L(+) lactate in the left arm was not measured during the infusions. However the mean arterial ^{14}C-L(+)-lactate concentration in the left arm during the last 4 min of the infusions could be calculated to be approximately 3/4 of the arterial concentration immediately after the end of the infusions. In these calculations it was assumed that the arterial concentration of ^{14}C-L(+)-lactate rose according to the equation $\square - \square\ (1 - e^{bt})$ where t denotes time in minutes and b is a constant. This assumption was supported by results from a complementary study including two experiments, from which b could be calculated to be about 0 1

Y revealed a biphasic course when plotted semilogarithmically against time. This function was fitted to a biexponential course and the quantity of $^{1}CO_2$ released was calculated after separate integration of the two phases.

Results

The results from the first period of infusion (infusion 1) after about 10 min of exercise comprise data from series 1 and 2 (n = 15) The results from the second period of infusion in series 1 (infusion 2) after about 40 min of exercise, are based on 9 experiments. One of the experiments in series 1 had to be interrupted after the first period of infusion owing to a technical mishap

Muscle blood flow and oxygen uptake (Table 5)

The calculated *muscle blood flow* was slightly greater ($p < 0.01$) after 40 min than after 10 min of exercise. It was positively correlated ($r = 0.5^{2**}$) to the variability in exercise pattern, for definition see legend of Fig 5 No significant difference existed in respect of *oxygen uptake* between 10 and 40 min.

TABLE 5 Data btained t normal arterial concentration of total lactate on uptake, release and fractional oxidation of lactat the brachial artery blood flow and the metabolism of oxygen, CO_2 nd pyruvate in the forearm after boot 10 min and 40 min of exercise p denotes the probability that the difference between 10 and 40 min of exercise was caused by random factors. The $p > 0.05$ values are omitted. a = rterial, v = deep venous concentrations, $\dot{Q}_{O_2}$ = forearm muscle oxygen uptake $\dot{Q}_{CO_2}$ = forearm muscle production of CO_2. For definition of the symbols T — T and variability i exercise pattern see Fig 5.

V riable	10 min		40 min		p
	n	Mea ±SD	n	Mean±SD	
Forearm muscle blood flow ml/min	15	301±46	9	384±62	<0.01
Hemoglobin$_a$ g/l	15	142±9	9	135±8	
$S_v O_2$ %	14	34 4±5 7	9	38.7±7.5	
O_2-content$_{(a-v)}$, ml/l	14	124±12	9	109±14	<0.05
$\dot{Q}\, O_2$, ml/ml	14	37±8	9	42±8	
Lactate					
arterial conc., mmoles/l	15	1 09±0.33	9	0.98±0.36	
net release mmoles/l	15	1.58±0.96	9	0.42±0.28	<0.01
rat of net release mmoles/mm	15	0.48±0.34	9	0.16±0.11	<0.05
fractional uptak of ^{14}C-L(+)-					
lactate	15	0.32±0.08	9	0.30±0.07	
uptake mmoles/l	15	0.33±0.09	9	0.29±0.11	
rate of uptake, mmoles/min	15	0.10±0.03	9	0.11±0.05	
fractional oxidation of C-L(+)					
lactate	15	0.38±0 16	9	0.52±0.19	
Pyruvate$_a$, mmoles/l	15	0.07±0.02	9	0.07±0.01	
Pyruvate$_{(v-a)}$, mmoles/l	15	0.04±0 03	9	0.02±0 01	<0.05
$p_a O_2$, mm Hg	15	100±6	9	101±6	
$p_v O_2$, mm Hg	15	25.6±3.5	9	25.0±4.3	
pH_a	15	7 40±0 02	9	7 40±0.03	
pH_v	15	7.26±0.04	9	7.33±0 03	<0 001
Standard bicarbonate$_a$, meq/l	15	24 6±1 4	9	23.8±0.9	
Standard bicarbonate meq/l	15	22.8±1.2	9	22.9±0.7	
p_aCO_2, mm Hg	15	41 6±4.0	9	38.4±3.4	
p_vCO_2, mm Hg	15	61 4±6.6	9	47 6±5.7	<0.001
CO_2-content$_a$, ml/l	15	451±34	9	435±36	
CO_2-content$_{(v-a)}$, ml/l	15	109±27	9	91±26	
$\dot{Q}\, CO_2$, ml/min	15	33±11	9	34±8	
RQ			9	0.84±0.16	
$T_a/(T + T_a)$	9	0 38±0 11			—
$(T + T)/(T + T)$	9	0.27±0.08			—
Variability in exercise pattern	9	0.35±0.10			—

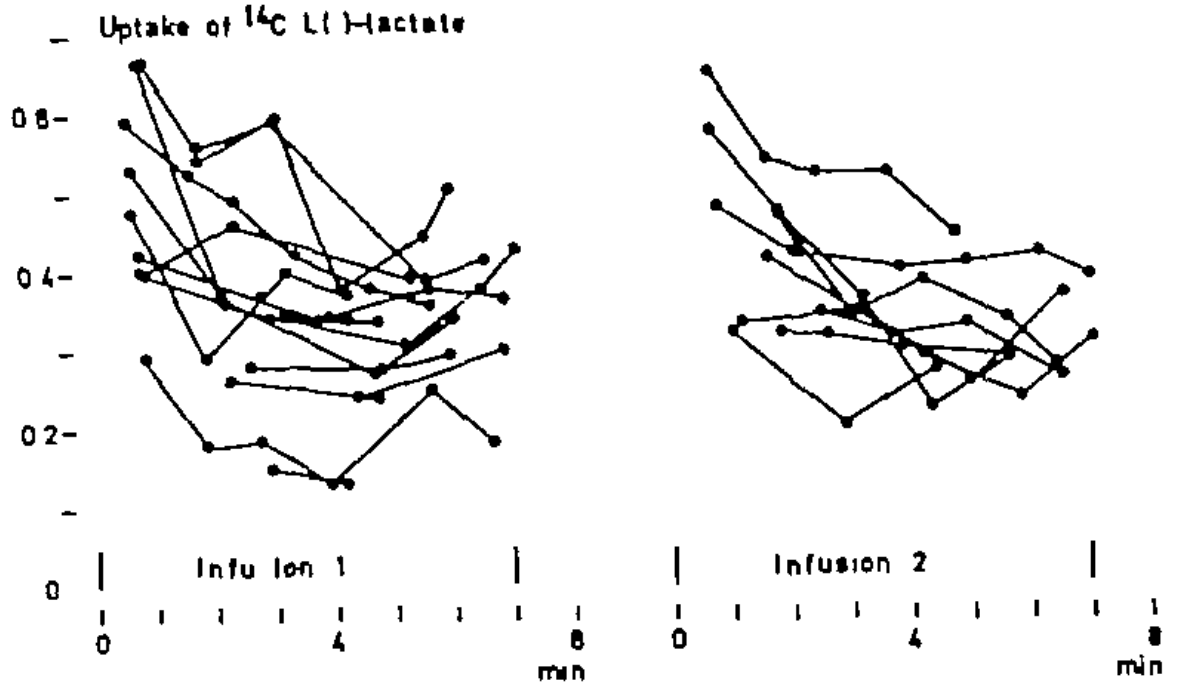


Fig. 8. Uptake of C-L(+)-lactat during infusion expressed as fraction of the arterial concentration of C-L(+)-lactate. Individual values from infusion 1 nd 2 s normal arterial concentration of total lactat are given.

Release of lactate and pyruvate (Table 5)

The lactate concentration in the arterial blood was the same on both occasions of observation. The *net r lease of lactate* was 1.6 ± 1.0 and 0.4 ± 0.3 mmoles/l after 10 and 40 min of exercise respectively. The difference was significant ($p < 0.01$). This means that the *rate of net release of lactate* from the forearm muscles diminished ($p < 0.05$) during the exercise period. For *pyruvate* no significant differences were measured between 10 and 40 min as regards arterial concentration or rate of release.

Uptake of lactate (Fig. 8, 9, Table 5)

The uptake of ^{14}C-L(+) lactate was initially high during the infusions but fell successively and reached a constant level after about 4 min of infusion on both occasions. The *fractional uptake of* ^{14}C *L(+)-lactate* was similar ($p > 0.05$) in infusions 1 and 2 (0.32 ± 0.06 and 0.30 ± 0.07 respectively). No significant difference existed between 10 and 40 min of exercise as regards *uptake of lactate* and *rate of lactate uptake* in forearm muscle. Both were positively correlated to the arterial lactate concentration ($r = 0.68^{**}$ and $r = 0.57^{**}$ respectively). Within the limited range of variation for the arterial lactate concentration there was no significant deviation from a linear relation.

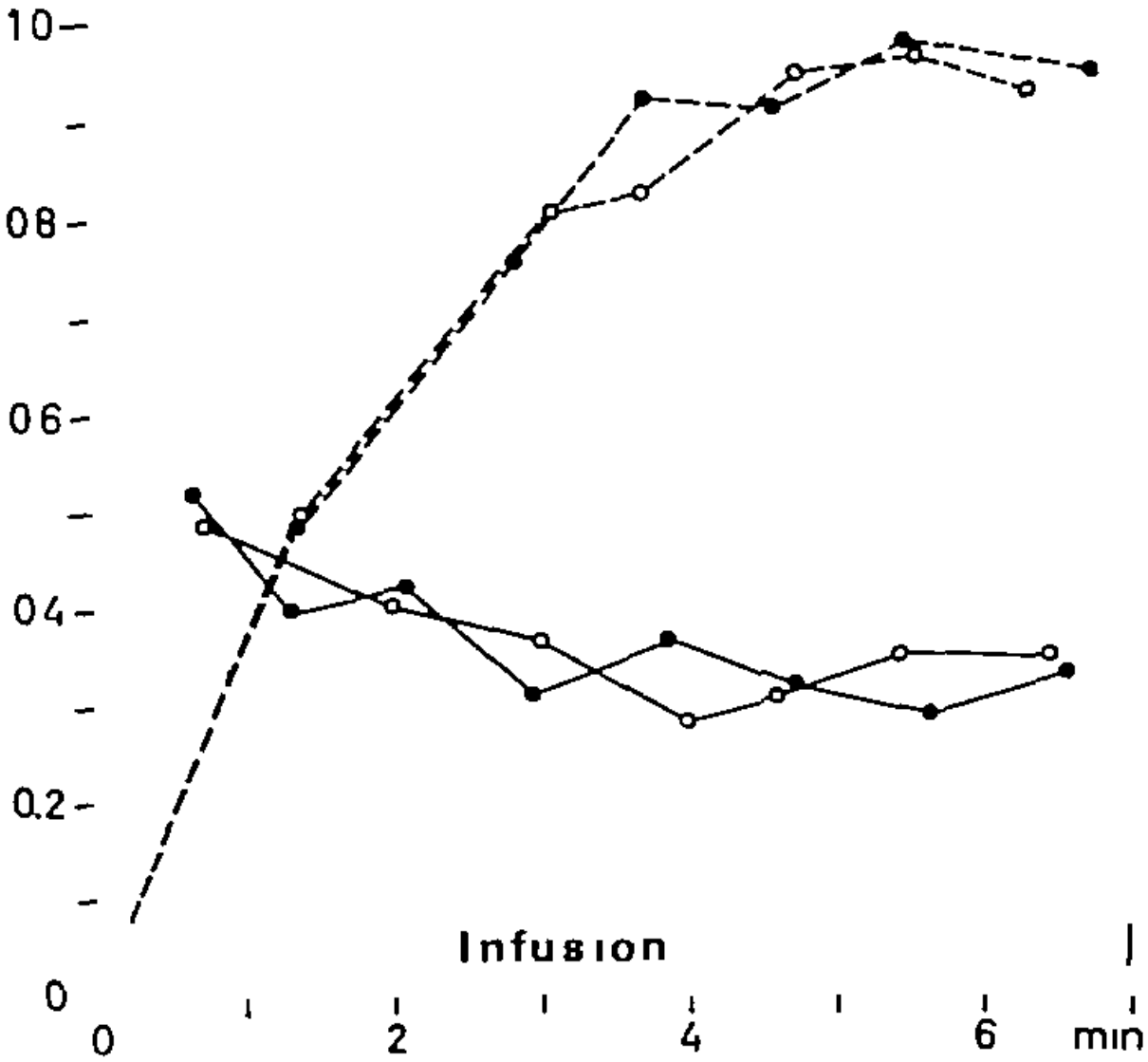


Fig 9. Uptake of ^{14}C-L(+)-lactate during infusion expressed as fraction of the arterial concentration of ^{14}C-L(+)-lactat (○—○, ●—●) and release of $^{14}CO_2$, calculated as fraction of the release t steady state during the infusion period (○---○, ● --●) Mean values from infusion 1 (○—○, ○- -○) and infusion 2 (●—● ●--●) at normal arterial concentration of total lactate are given. This figure shows that the uptake of ^{14}C-L(+) -lactate and the production of CO_2 normally reached constant level during the infusions.

No significant correlation existed in this material between uptake and net release of lactate.

Release of ^{14}CO during infusion (Fig 9 10 Table 5)

The (v—a) difference of CO_2 rose rapidly at the beginning of the infusions and reached a constant level after an average of about 5 min of infusion. The conditions were essentially similar in infusions 1 and 2. The fractional oxidation of C-L(+) lactate was 0.38 ± 0.16 and 0.52 ± 0.19 in infusions 1 and 2 respectively

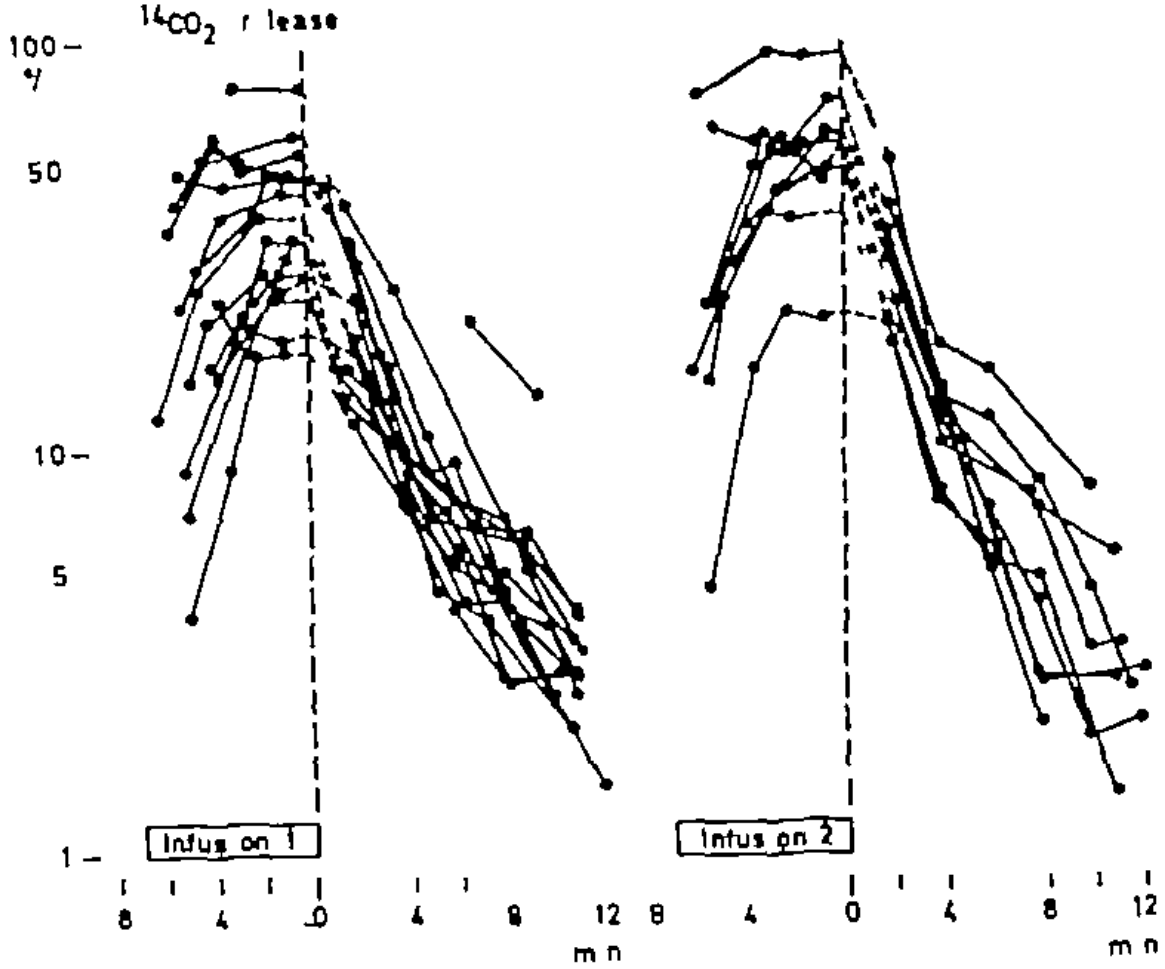


Fig. 10 Semilogarithmic representation of release of CO_2 during and after infusion ex pressed as fraction of the uptake of C-L(+)-lactat t steady stat during infusion Individual lues from infusions 1 nd 2 at normal arterial co centration of total lactat re gi en The calculated fractional oxidation is plotted on the y-axis and joined to the surrounding bser tions by broken lines.

Radioactivity in perchloric acid extract and fat extract of blood

The total content of radioactivity as well as the concentration of ^{14}C-L(+) lactate in a perchloric acid extract (PCA extract) were measured in four of the experiments at the end of and about 1 and 10 min after the infusions. No difference between infusions 1 and 2 was observable as regards the proportion of the total radioactivity in the PCA extracts recovered as ^{14}C-L(+) lactate the results from the two infusions have therefore been combined. During infusion all radioactivity in the PCA extracts in arterial blood was in the form of ^{14}C-L(+)-lactate In venous blood, however other ^{14}C-labelled substances than ^{14}C-L(+) lactate also existed constituting 5—10 % of the total radioactivity in the extract and about 20 % of the simultaneous uptake of ^{14}C-

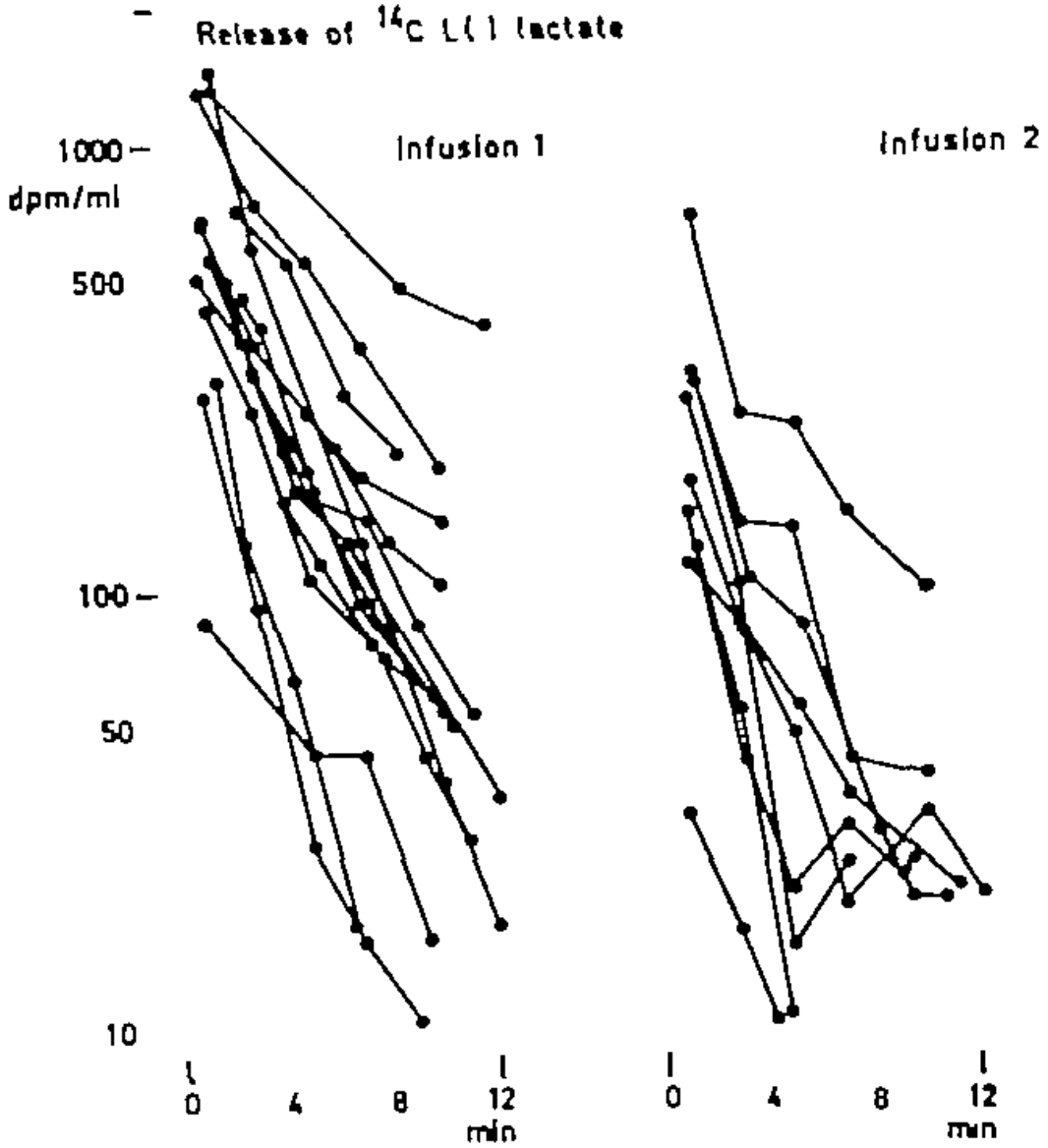


Fig 11. Semilogarithmic representation of release of ^{14}C-L(+)-lactate after infusion. I dividual values from infusions 1 and 2 t normal arterial concentration of total lactate are given.

L(+)-lactate. This finding indicates that about 20 % of the radioactivity from the ^{14}C-L(+) lactate extracted was released in the form of other ^{14}C-labelled metabolites.

One and ten minutes after the infusions ^{14}C-L(+) lactate constituted only about 60 and 50 % respectively of the total content of ^{14}C in PCA extract of arterial blood. The corresponding values for the venous blood were about 10 % higher indicating an uptake of other ^{14}C-labelled PCA-extractable metabolites than ^{14}C-L(+) lactate.

No significant incorporation of C in fat-extractable (Dole and Meinertz 1960) substances in arterial and venous blood was measured (one experiment)

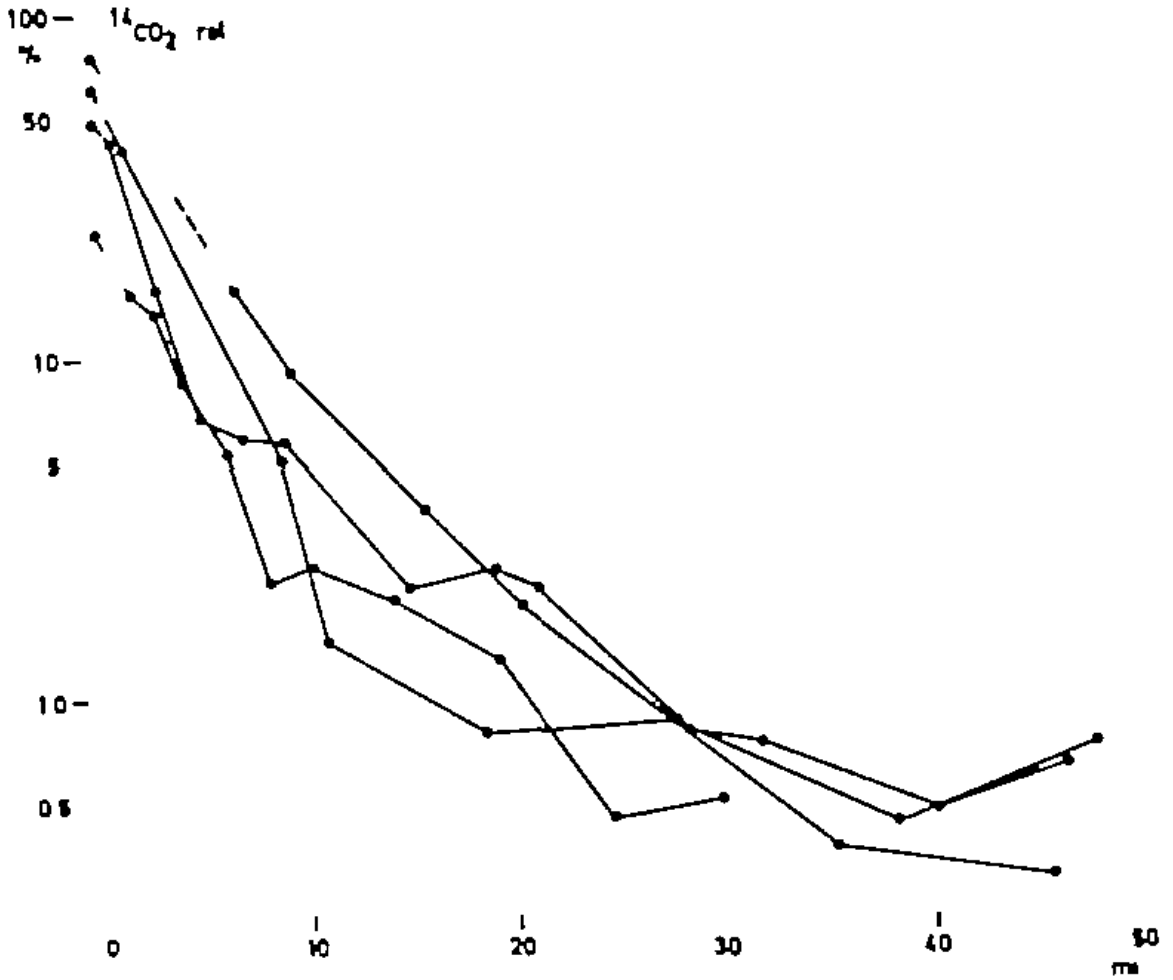


Fig 12 Semilogarithmic representation of release of $^{14}CO_2$ after infusion from the ^{14}C-L (+) lactate ven p by forearm muscle during the infusions. Y_{rf}((a—v) ^{14}C-La)) for the four mples experiments in series 2 are given for explanation see text. The calculated fractional oxidation of the ^{14}C-L(+)-lactat extracted during infusion is plotted and joined to the curves by broken lines.

Rel ase f $^{14}CO_2$ and ^{14}C L(+)-lactate after the infusions of ^{14}C L(+)-lactate (Fig 10 11 12, 13)

The release of CO_2 from the forearm muscles 0—12 min after an infusion of ^{14}C-L(+)-lactate revealed no clearly distinguishable multiphase course when plotted semilogarithmically against time for either of the infusion periods (Fig 10) Within the prolonged observation period in series 2, however the release of $^{14}CO_2$ from the ^{14}C-L(+) lactate taken up by the forearm muscles during the infusions (Y) exhibited a least biphasic course in semilogarithmic plotting against time (Fig 12) The appearance of at least one slow phase supported the assumption that a part of the ^{14}C from the extracted ^{14}C-L(+)-lactate was incorporated into metabolites with a slow turnover rate The quantity of

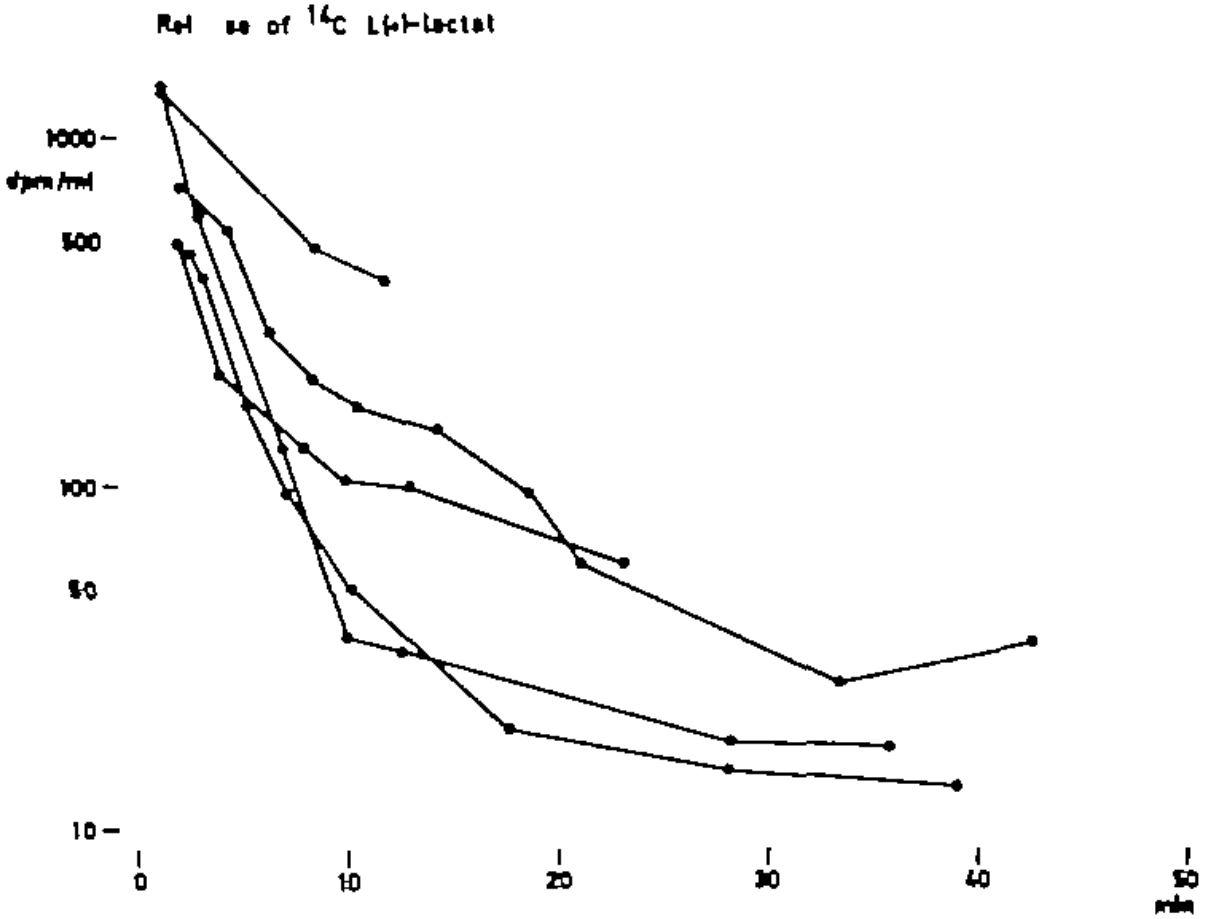


Fig 13 Semilogarithmic representation of release of ^{14}C-L(+)-lactate after infusion. Values from series 2.

$^{14}CO_2$ released corresponding to the slower phase amounted to about 70 % of the ^{14}C-L(+)-lactate taken up by the muscles during the infusion. The latter was calculated as the product of the (a—v) difference of ^{14}C-L(+) lactate at steady state during infusion, the forearm muscle blood flow and the infusion time.

The release of ^{14}C-L(+)-lactate after the infusions exhibited mainly the same course as that of $^{14}CO_2$ and there was no obvious difference in the elimination rate. However in contrast to the conditions for $^{14}CO_2$ there was no (v—a) difference of ^{14}C-L(+) lactate over the left forearm.

Discussion

The forearm as experimental model

In man the forearm muscle offers many advantages for quantitative studies of substrate uptake and utilization. The arterial blood supply of the forearm normally takes place through a single dominating artery the flow of which

can be determined during exercise (Wahren 1966) The part of the flow which goes to the muscles during exercise can also be determined indirectly with sufficient accuracy Furthermore practically pure muscle vein blood can be obtained from deep veins of the forearm (Coles *et al* 1958 Idbohrn and Wahren 1964) after percutaneous catheterization of these veins. Fick s principle can only be applied on the assumption that the sampled venous blood is representative of the entire area to which the blood flow determination related (Zierler 1961) That this may be considered to be the case as regards the oxygen content in the deep venous blood was shown by Wahren (1966) Whether the same applies to lactate and other metabolites is not known As the turnover of lactate is not necessarily associated with oxidative metabolism, a condition shown to be applicable to oxygen cannot unreservedly be extended also to lactate The quantitative calculations concerning lactate turnover in this study in which the blood flow is a variable must for this reason be regarded as approximate

The results as regards blood flow and oxygen uptake during lengthy exercise are entirely in agreement with earlier observations by Wahren and Hagenfeldt (1968) The reason why the forearm blood flow is greater after 40 than after 10 min of exercise is not fully clear but it is of importance for the calculations of rate of uptake and release of metabolites in forearm muscle that the increase in forearm blood flow is not caused by an increased cutaneous blood flow In fact, in a study using ^{133}Xe clearance technique it was shown that no changes of significance took place in the cutaneous blood flow during a 60 min forearm exercise (Jorfeldt and Wahren 1970)

Under the prevailing experimental conditions another type of variation in the forearm blood flow was noted namely random variations during the infusions and thereby at constant rate of infusion, also in the arterial concentration and in the fractional uptake of ^{1} C-L(+) lactate. But nor should this type of variation either have influenced the calculated uptake and release of lactate, inasmuch as the calculations are mean values of at least two determinations.

Release and uptake of lactate

Under the prevailing experimental conditions there was a considerable net release of lactate from forearm muscle The rate of net release was greater after 10 than after 40 min of exercise These results are fully in agreement with earlier observations (Wahren and Hagenfeldt 1968)

The fact that the (a—v) difference of ^{14}C-L(+) lactate attained a constant level during the infusions shows that there is a continuous uptake in muscle of lactate from the blood During the first part of the infusion periods the (a—v) difference of ^{14}C-L(+) lactate fell successively which together with a de-

creasing (v—a) difference after the infusions, suggests that an exchange of lactate takes place between blood and muscle.

Metabolism of lactate taken up

Within 1—2 min after the start of an infusion of ^{14}C-L(+) lactate into the brachial artery a considerable release of $^{14}CO_2$ was measured. This shows that a rapid oxidation of the ^{14}C-L(+)-lactate taken up from the blood occurs in forearm muscle during exercise. At the end of the infusions this oxidation was in equilibrium with the uptake, as is evident from the fact that a constant (a—v) difference was attained in respect both of ^{14}C-L(+) lactate and of $^{14}CO_2$.

After 10 or 40 min of exercise only 38 and 52 % respectively of the radioactivity of the ^{14}C-L(+) lactate extracted was recovered as $^{14}CO_2$. There must therefore be also other metabolic routes for the lactate extracted than by direct oxidation to carbon dioxide. Thus 70 per cent of the labelled carbon from ^{14}C-L(+) lactate was recovered as other PCA-extractable metabolites. No definite release of fat-extractable ^{14}C-labelled metabolites was observed. The remaining discrepancy between uptake of labelled lactate and release of labelled metabolites therefrom can be explained by the fact that, in the studied area in the forearm, a metabolism of ^{14}C-L(+) lactate took place, with incorporation of ^{14}C in one or more metabolite pools with slow turnover. This assumption is supported by the results from series 2, in which the $^{14}CO_2$ release after the infusions from the labelled lactate taken up during the infusions exhibited a least biexponential course. It is possible that the rapid phase is caused by ^{14}C-L(+) lactate, which via pyruvate, is directly oxidized in the tricarboxylic acid cycle, and the slower phase by oxidation of ^{14}C-labelled metabolites from pools with slower turnover.

About 40 % of the radioactivity of the ^{14}C-L(+) lactate extracted during the first infusion period was calculated to have been incorporated into such pools of metabolites with slow turnover rate. However, the ^{14}C recovered as $^{14}CO_2$ corresponding to the slow phase of the $^{14}CO_2$ release after the infusions was only approximately 20 %. The fate of the remaining 20 % of the ^{14}C is unclear. It cannot be excluded however that there was another slow phase of the $^{14}CO_2$ elimination which was not detected.

The experiments and their results offer no means of directly assessing which metabolites were labelled with ^{14}C from the ^{14}C-L(+) lactate extracted. Incorporation of ^{14}C in glycogen and a muscular triglyceride pool may be regarded as conceivable possibilities. This presupposes an exchange of ^{14}C upstream in the glycolysis, either through a reversal of the pyruvate kinase reaction or through the dicarboxylic acid shuttle. The first alternative seems to be relevant for skeletal muscle (Lardy and Ziegler 1945, Krimsky 1959, Utter

1959) but muscle seems to lack the enzymatic requirements for the latter alternative (Utter 1959 Salas *et al* 1964) The possibility of labelling of ^{14}C in skeletal muscle glycogen from ^{14}C-pyruvate and ^{14}C-L(+) lactate has been demonstrated in animal experiments (Hlatt *et al.* 1958 Koeppe *et al.* 1964) Enzymatic conditions exist for ^{14}C-labelling of the glycerol fraction of the triglycerides. Hagenfeldt and Wahren (1968) have suggested that some esterification of FFA in forearm muscle probably takes place during exercise and that oxidation of glycerol occurs in muscle.

The significance of white and of red muscle fibres

It should be observed that the results are summation values from the various units within the part of the forearm muscle which is drained by the catheterized deep forearm vein The bulk of the contraction work during this type of exercise is performed by the flexor muscles while other muscle groups probably perform static work for fixation of the forearm. Under the prevailing conditions the part of the forearm muscle concerned is probably fairly heterogeneous, not only between but also within the individual muscles and uptake of lactate fractional oxidation of extracted ^{14}C-L(+) lactate and net release of lactate may presumably shift between the units. Of interest in this connection is the simultaneous occurrence of red and white muscle fibres within an individual muscle The white fibres and certain glycolytically active red fibres are activated chiefly at the start of submaximal exercise (Kugelberg and Edström 1968, Grimby and Hannerz 1968) This may explain the considerable net release of lactate at the start of the exercise period. The greater part of the lactate uptake and the oxidation of the extracted lactate however probably take place in red muscle fibres which are adapted for a more significant oxidative metabolism (Keul, Doll and Keppler 1969) The possible significance of red and white muscle fibres is discussed in greater detail in Chapter IV

CHAPTER III D

Uptake, Release and Metabolism of Lactate at Elevated Arterial Lactate Concentration

At high arterial concentrations of lactate there may be a net uptake of lactate in exercising muscle (Stainsby and Welch 1966 Freyschuss and Strandell 1967) The present study was performed in order to determine to what extent the uptake, release and fractional oxidation of lactate in forearm muscle are influenced by the arterial concentration of total lactate.

Subjects

The results in this chapter are based on data from six volunteers aged 21—27 years. They were all healthy firemen who had gone through regulated health checks. Arm dimensions of the subjects are given in Table 6.

Procedure

The studies were made in the morning after the subjects had fasted for at least 12 hours. The right forearm was used in all cases. Catheterization was done in the same way as in the studies reported in Chapter III-C. Roughly 30 min before the start of exercise an infusion of unlabelled hyperosmotic sodium lactate was commenced through a catheter placed with the tip in the upper vena cava (see Chapter III A) Before the infusion a priming dose of about 15 mmoles was given, and thereafter about 3.5 mmoles/min was infused until the end of exercise. Unless otherwise stated, the procedure during the exercise and for the calculations was identical to that described for series 1 in Chapter III-C.

TABLE 6. Arm dimensions of the subjects.

	n	Mean ± SD
Forearm volume, ml	6	1240 ± 200
Skinfold thickness, mm	6	3.9 ± 0.4
Wrist circumference cm	6	17.8 ± 0.9
Isometric strength kp	5	57 4 ± 6.0

TABLE 7 Data btained t elevated rterial concentration of total lactate on uptake release oxygen, CO and pyruvate In the forearm after bout 10 min and 40 min of the first Infusions (p_1) and the second infusions (p_2) at normal in relation to ele $p > 0.05$ alues are omitted = arterial, v = deep venous concentrations, definition of the symbols T —T and variability in exercise pattern see Fig 5

Variable	n	10 min Mean ± SD
Forearm muscle blood flow ml/min	6	302 ± 75
Hemoglobin$_a$ g/l	6	132 ± 10
S_vO_2, /o	6	37 7 ± 4.8
O_2-content$_{(a-v)}$ ml/l	6	107 ± 14
$\dot{Q}_{O_2}$ ml/min	6	32 ± 8
Lactate		
arterial conc. mmoles/l	6	3.35 ± 0.54
net release mmoles/l	6	0 65 ± 0 47
rate of net release, mmoles/min	6	0.18 ± 0 12
fractional uptake of ^{14}C-L(+)-lactate	6	0.23 ± 0.05
uptake, mmoles/l	6	0.76 ± 0.22
rat of uptake mmoles/min	6	0.23 ± 0.10
fractional oxidation of C-L(+)-lactate	6	0.52 ± 0 19
Pyruvat $_a$, mmoles/l	6	0 18 ± 0 02
Pyruvate$_{(v-a)}$, mmoles/l	6	0 01 ± 0 04
p_a O mm Hg	6	96 ± 9
p_v O_2, mm Hg	6	26.5 ± 5.5
pH_a	6	7 43 ± 0.02
pH	6	7 31 ± 0 03
Standard bicarbonate$_a$, meq/l	6	28.0 ± 1 4
Standard bicarbonat $_v$, meq/l	6	26.7 ± 1.6
p_a CO mm Hg	6	44.3 ± 4 1
p_v CO_2, mm Hg	6	64 0 ± 7.-
CO_2-content$_a$, ml/l	6	507 ± 21
CO_2-content$_{(v-a)}$, ml/l	6	91.2 ± 25.2
$\dot{Q}_{CO_2}$, ml/min	6	27 ± 8
RQ		
$T_a/(T + T)$	6	0.26 ± 0.09
$(T + T)/(T + T)$	6	0 18 ± 0.05
V riability in exercise pattern	6	0 43 ± 0 17

Control studies

Three preliminary experiments with constant intravenous infusion of sodium lactate after a priming dose showed that a constant arterial lactate level had been reached after about 30 min. This is in agreement with earlier studies (Fiorentini and Faini 1967) In the subsequent experiments with ^{14}C-L(+)

and fractional oxidation of lactate, the brachial artery blood flow and the metabolism of exercise. The probability that the difference between infusions 1 and 2 (p_{1-2}) and between rated arterial concentration of total lactate was caused by random factors is given. The $\dot{Q}_{O_2}$ = forearm muscle oxygen uptake $\dot{Q}_{CO_2}$ = forearm muscl production of CO_2. For

n	40 min Mean ± SD	p_{1-2}	p_1	p_2
6	322 ± 96			
6	123 ± 9		< 0 05	< 0.05
6	41.8 ± 4.4			
6	91.5 ± 9.8	< 0.05	< 0.05	< 0 05
6	29 ± 8			< 0.05
6	3.36 ± 0.54		< 0 001	< 0 001
6	0.14 ± 0.29	< 0 05	< 0.05	
6	0.05 ± 0 11		< 0 05	
6	0.23 ± 0 04		< 0 05	
6	0 76 ± 0.14		< 0 001	< 0.001
6	0.25 ± 0 09		< 0 001	< 0.01
6	0.48 ± 0.10			
6	0 19 ± 0 04		< 0.001	< 0.001
6	—0.04 ± 0 06			< 0.05
6	89 ± 13			< 0.05
6	26.7 ± 4 1			
6	7 47 ± 0 03	< 0 05	< 0 001	< 0.001
6	7 41 ± 0 05	< 0 01	< 0.01	< 0.01
6	30.0 ± 1.3	< 0.05	< 0.001	< 0.001
6	30 5 ± 1 4	< 0.01	< 0 001	< 0.001
6	43.2 ± 5 0			< 0 05
6	54 0 ± 9 7			
6	546 ± 39		< 0 01	< 0.001
6	68.7 ± 29 4			
6	23 ± 11			< 0.05
5	0.83 ± 0.19			
		—	< 0.05	—
		—	< 0.05	—
		—		—

lactate, therefore, the exercise period was not started until after 30 min of Lv lactate infusion.

Two control experiments were made in order to see whether the rising bicarbonate concentration following on the combustion of the sodium lactate in the body (Schwartz 1962) affected the release, uptake and fractional oxida

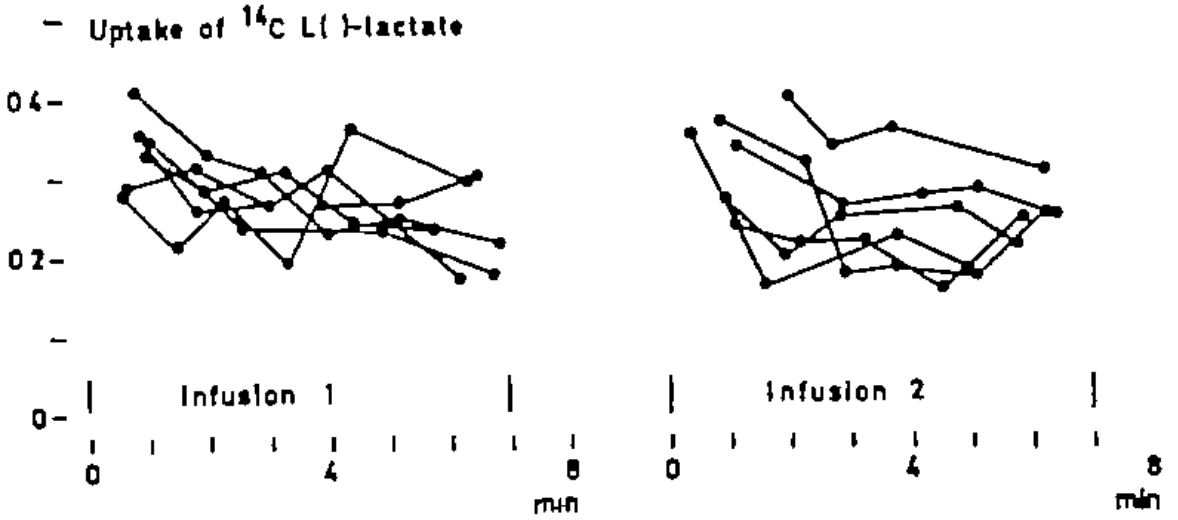


Fig 14 Uptake of ^{14}C-L(+) lactat during infusion expressed as fraction of th arterial concentration of ^{14}C-L(+) lactat I dividual values from infusions 1 and 2 at increased arterial concentration of total lactate re gi ven.

tion of lactate in forearm muscle during exercise as at diffusion equilibrium the ratio between intra and extracellular concentrations of lactate and bicar bonate may be assumed to be the same (Jacobs 1940 Granholm *et al.* 1968) Two identical experiments were made on each of two subjects with 1 hour s interval. Apart from the intravenous lactate infusion, each experiment was identical to the first half of the experiments in series 1 During the period of rest between the tv o experiments sodium bicarbonate was infused intravenously (roughly 100 meq) The infusion was adjusted so that in the second exer cise period the subjects had roughly the same bicarbonate concentration in plasma as in the experiments with infusion of sodium lactate (mean value for standard bicarbonate 30 0 and 29 0 meq/l respectively) The results showed no obvious difference between the two periods of exercise in respect of rate of net release of lactate (mean 0 29 and 0 25 mmoles/min, respectively) fractional uptake of ^{14}C-L(+) lactate (mean 31 and 36 % respectively) and fractional oxidation of ^{14}C-L(+) lactate (mean 53 and 55 % respectively)

Results

All statistical comparisons between 10 min of exercise (infusion 1) and 40 min (infusion 2) are based on intraindividual comparisons of the respective varia bles. In the comparisons between results obtained at normal (Chapter III-C) and elevated arterial lactate concentration the results for oxygen consumption and fractional uptake of ^{14}C-L(+) lactate from 10 and 40 min of exercise have been combined since no significant difference existed between the two in fusions in any of the investigations.

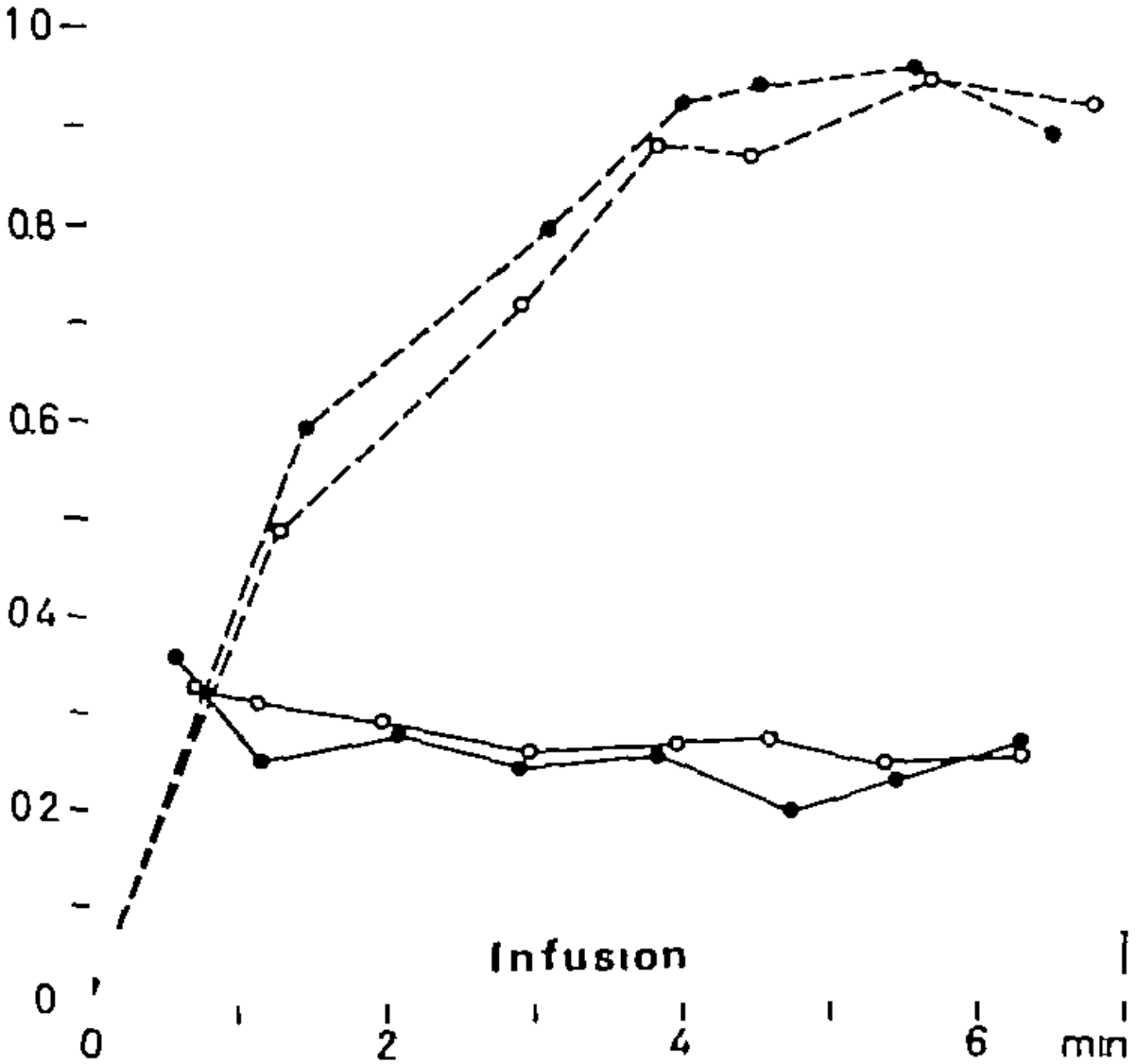


Fig. 15. Uptake of ^{14}C-L(+)-lactate during infusion, expressed as fraction of the arterial concentration of C-L(+)-lactate (○—○, ●—●) and release of $^{14}CO_2$, calculated as fraction of the release at steady stat during the infusion period (○- -○, ●—●) Mean values from infusion 1 (○—○ ○- -○) and infusion 2 (●—● ●--●) t increased arterial concentration of total lactate are given. This figure shows that the uptake of ^{14}C-L(+)-lactate and the production of $^{14}CO_2$ normally reached constant level during the infusions.

Muscle blood flow and oxygen uptake (Table 7)

No significant difference existed between 10 and 40 min as regards the calculated *muscle blood flow* Combining the results from 10 and 40 min, the calculated muscle blood flow was negatively correlated ($r = -0.75^{**}$) to the arterial hemoglobin concentration. In this series no correlation was found between the muscl blood flow and the variability in exercise pattern. The oxygen (a—v) difference was less ($p<0.05$) at 40 than at 10 min. The difference between the 10th and 40th minute as regards *oxygen consumption* in forearm muscle was, however not significant. The oxygen consumption in forearm

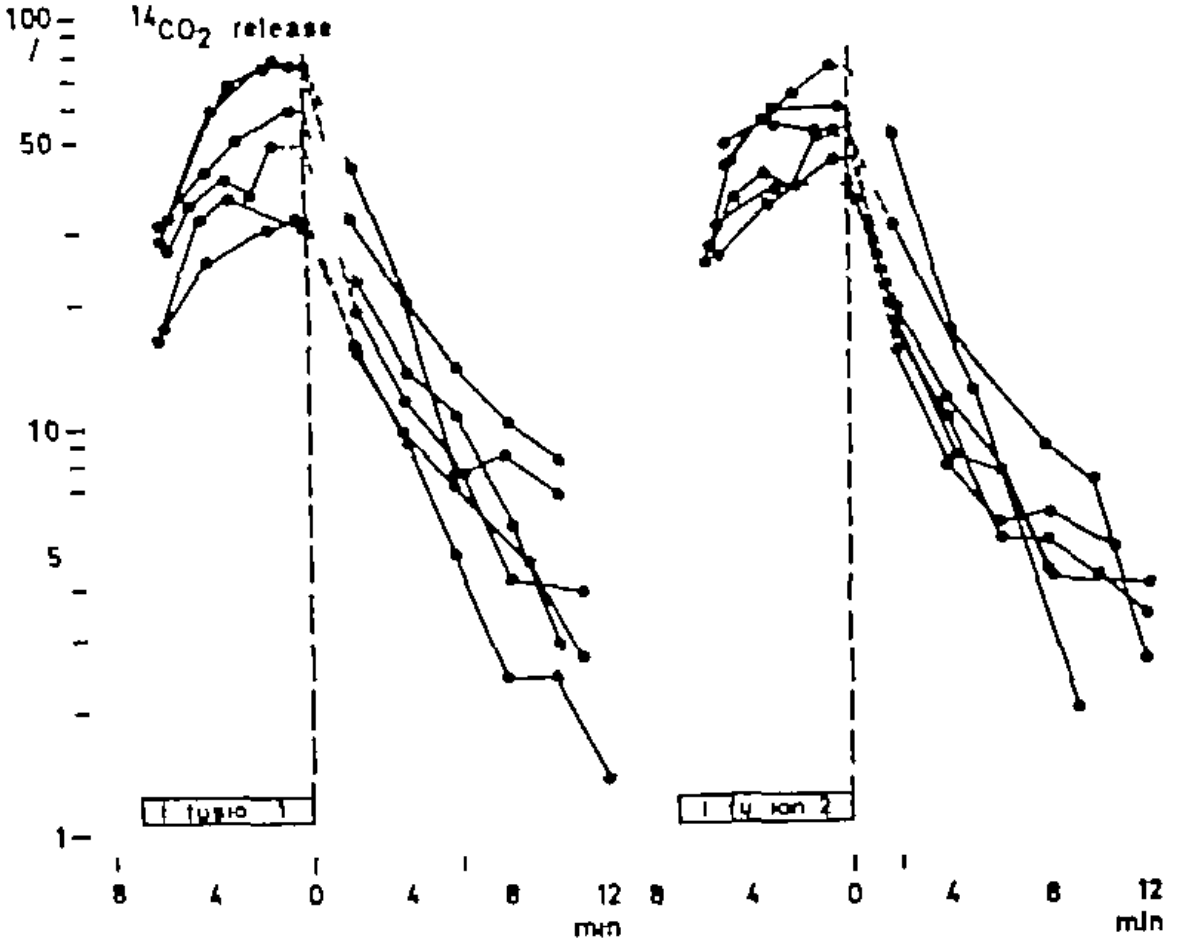


Fig 16 Semilogarithmic representation of release of CO during and after infusion expressed as f ction of th uptake of ^{14}C-L(+) lactate at steady sta during infusion. Individual values from infusions 1 and 2 at increased arterial concentration of total lactate are given. The calculated fractional oxidation is pl tted on the y-axis and joined to the surrounding observ ions by broken lines.

mu s le was significantly lower ($p<0.01$) in these experiments than at normal rterial lactate concentration.

Releas f lactate and pyruvate (Table 7)

The lactate concentration in the arterial blood was the same (3.4±0.5 mmoles/l) after 10 and 40 min exercise. The *rate of net release of lactate* was not significantly different at 10 as compared to 40 min of exercise. In two cases in which the arterial lactate concentration was greater than 4 mmoles/l a net uptake of lactate was recorded (1 after 10 min and 1 after 40 min). After 10 min of exercise both the net release and the rate of net release of lactate were lower ($p<0.05$) in this series than in the studies at normal arterial lactate concentration. No significant difference on the other hand existed in these respects after 40 min of exercise. After about 55 min of exercise a *net uptake of*

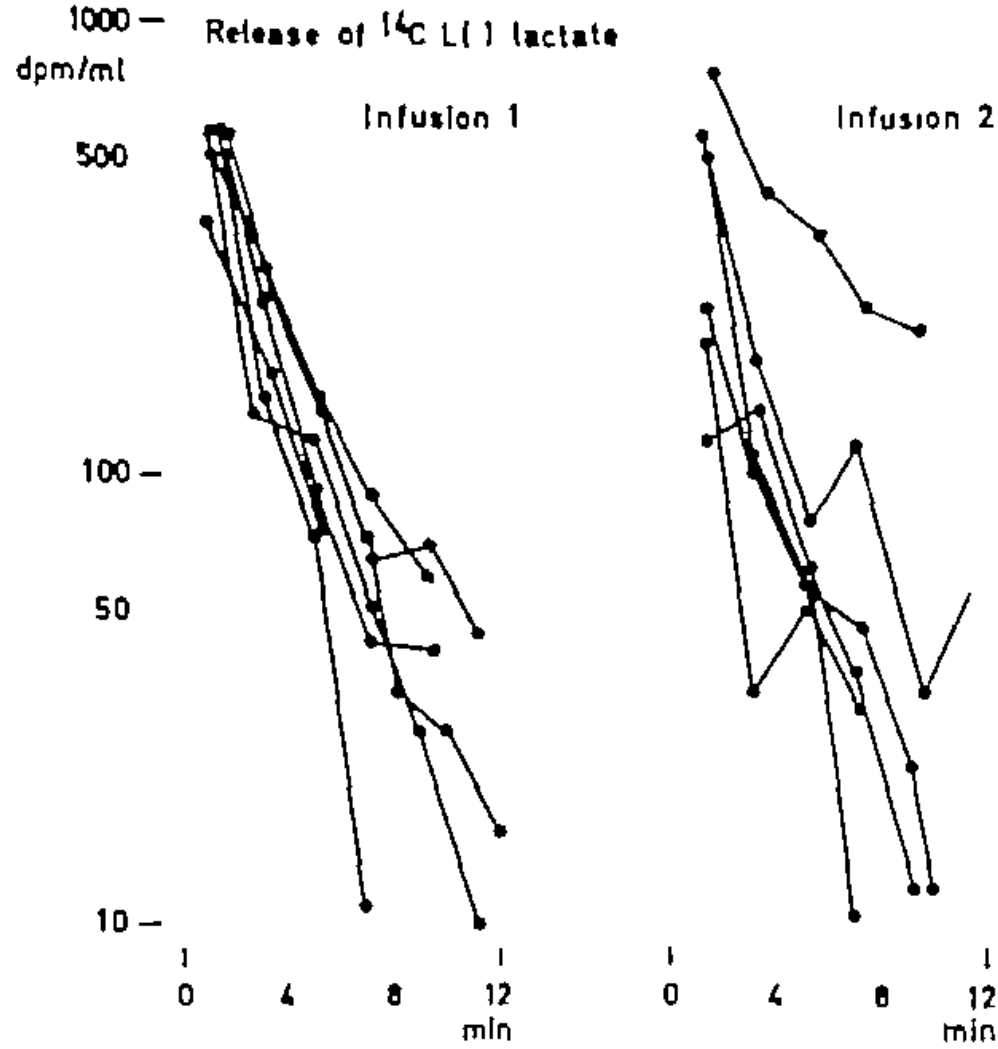


Fig 17 Semilogarithmic representation of release of C-L(+)-lactate after infusion. Individual values from infusions 1 and 2 t elevated arterial concentration of total lactate are given.

lactate (mean 0 09 ± 0 16 mmoles/l) was measured in all subjects. For pyruvate a net uptake was measured in two cases after 10 min and in two further cases after 40 min.

Uptake of lactate (Fig 14 15 Table 7)

In this series, as in the study at normal arterial lactate concentration, the *fractional uptake of* ^{1} *C I (+)-lactate* reached a constant level after about 4 min of infusion both in infusion 1 and in infusion 2. The fractional uptake of ^{14}C-L(+) lactate was the same in both infusions but significantly lower ($p < 0.01$) than at normal arterial lactate concentration. No significant difference between 10 and 40 min of exercise existed in respect of *uptake of lactate* or *rate of lactate uptake*. Both variables were positively correlated to the arterial

lactate concentration and the muscle blood flow there was an apparently linear relation ($r = 0.92^{***}$ $n = 36$) (Fig 19) The maximal rate of lactate uptake calculated from the y-intercept was 0 65 mmoles/min, which corresponds to approx. 0 08 mmoles $\times$ min $^{-1} \times 100$ ml $^{-1}$ muscle This value was calculated for an estimated muscle fraction of 0 6 of the forearm volume (Abramson and Ferris 1940 Cooper Edholm and Mottram 1955)

The fractional oxidation of the extracted lactate was negatively correlated ($r = -0.65^{***}$ $n = 36$) to the fractional uptake of ^{14}C-L(+) lactate The net release of lactate from forearm muscle was positively correlated ($r = 0.55^{**}$ $n = 36$) to the oxygen uptake

Discussion

For assessing the metabolic effects in forearm muscle of the artificially elevated arterial lactate concentration it must be noted that a high arterial lactate concentration probably affects the supply of other blood-borne substrates to the muscles. Issekutz *et al.* (1965) in experiments on dogs showed that an infusion of sodium lactate of the same relative magnitude as in this study results in a pronounced lowering of the plasma concentration of FFA and in a reduction of the rate of oxidation of FFA and glucose in plasma. In contrast to these findings Svedmyr (1966) found in man a slight elevation of the plasma concentration of FFA on infusion of sodium lactate The rate of infusion used by him, however produced a slightly less pronounced rise of blood lactate than in the present study The abovementioned factors however have presumably been of subordinate importance in the present experiments, as the same RQ was measured at ordinary and elevated lactate concentration after 40 min of exercise It is noted, however that the methodological errors in the RQ determinations are of such magnitude that minor deviations between the relative proportions of the various substrates in the total oxidation cannot be detected. The lower oxygen consumption at elevated arterial lactate concentration may possibly be interpreted as a sign that the carbohydrate oxidation constituted a greater proportion of the oxidative metabolism since the energy recovered per mole oxygen is somewhat greater in carbohydrate oxidation than *in fat oxidation*.

The results permit no conclusions concerning the mechanism underlying the lower net production of lactate in forearm muscle at elevated arterial lactate concentration. The following explanations, however may be conceivable

1) In muscle fibres with a net production of lactate the intracellular lactate concentration must rise in step with the arterial in order that an outward directed diffusion gradient may be maintained The high intracellular lactate

concentration which thus arises may conceivably inhibit the formation of lactate through one or more feedback processes.

2) Red muscle fibres, which appear to have the morphological and enzymatic prerequisites for utilization of exogenous lactate (see Discussion in Chapter IV) take up a quantity of lactate which in analogy with the myocardium (Goodale, Olson and Hackel 1959) is related to the arterial lactate concentration

The latter alternative appears to be the most probable explanation of the net uptake of lactate recorded after 55 min of exercise

The rate of lactate uptake rose with increased supply of lactate to the muscles. The curvilinear relation between these variables may indicate the existence of saturation kinetics. Whether the rate limitation is caused by a limited rate of transport for lactate through the cell membrane or a limited rate of metabolism of the extracted lactate cannot be definitely decided solely on the basis of the present results. Other investigations of the difference of concentration of lactate between skeletal muscle and blood in dog (Karlsson, Personal communication) and of the lactate transport conditions in hypothermal myocardium (Kübler *et al.* 1966) suggest that the rate of lactate transport through the cell membrane is restricted. Nor solely on the basis of the present results, can one draw any definite conclusions concerning the negative regression of the fractional oxidation of extracted lactate on the fractional lactate uptake. A conceivable mechanism is that, in the tricarboxylic acid cycle, the rate of direct oxidation of ^{14}C-L(+)-lactate taken up was mainly constant and that the surplus ^{14}C-L(+) lactate extracted was metabolized by other means. Further investigations are required, however to cast more light on these conditions.

CHAPTER IV

General Discussion

The use of labelled lactate enables a study to be made of the metabolism and turnover of lactate in the body as a whole and in individual organs and tissues. The rate of turnover of lactat is high however and incorporation of the label into other metabolites occurs rapidly It is therefore necessary in studies of this type to make use of a specific method of analysis for labelled lactate. The method described in this study has proved well adapted for studying the lactate turnover in forearm muscle during exercise. The recovery obtained by this method is not affected by th total lactate concentration in blood within physiological limits and the method hould therefore be usable also in turnover rate studies during physical exercise or in conditions of pathologically elevated lactate concentr tion. The necessity of a specific method of analysis for these studies is illustrated by the results reported in Chapter III-C. During a 5—10 min period of infusion of L(+)-lactate-1 ^{14}C into the brachial artery it was found that about 10 % of the radioactivity in the venous blood coming from the working muscles was incorporated into other non-volatile metabolites than labelled lactate Immediately after the infusion the corresponding value for arterial blood was about 50 %. In the light of the foregoing statements the assumption that all radioactivity in the blood after infusion of ^{14}C-L(+) lactate ith the exception of ^{14}CO consists of ^{14}C-L(+) lactate (Drury Wick and Morita 1955) must be regarded as an approximation.

The results of this study show that an exchange of lactate betwern the blood and skeletal muscle during exercise takes place in both directions and that the exogenous lactate is partly catabolized in muscle, inter alia to CO_2. These in vivo studies on man confirm earlier results from animal experiments (Drury and Wick 1956 Omachi and Lifson 1956) The net release of lactate from forearm muscle falls successively during prolonged exercise, whereas the lactate uptake appears to be independent of the length of the exercise period but dependent on the arterial lactate concentration. A possible explanation of this circumstance may be that the individual muscles are made up of red and white fibres which have different metabolic patterns (for survey see Keul Doll and Keppler 1969) The white fibres have a large content of glycogen and high activity of glycolytic enzymes. Their LDH is of M type, which has a high pyruvate to-lactate reducing activity even at high pyruvate concentrations (Daw

son, Goodfriend and Kaplan 1964) These fibres are thus adapted for resynthesizing their ATP to a large extent through glycolysis, with consequent production of lactate. The red fibres on the other hand are adapted for resynthesizing ATP chiefly through oxidative metabolism. They have an abundant capillary network, high myoglobin content, numerous mitochondria and high activities of the enzymes which are essential for complete oxidation of carbohydrates and free fatty acids. Their LDH is of H type, i.e. the same type as in heart muscle. This LDH is inhibited by pyruvate, thus favouring oxidation of the pyruvate in the tricarboxylic acid cycle (Dawson Goodfriend and Kaplan 1964)

In a study of the anterior tibial muscle of the rat Kugelberg and Edström (1968) were able to distinguish, apart from white fibres (A fibres) two types of red fibres (B and C fibres) According to these authors there are no pronounced differences in glycogen content between A B and C fibres. On the other hand they found a difference in glycogen breakdown on stimulation, the breakdown being very rapid in A fibres, rapid in B and apparently non-existent in C fibres. The B fibres thus have a significant anaerobic as well as aerobic metabolism and from this aspect are an intermediate form between A and C fibres. The same authors showed that a motor unit consists of only one kind of fibres, phasic units of white fibres, tonic units of red fibres. The recruitment of the motor units varies with time. In a study of the anterior tibial muscle of man Grimby and Hannerz (1968) found that, on rapid contraction of a resting muscle, a group of motor units (A units) is initially activated but on continued contraction is quickly relieved by another group (B units) On rapid contraction of a muscle which has recently been active, a direct activation of B units takes place. On prolonged isometric contraction these authors saw in some cases an activation of an additional unit (C unit)

These circumstances offer a conceivable explanation of the process of lactate release from the muscle during exercise. The very early release of lactate from the forearm muscles on changing from total rest to exercise (Pernow and Wahren 1962) may possibly be due to activation of A fibres. On continued intermittent exercise at submaximal level these fibres are probably not activated. But if the work intensity exceeds a given level, a reactivation of A fibres may conceivably take place. In such case this may contribute to the rapid rise of blood lactate at work intensities exceeding 75 % of maximum (Hermansen and Saltin 1966) and to the marked rise of lactate production in forearm muscle when the work intensity is increased from 5 to 10 kpm/min (Wahren 1966) The marked lowering of the rate of net release during prolonged exercise as found by Wahren and Hagenfeldt (1968) and verified in the present study may possibly be explained by a successive emptying of the glycogen con

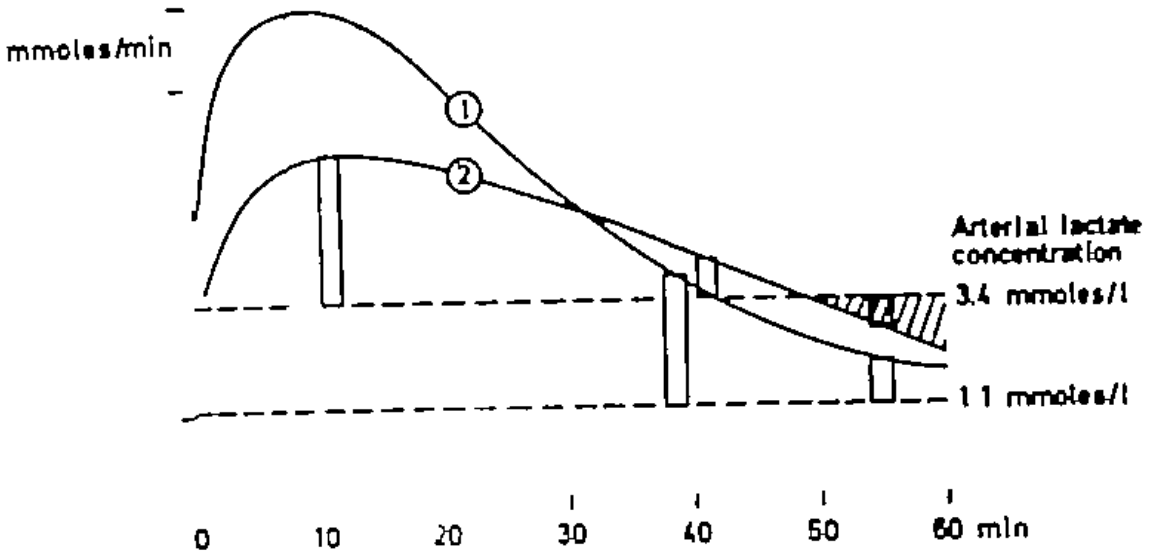


Fig 20 Schematic representation of rates of lactate uptake (— — —) net release (open brackets) or net uptake (shaded area and bracket) and calculated total production (——) during exercise at normal (1) and elevated (2) arterial concentrations of total lactate.

tent of the glycolytic fibres (cf Saltin and Hermansen 1967) and/or non activation of these fibres with successive changeover to C fibre activity The immediate oxidation of the lactate taken up in forearm muscle during exercise probably takes place mostly in red fibres. This assumption is confirmed by results of in vitro experiments in non-stimulated muscle which showed that the quantity of lactate 1 ^{14}C recovered as $^{14}CO_2$ was greater for red than for white muscle (Bär and Blanchaer 1965) Furthermore the LDH in the red fibres is better adapted for oxidation of lactate to pyruvate than the LDH in the white fibres. It may be assumed that in some of the red fibres, in conformity with the condition in the heart muscle (Goodale Olson and Hackel 1959 Keul *et al* 1965) a net uptake of lactate takes place which is related to the arterial lactate concentration. Whether the part of the ^{14}C-L(+) lactate uptake which is not immediately oxidized is metabolized preferentially in red or white fibres cannot be decided. In vitro studies indicate that the incorporation of ^{14}C in glycogen from DL-Lactate 1 C is equal in red and white muscle (Bär and Blanchaer 1965) These circumstances suggest that the relation between release and uptake of lactate differs in the different types of fibres. Some uptake of lactate, as determined in this study may however take place in all cells with an intracellular lactate turnover

A conceivable interpretation of the findings of simultaneous uptake and release of lactate in forearm muscle is illustrated in Fig 20 At a normal arterial lactate concentration (curve 1 Fig 20) the rate of production is greater than

the rate of uptake, so that a net release of lactate is recorded during the entire period of exercise. The successively diminishing rate of net release during the exercise reflects a reduced lactate production. At a higher arterial lactate concentration (curve 2, Fig 20) the rate of lactate uptake is increased. In this case, too the rate of production is greater than the rate of uptake during the greater part of the exercise period, so that a net release of lactate is recorded. At the end of the exercise period however the rate of lactate production within the forearm has fallen to so low a level that it is less than the rate of uptake and therefore a net uptake is recorded.

The fact that it would seem that a rapid exchange of lactate can take place across the cell membrane, and that the histological picture shows a close contact between red and white fibres (Kugelberg and Edström 1968) makes it possible that lactate released by white fibres can be partially taken up and oxidized by red fibres.

The rate of lactate uptake was better correlated to the product of the arterial lactate concentration and the muscle blood flow than to the arterial lactate concentration alone A possible explanation of this circumstance may be that a larger blood flow implies a larger open capillary surface and therefore more favourable conditions for uptake of lactate. This finding fits in with earlier investigations by Rämmal and Ström (1949) and Freyschuss and Strandell (1967) who at elevated blood lactate level, showed that the lactate utilization in the body was more rapid and the calculated rate of uptake of lactate in the legs greater respectively when the subjects were performing light leg exercise than at rest. The rapid exchange of lactate found between blood and skeletal muscle and the fact that under certain conditions a net uptake of lactate occurs in working skeletal muscle indicate that the skeletal musculature, which represents about 40 % of the body weight (Andres, Cader and Zierler 1956) may have a quantitative significance for the lactate catabolism in the body

CHAPTER V

Summary

The uptake and release of lactate in forearm muscle has been studied in young healthy volunteers at different times during prolonged exercise at both normal and artificially elevated arterial lactate concentration. *L(+) lactate-1 ^{14}C* has been used as tracer substance. An enzymatic method for determination of ^{14}C-L(+) lactate has been evolved.

1 *Method of analysis*

The lactate in a perchloric acid extract of blood is oxidized to pyruvate by using LDH and with NAD as proton acceptor. Hydrazine sulphate binds the pyruvate produced. After adjustment of the pH and addition of phenylhydrazine hydrochloride and pyruvate as carrier the pyruvate is precipitated as phenylhydrazone, washed, filtered off and the radioactivity counted in a liquid scintillation counter. A blank is analysed in an identical way but without LDH and NAD. The difference in radioactivity is considered to originate from *L(+)-lactate* since the *LDH* used is specific for that stereo-isomer. The recovery is 85.2 ± 4.0 (mean ± SD) and the coefficient of variation 2.8 % and 7.2 % at more than or less than 1000 dpm/ml blood respectively.

2. *Metabolic studies*

The subjects performed during 60 min a rhythmic, isotonic forearm exercise on a hand ergometer at a work intensity of 10 kpm/min and with one contraction per sec. Six subjects were given an intravenous infusion of sodium lactate, whereby an arterial lactate concentration of about 3.5 mmoles/l was obtained during the entire investigation. The forearm blood flow was determined by indicator dilution technique with continuous infusion of dye, which also contained the radioactive lactate.

The net release of lactate was higher after 10 min of exercise than after 40 min. If the exercise was performed at an elevated arterial lactate concentration the net release was lower and at the end of the period of exercise, changed to a net uptake. Simultaneously with the net release there was an uptake of lactate in forearm muscle. The uptake was the same at 10 and 40 min and amounted to 31 and 23 % respectively of the arterial lactate at normal and elevated arterial lactate concentration. The results indicate that a rapid ex

change of lactate takes place in both directions between blood and muscle. Only 30—50 % of the ^{14}C-L(+) lactate taken up in the muscles underwent immediate oxidation to $^{14}CO_2$. About 20 % was released from the muscle in the form of other metabolites. The release of $^{14}CO_2$ after the infusion of ^{14}C-L(+) lactate exhibited a biexponential course, which indicates that the remainder of the lactate uptake was incorporated into pools with slow turnover. The rate of lactate uptake has been best correlated to the lactate inflow into the muscle expressed as the product of blood flow and arterial lactate concentration, and the relation indicates a saturation kinetics mechanism for the uptake of lactate.

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An experimental study

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CONTENTS

I INTRODUCTION 5

II THE EFFECTS OF INCREASED CSF PRESSURE ON CEREBRAL BLOOD FLOW 7

a Methodological considerations 7
b Summary of main results 8
c Discussion 9

III THE EFFECTS OF INCREASED CSF PRESSURE ON CEREBRAL ENERGY METABOLISM 13

a Biochemical background 13
b Methodological considerations 14
c Summary of main results 15
d Discussion 17

IV GENERAL SUMMARY AND CONCLUSIONS 21

V LIST OF ABBREVIATIONS USED 24

ACKNOWLEDGEMENTS 26

REFERENCES 27

The present thesis is based on the following five papers

I Höggendal E J Löfgren N J Nilsson and N N Zwetnow

Effects of varied cerebrospinal fluid pressure on cerebral blood flow in dogs Acta physiol scand 1970 In press

II Höggendal E J Löfgren N J Nilsson and N N Zwetnow

Prolonged cerebral hyperemia after periods of increased cerebrospinal fluid pressure in dogs Acta physiol scand 1970 In press

III Kjällquist Å B K Siesjö and N N Zwetnow

Effects of increased intracranial pressure on cerebral blood flow and on cerebrospinal fluid HCO_3^- pH lactate and pyruvate Acta physiol scand 1969 75 345–35.

IV Zwetnow N N

The influence of an increased intracranial pressure on the lactate pyruvate bicarbonate phosphocreatine ATP ADP and AMP concentrations of the cerebral cortex of dogs Acta physiol scand 1970 In press

V Siesjö B K and N N Zwetnow

Effects of increased cerebrospinal fluid pressure upon adenine nucleotides and upon lactate and pyruvate in rat brain tissue Acta neurol scand 1970 In press

In the text the papers are referred to by their Roman numerals

I INTRODUCTION

It is generally accepted that the brain is one of the tissues of the body which is most sensitive to a deficiency of its blood supply (for review see Mollwain 1966) This is due to the fact that the tissue has a high resting energy consumption and relatively low reserves of substrates and of high energy compounds Under normal circumstances the tissue utilizes glucose as the main substrate and the high energy consumption requires that the glucose is fully oxidated. Thus when oxygen is deficient the energy yield from glucolysis is insufficient both for the function of the tissue and for the maintenance of its structural integrity The tissue reserves of substrate (glucose and glycogen) and of high energy compounds (phosphocreatine and ATP) are sufficient to support function for only a few minutes and a complete interruption of the circulation will very quickly lead to irreversible tissue changes If the circulation is impaired but not abolished survival will be prolonged but the increased glucolysis and the ensuing lactacidosis will by itself represent a threat to the tissue

The brain possesses efficient mechanisms which adjust its blood supply relative to the needs of the tissue The blood flow is determined primarily by two factors i.e. the driving perfusion pressure and the vascular resistance An increased metabolic demand may through locally situated regulatory mechanisms, elicit a vasodilation which results in an appropriate cerebral blood flow A more dramatic vasodilatation however may occur in situations which are accompanied by decreased perfusion pressures In these situations it will depend upon the efficiency of the flow regulation if the brain may be saved from hypoxic injury

Variations in the cerebrospinal fluid (CSF) pressure – a commonly occurring phenomenon in intracranial diseases (see Lundberg 1960) – represent a potential threat to the cerebral circulation primarily and to the energy metabolism of the tissue secondarily While a decrease in CSF pressure has been said to provoke various vascular changes (arteriolar constriction local arterial vasospasms dilation of subarachnoid veins) and clinically been considered of importance ("intracranial hypotension syndrome" see Schaltenbrand 1940 Sarteschi 1951 Shenkin and Fimeson 1958) its effect on cerebral blood flow (CBF) has not been evaluated experimentally A considerable interest however has been focused on the effect of an increase in CSF pressure on CBF In such a situation the cerebral perfusion pressure is decreased pari passu with the increase

of pressure in the collapsible subarachnoidal veins occurring simultaneously to the increase in the CSF pressure and maintenance of the cerebral blood flow becomes dependent upon dilatation of the resistance vessels There is a great number of studies of the effect of an increased intracranial pressure on cerebral blood flow and cerebral function (for review see Paper I Langfitt 1968 Zwetnow 1966)

The clinical studies performed have indicated that the cerebral vessels can autoregulate when the intracranial pressure is increased but the quantitative data obtained have been somewhat conflicting Thus Kety et al (1948) showed that in patients with brain tumors the cerebral blood flow decreased when the intracranial pressure exceeded about 450 mm H_2O On the other hand Ryder et al, (1951) as well as Greenfield and Tindall (1965) showed that cerebrospinal fluid pressure could be increased acutely to 1000 mm H_2O before clinical symtoms appeared or before the cerebral blood flow decreased. The differences in results between these studies may be due both to the duration of the intracranial hypertension and to the nature of the processes leading to the hypertension since these may well interfere with the normal regulation of the CBF

It is to be expected that the quantitative relations between the intracranial pressure and the cerebral blood flow can be better evaluated in animal experiments There are a number of qualitative and semiquantitative studies showing that cerebral vasodilation occurs when the cerebrospinal fluid pressure is increased (Wolff and Forbes 1928 Wolff and Blumgart 1929 Fog 1934 Ludwigs and Wiemers 1953 see also Heiskanen 1964 and Langfitt 1968 for review) and it has also been shown that restitution of a normal CSF pressure is followed by a shortlasting increase of the flow above the control values ("reactive hyperemia" see Noell and Schneider 1948) However in spite of information of this type from a number of studies quantitative data have been lacking defining the level of CSF pressure which leads to a decrease of flow and no study has been made of the effects of intracranial pressure upon the cerebral energy metabolism

The present study is an attempt to assess quantitatively the influence of an increased CSF pressure on the cerebral blood flow both during and after the hypertension (Papers I and II) as well as on the energy metabolism of the tissue The latter was studied by analysing lactate pyruvate and bicarbonate concentrations in brain tissue (Papers IV and V) and in CSF (Paper III) as well as the tissue concentrations of phosphocreatine ATP ADP and AMP (Papers IV and V) The results of the studies will be presented by considering first the relation between the CSF pressure and the cerebral blood flow and then the relation between the CSF pressure and the cerebral energy metabolism

II THE EFFECTS OF INCREASED CSF PRESSURE ON CEREBRAL BLOOD FLOW

The influence of experimental increases in the CSF pressure on the cerebral blood flow (CBF) of dogs was studied in Papers I and II which also contain details of different experimental procedures and techniques CBF measurements were also carried out in several experiments in papers III and IV and served the purpose of defining the cerebral circulatory state of the animals in which chemical analyses were made of cerebral energy metabolism

a Methodological considerations

With the method used for increasing the CSF pressure (P_{CSF}) a generalized increase of the intracranial pressure (ICP) was produced as shown in Paper I This generalized increase in pressure may be of importance when evaluating the effects on flow since it has been found (Langfitt et al, 1965 Huber et al 1965 Baldy-Moulinier and Frerebeau 1968 Easton and Pálvölgyi 1968) that if the method used for increasing the pressure gives rise to local pressure effects autoregulation may be absent (see below) or deficient The method also allows the intracranial pressure to be almost instantaneously raised and to be accurately controlled even during longlasting experiments However since an increased CSF pressure usually leads to an increased absorption of CSF (see Davson 1967 Andersson and Löfgren 1965) there will be a flow of artificial CSF over the convexities of the hemispheres allowing a diffusion of ions into the intercellular spaces of the brain (Rall Oppelt and Patlak 1962 Fencl Miller and Pappenheimer 1966) It was thus possible that vascular or metabolic effects would be elicited by the artificial CSF per se if it differed in composition from that of the native CSF However in all instances the potassium magnesium calcium and bicarbonate concentrations of the artificial CSF were very close to those of dog CSF in the control state Moreover control experiments with infusion of dextrane blood or CSF from a donor dog into the cisterna magna did not significantly influence the reactive hyperemia elicited by increased intracranial pressure under otherwise comparable conditions (Paper II)

The CBF method used (Lassen et al. 1963 see also Höggendal et al 1965) is based on the tissue clearance of an inert gas – xenon^{133} or krypton^{85} – injected into the vertebral artery The method requires only moderate surgical preparation and no blood sampling thus restricting the traumatic influence on the cerebral vascular bed to a minimum It allows repeated measurements at intervals of 10 – 15 min to be made but since it requires a steady state with regard to flow during this period rapid changes in flow are difficult to assess quantitatively

The CBF values varied from animal to animal probably chiefly due to a biological scatter In the particular animal however a virtual constancy in flow was found when induced CSF pressure levels of different heights were kept unchanged for hours Thus using each animal as its own control the study of CBF under the defined steady state conditions of pressure and flow was considered fully possible (for accuracy of flow method see Höggendal 1965)

In all experiments CBF was estimated from the fast component of the clearance curve The values thus represent cortical flow mainly (Lassen et al 1963 Höggendal et al 1965) In addition total cerebral blood flow was also calculated in all cases by means of the height–over–area method (see Höedt-Rasmussen et al, 1966) The main emphasis was placed on the results obtained with the first mentioned method but the conclusions drawn from these data were usually not much different from those based on total CBF determinations

b Summary of main results

The main task of Paper I was to study the cerebral blood flow when the CSF pressure was raised stepwise from –15 mm Hg to +150 mm Hg The cerebral blood flow was consistently found to remain virtually unchanged until a CSF pressure of about 100 mm Hg was reached corresponding to a cerebral perfusion pressure (for definition see above) of 30–50 mm Hg At pressures above 100 mm Hg however the CBF fell progressively approaching flow values as low as 10 – 20 ml/100 g/min If at such low flow values the cerebrospinal fluid pressure was raised still further a rapid fall in systemic pressure usually occurred and the animals succumbed The well-known vasoactive influence of hypercapnia and hypocapnia on CBF was demonstrated to be present even at high intracranial pressure Even when the cerebral perfusion pressures were lowered down to 20 – 30 mm Hg the vasodilatory effect of CO_2 still seemed to operate A compensatory blood pressure rise (Cushing effect) was ob–

served when the CBF pressure exceeded 100 mm Hg The main object of the experiments described in Paper II was to give a quantitative description of the reactive cerebral hyperemia elicited when an elevated cerebrospinal fluid pressure was brought back to normal The cerebral hyperemia usually ensued upon periods of induced cerebrospinal fluid pressure exceeding about 50 mm Hg corresponding to a cerebral perfusion pressure of 70–80 mm Hg as well as upon periods of reduced cerebral blood flow In some cases however a hyperemia was also seen without a prior flow reduction. An impressive feature of the hyperemia was its long duration in some experiments lasting for several hours Reversibility of the hyperemia was the rule though the time before the overshoot in flow was restored to normal varied greatly

c Discussion

The results of Paper I clearly indicate two mechanisms which operate to maintain the CBF at increased CSF pressures as well as their relative efficiency Obviously the changes in cerebrovascular resistance play the main part operating efficiently as long as the cerebral perfusion pressure exceeds a level of 30–50 mm Hg Later studies with a serio–angiographical x-ray technique (Alba et al 1967) have confirmed these results

The second flow-compensating mechanism – the increase in systemic pressure (the Cushing effect see Cushing 1901) – was called into action at CSF pressures exceeding about 100 mm Hg when there was already a measurable decrease in CBF No overlapping seemed to occur between the two flow-compensating mechanisms An exhaustion of the autoregulatory mechanism seemed to be a prerequisite for the Cushing effect to appear Moreover it was evident that while the cerebral vasodilatation very effectively kept the CBF up the Cushing effect was never able to restore it to normal only to diminish the degree of flow reduction. Evidence is accumulating that ischemia or hypoxia in the brain stem is the factor eliciting the Cushing effect (see Sagawa 1967 and paper V) If so the present results make it probable that the blood flow in the brain stem decreases simultaneously with the flow in the grey matter areas of the cerebral hemispheres The Cushing effect appearing in this situation may thus be taken as a clearcut and ominous sign of a general ischemia of the brain

It may seem odd that the cerebral blood flow was kept virtually unchanged up to CSF pressures around 100 mm Hg in our experiments while rather

small rises in CSF pressures brought about by intracranial expansive lesions tend to reduce the CBF both in clinical cases (Kety et al 1948 Easton and Pálvölgyi 1968) and in animal experiments (Huber et al 1965 Langfitt et al. 1965) It would thus seem that a decreased flow may result from an intracranial expansivity as such or from regional increases in the intracranial pressure which only to a moderate extent affect the cerebrospinal fluid pressure Another explanation might be that a long-lasting CSF pressure of moderate height might have special effects not seen in acute situations Decreases in the flow values were however not observed in our experiments when constant levels of increased CSF pressure were induced and kept for periods up to 6 hours

It may here be emphasized that reduced CBF at high CSF pressures not necessarily means maximal vasodilatation. Thus experiments where CSF pressure was raised in hypercapnic animals suggested that CO_2 could dilate the vessels further in a situation when maximal vasodilatation was assumed to be attained due to the markedly reduced perfusion pressure (20–30 mm Hg) This finding, which has later been confirmed (Ekström Jodal et al 1969) suggests that the vasodilatory mechanisms during autoregulation and during CO_2 administration are different.

In Paper II an attempt was made to perform a closer study of the increase in cerebral blood flow which followed when the increased CSF pressure was brought back to normal The existence of a reactive hyperemia after periods of increased CSF pressure was anticipated by earlier workers (Wolff and Forbes 1928) and it was qualitatively studied by Noell and Schneider (1948) and by Ludwigs and Wiemers (1953) A more systematical study of this phenomenon has however so far been lacking

The main features of the cerebral hyperemia (schematically outlined in fig 1) followed essentially the same general pattern as has been observed in other tissues or body organs (see Barcroft 1963 Greenfield 1963 Gregg and Fisher 1963)

The cerebral hyperemia was usually reversible although in contrast to hyperemias in most other tissues it sometimes took several hours to bring the flow back to normal The first blood flow value measured during the hyperemia was always the largest one and this could amount to 5 – 6 times the control flow value Usually the blood flow measured prior to the hyperemia was diminished compared to the control value, but a measurable hyperemia was occasionally seen without a preceding reduction of CBF The flow debt was usually overpaid

It should be pointed out that the calculated vascular resistances during the last phase of the intracranial hypertension and during the first minutes of

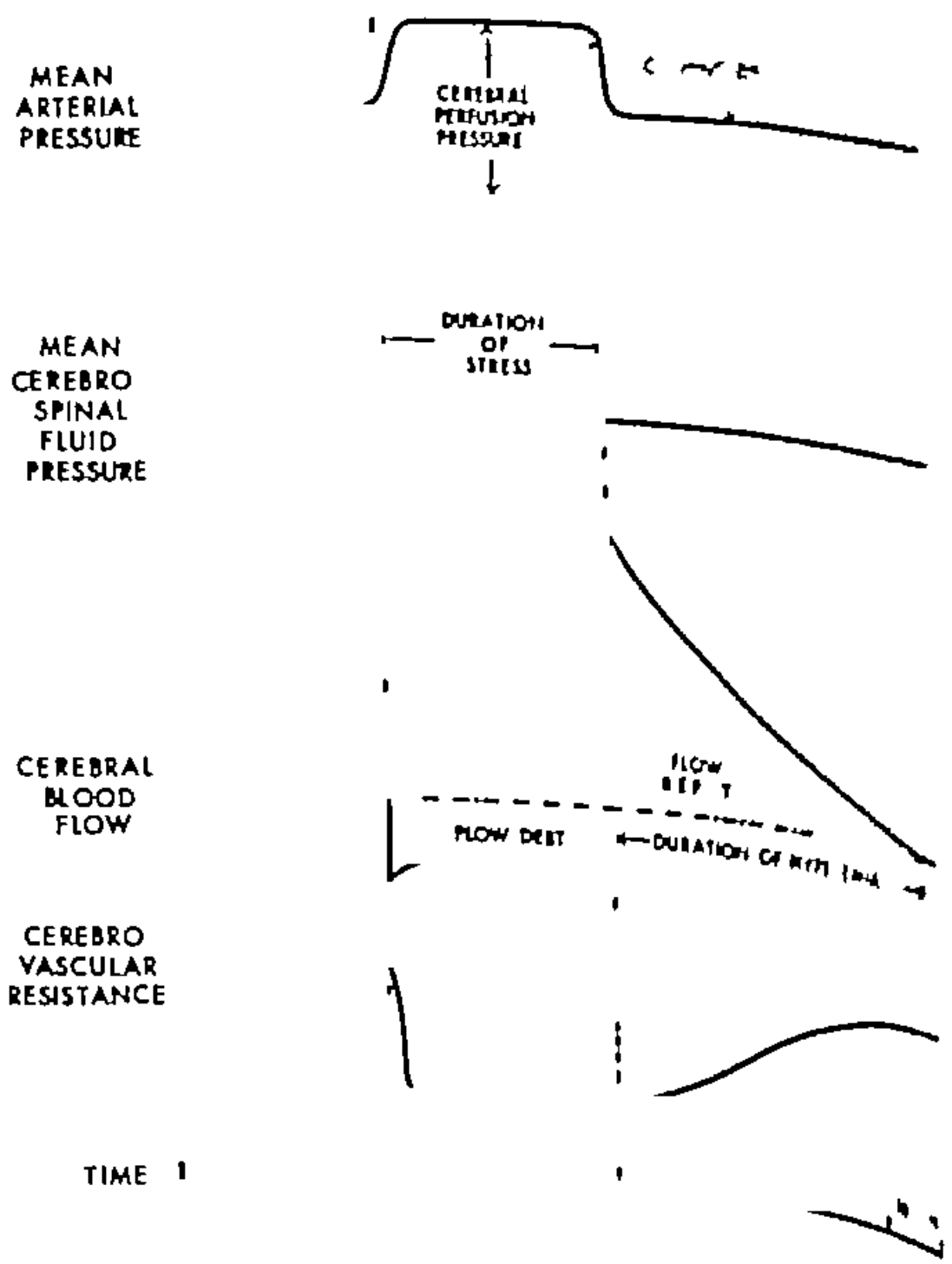


Fig 1 Schematical description of the changes in the pertinent hemodynamic parameters during an induced square wave increase in CSF pressure (For details see text)

the hyperemia respectively differed very little Thus the vascular resistance calculated for the period immediately preceeding the normalization of pressure determined the peak value of the hyperemia which subsided concurrently with the increase of the resistance caused by the gradual constriction of the resistance vessels While the changes in vascular diameter occurring after small variations in cerebral perfusion pressure have been reported to occur quite rapidly i e within a few minutes a slow regulation of the vascular resistance appears to be responsible for the prolonged hyperemia An overshoot of flow - a reactive hyperemia in its strict sense - must always be assumed to follow a period of reduced cerebral perfusion pressure even if this reduction is very shortlasting However after shortlasting and moderate reductions of the cerebral perfusion pressure the hyperemia may vanish so quickly that it cannot be adequately measured with steady state flow methods (see e g Kjällquist Siesjö and Zwetnow 1969) Such a hyperemia may merely reflect the preceeding dilatation of the vessels and has presumably a pathophysiological implication which is different from the longlasting hyperemia usually seen after prolonged and marked reductions of the cerebral perfusion pressure It would thus appear most logical to assess the hyperemia in terms of its duration which in most situations would give important information on the severity of the disturbance of tissue metabolism (see Lassen 1966) The long-lasting and slowly subsiding cerebral hyperemia is probably closely related to the phenomenon of cerebral vasoparalysis which may be produced experimentally in animals using intracranial expanding lesions or repeated impacts to the skull (Langfitt 1968)

III THE EFFECTS OF INCREASED CSF PRESSURE ON THE CEREBRAL ENERGY METABOLISM

The object of the experiments described in Paper III IV and V was to study the effects of increased intracranial pressure on the energy metabolism of the tissue as reflected in the tissue concentrations of phosphocreatine ATP ADP AMP lactate pyruvate and bicarbonate and in the CSF concentrations of lactate pyruvate and bicarbonate The importance of these parameters in the energy metabolism of the tissue will be evident from the following arguments

a Biochemical background

As mentioned in the introduction the tissue can only obtain an adequate amount of high energy compounds by oxidating glucose to CO_2 and water The necessary amount of ATP is obtained when the electrons obtained from the oxidation of pyruvate are passed along the electron transport chain to be finally accepted by oxygen In a hypoxic situation irrespective of its cause a series of characteristic biochemical events will follow Thus when less oxygen is available to accept the electrons the various redox systems will shift toward their reduced states At a certain degree of reduction the phosphorylation of ADP will slow down and there will be a tendency for the ATP concentration to decrease However due to the operation of the creatine phosphokinase reaction the ATP levels will be maintained at the expense of the phosphocreatine concentrations The ATP concentrations can also be upheld through the myokinase reaction but this will lead to an increase also in the AMP concentrations Consequently it is to be expected that a moderate hypoxic condition will be accompanied by decreased phosphocreatine concentrations and increased AMP concentrations However it is possible that a certain degree of reduction of the electron carrier systems can occur before there are any measurable changes in the concentrations of the phosphorylated compounds In such a case a chemical diagnosis of tissue hypoxia will depend on methods which can detect a change in the oxidation-reduction state of the carriers A convenient method of measuring such a change is to analyze the components of a system which exists in a steady state relationship with a carrier system such as the $NADH/NAD^+$

couple Of such systems the lactate/pyruvate system lends itself best to analyses and this system also has the advantage that its components are diffusible through cell membranes Accordingly an increase in the lactate/pyruvate ratio of the cytoplasm will be transmitted to the extracellular fluid (Huckabee 1958 Hohorst Kreuz and Bücher 1959 Hohorst 1960 Siesjö Kjällquist and Zwetnow 1968) When evaluating the redox state from lactate/pyruvate ratios it must be kept in mind though that these ratios depend not only on the cytoplasmatic NADH/NAD^+ ratio but also on the hydrogen ion activity according to the relation

$$\frac{NADH}{NAD^+} = \frac{Lactate}{Pyruvate} \frac{K}{(H^+)}$$

A decrease in the pH of the system will thus by itself tend to increase the lactate/pyruvate ratio A proper evaluation of the NADH/NAD^+ ratio requires knowledge both of the intracellular lactate/pyruvate ratio and of the intracellular pH However it is not certain that such a calculation will reveal a moderate increase of the NADH/NAD^+ ratio due to inevitable variations in the calculated values It could thus be desirable to evaluate the redox state from the lactate and pyruvate concentrations alone This can be done qualitatively since there are many reasons to believe that a decrease in the intracellular pH by itself increases the lactate/pyruvate ratio and decreases the lactate concentration (Leusen Lacroix and Deemester 1957 Granholm and Siesjö 1969 Kjällquist Nardini and Siesjö 1969) Thus if there is an increase in the lactate/pyruvate ratio and a concomitant increase in the lactate concentration a true rise in the NADH/NAD^+ ratio is probably present

b Methodological considerations

In the experiments in which the influence of the increased CSF pressure on the CSF lactate pyruvate and bicarbonate concentrations was studied the infusion of an artificial CSF made it difficult to study the changes in the native CSF The distortion of the true lactate pyruvate and bicarbonate concentrations should be grossly proportional to the amount of CSF infused and thus both to the level of intracranial pressure and to the duration of the pressure period The error was minimized by calculating the admixture of artificial CSF to the samples from the dilution of added C^{14}-inulin. However since the outflux of lactate pyruvate bicarbonate and inulin from the CSF system will probably not be equal the procedure

will only give approximate concentrations whereas the true lactate/pyruvate ratios can be assumed to be unaffected In order to ascertain that changes in the lactate and pyruvate concentrations calculated represented true events they were verified by decreasing the cerebral perfusion pressure by means of controlled arterial bleeding both in the present experiments (Paper III) and in other experiments from the laboratory (Siesjö and Zwetnow 1970)

When evaluating the CSF results it must also be remembered that changes in the tissue concentrations of lactate pyruvate and bicarbonate will be reflected in the bulk CSF sampled from the large cavities with an inevitable lag Thus a transient increase in the lactic acid production in the tissue will show a delayed rise in the large cavity CSF fluid and the CSF may show an increased lactate concentration and an increased lactate/pyruvate ratio at a time when the tissue values are again approaching the normal ones (c f Kaasik Nilsson and Siesjö 1970b) When interpreting changes in the CSF concentrations of these ions it may be helpful to remember that the rate of free diffusion of e.g. bicarbonate ions in the intercellular channels is such that 50 per cent of a step change should be transmitted 10 mm in 10 min (Pappenheimer et al. 1965) and that 0 4 per cent of the CSF is renewed every minute (see Davson 1967)

The determination of tissue concentrations of bicarbonate lactate pyruvate ATP ADP AMP and phosphocreatine were performed with previously described titrimetric and enzymatic techniques (Pontén and Siesjö 1964 Hohorst Kreutz and Bücher 1959 Lowry et al. 1964) In all experiments the tissue was frozen in situ with a method described by Pontén 1966 (see also Granholm et al. 1968) and the tissue was extracted for the enzymatic analyses using the method described by Lowry et al 1964 This method yields optimal concentrations for the labile tissue phosphates with minimal autolysis (for discussion see Nilsson and Siesjö 1970) It should be remarked though that the AMP concentrations reported in Paper IV are slightly too high due to a contamination of the NADH with AMP (Lowry et al. 1964)

c Summary of the main results

In Paper III it was shown that increments in CSF pressure leading to cerebral perfusion pressure reductions to 40 mm Hg or lower and to a decrease in CBF and thus ultimately to a measurable reactive hyperemia also led to long-lasting and usually marked increases in the lactate concentrations and the lactate/pyruvate ratios as well as to

decreases in the bicarbonate concentration of the cerebrospinal fluid. Similar changes though to a less degree were usually observed also after such small increments in CSF pressure which did not seemingly alter CBF Reduction of the cerebral perfusion pressure brought about by controlled bleeding produced in essence the same metabolical findings

In Paper IV the influence of a 15 min reduction of the cerebral perfusion pressure to 30 – 35 mm Hg on labile metabolites and on bicarbonate concentrations of brain tissue were studied There were variable but usually marked increases in the tissue lactate and pyruvate concentrations as well as in the lactate/pyruvate ratios corresponding decreases in the bicarbonate concentrations and marked decreases in the ATP and the phosphocreatine concentrations Resting values for all metabolites measured were not approached until 30 – 90 min after restitution of a normal perfusion pressure CBF measured in some of the animals generally showed reduced values during the period of increased CSF pressure and a reactive hyperemia upon restoration of the CSF pressure to normal The duration of the hyperemia grossly coincided with the disturbance of the energy metabolites

In Paper V the cerebrospinal fluid pressure was progressively raised in rats and studies were made of energy metabolites and of bicarbonate in parts of the tissue designated as supratentorial parts cerebral hemispheres cerebellum and the brain stem No significant changes in phosphocreatine ATP ADP or AMP concentrations and no marked changes in the lactate concentrations were found until the induced intracranial hypertension had decreased the cerebral perfusion pressure to about 30 mm Hg There was a small increase in the lactate concentrations and in the lactate/pyruvate ratio of supratentorial structures and of the cerebellum but not of the brain stem at perfusion pressures which were presumably within the CBF autoregulation range (50 – 100 mm Hg) From the measured total CO_2 contents and from the arterial CO_2 tensions "intracellular pH" and the intracellular bicarbonate concentrations were calculated These figures showed that there was no significant non-respiratory acidosis in the intracellular space until the cerebral perfusion pressure had been reduced below 30 mm Hg

d Discussion of results

The results of Paper III showed that any increase in the CSF pressure which decreased the cerebral perfusion pressure to 40 mm Hg or lower gave rise to marked and longlasting increases in the CSF lactate/pyruvate ratios and in the lactate concentrations as well as to an approximately stoichiometrical decrease in the bicarbonate concentrations The CSF changes were longlasting and could persist for hours after the CSF pressure had been normalized It was also apparent that the reactive hyperemia was unrelated to the extracellular lactacidosis since in some experiments the latter was found to outlast the hyperemia by hours The fact that the CSF lactacidosis sometimes could persist for such long periods makes it entirely unprobable that the lactacidosis of the tissue proper had vanished (see above) and it was thus apparent that a persisting extracellular lactacidosis was compatible with a normal flow Thus even if we assume that the reactive hyperemia is partly caused by the metabolic consequences of the tissue hypoxia it was clear that the flow could return to normal in spite of a remaining extracellular lactacidosis

An unexpected finding was that the typical changes in the CSF lactate pyruvate and bicarbonate concentration were usually seen also after such moderate increases in the P_{CSF} which gave a CPP still within the autoregulation range. These findings were tentatively interpreted as being caused by a transient decrease in flow when the P_{CSF} was increased (see Ludwigs and Wiemers 1953) but a definite answer required tissue analyses (see below)

The longlasting lactacidosis and the longlasting increase in the lactate/ /pyruvate ratio in the CSF recorded after an increase in the ICP (Paper III) could evidently either be due to a slow clearing of lactate from the bulk CSF or to a longlasting disturbance of the oxidative metabolism of the tissue In order to study this problem tissue analyses were carried out at various periods after a 15 min reduction of the CPP to 30 - 35 mm Hg (Paper IV) Although this lowering of the CPP was chosen to give a reproducible reduction of the CBF the results were sufficiently variable to suggest that even these low pressures were just at the limits of the autoregulatory ability of the cerebral circulation. However the main finding of the experiments was the longlasting changes in the metabolites studied Thus it was apparent that the decrease in the CPP gave rise to a cellular lactacidosis which did not subside until 1 - 1 5 hours after the CSF pressure had been brought back to normal There was also a strikingly slow rephosphorylation of ATP and phosphocreatine These results strongly indicate that the oxidative metabolism of the tissue was disturbed for long periods after the normalization of the ICP Thus it has been shown that the tissue phosphates were restituted within 2-5 min after a period

of asphyxia with equally large or larger metabolic changes (Kaasik Nilsson and Siesjö 1970a) The slow restitution of the tissue metabolism after a decrease in the cerebral perfusion pressure may thus be due either to a damage to enzymatic systems involved in the oxidative metabolism or to temporary occlusion of certain capillary channels

The gross correlation of the restitution of flow and energy metabolites respectively would seem to have a bearing on the local control of the cerebral circulation Generally local control of the circulation has been attributed to different factors (myogenic activity acid metabolites adenine nucleotides extra- and intracellular pH increased osmolarity potassium ions and many others see Mellander and Johansson 1968) The extent to which these factors contribute to the reactive hyperemia is not known in detail.

As mentioned above a marked and long-lasting hyperemia ensued when the cerebral perfusion pressure was decreased to such values which gave marked reductions in flow (paper II) Such reductions in perfusion pressure were usually accompanied also by marked changes in the lactate and pyruvate concentrations of both CSF and tissue (Papers III and IV) suggesting that a pronounced hyperemia was elicited only in those cases in which there was a definite disturbance of the oxidative metabolism of the tissue This fact and the gross agreement between the duration of the hyperemia and the duration of the metabolic changes in Paper IV makes it tempting to assume that the longlasting vasodilatation during the reactive hyperemia had a metabolic cause However in our experiments as well as in those of Ekström-Jodal et al, (1969) a reactive hyperemia could also be found without a previous flow reduction and it can even occur at increased flow levels where metabolic changes could hardly be expected to occur (Ekström-Jodal et al, 1969) At this point it thus seems as if both metabolic and myogenic factors may be responsible for the hyperemic response after periods of increased CSF pressure (see Folkow 1962 1964)

In order to study the possible occurrence of minor metabolic changes at moderate increments in CSF pressure (see discussion of Paper III above) a series of experiments were carried out in rats with reductions of the cerebral perfusion pressure within the autoregulatory range established in the dog experiments The CSF pressure was increased in the same manner as in the dog experiments by infusing artificial CSF into the cisterna magna The results were consistent with the findings reported in papers I III and IV in the main points (see also Kjällquist et al 1969 and Siesjö and Zwetnow 1970) Thus although the cerebral perfusion pressure could be reduced to about 30 mm Hg before there were any measurable changes in the tissue concentrations of phosphocreatine ATP

ADP and AMP the lactate concentrations and the lactate/pyruvate ratios were increased at much higher CPP values

In an attempt to evaluate the effect of moderate increases in CSF pressure on different parts of the central nervous system the cerebellum the brain stem and the cerebral hemispheres were analyzed separately Lowering of the CPP to 50 - 100 mm Hg resulted in increased lactate concentrations as well as increased lactate/pyruvate ratios for all structures studied except for the brain stem The resistance of the brain stem to minor CPP reductions is compatible with the clinical reports in which the brain stem survives situations leading to the cell death in supratentorial structures

In order to substantiate that the CSF pressure effects are in reality an effect of CPP reduction and in order to make it possible to evaluate both CSF and tissue changes in a large series of experiments the cerebral perfusion pressure was reduced in rats by means of controlled bleeding so as to yield mean arterial pressures of 30 - 160 mm Hg (Siesjö and Zwetnow 1970) This series of experiments confirmed the results reported above Thus the cerebral perfusion pressure could be reduced to about 40 mm Hg before there were any measurable changes in the tissue concentrations of phosphocreatine ATP ADP and AMP However the lactate concentrations and the lactate/pyruvate ratio in both the extracellular and intracellular spaces appeared to increase continuously when the CPP was lowered within the pressure range mentioned

The increase of lactate concentrations and of the lactate/pyruvate ratios in both rat studies (Paper V and Siesjö and Zwetnow 1970) suggest that a moderate reduction of the cerebral perfusion pressure may lead to minor degrees of reductions in the NADH/NAD^+ system long before there are any changes in the phosphocreatine and the AMP concentrations It is thus possible that the lactate/pyruvate system may be suitable for analyses of minor degrees of tissue hypoxia provided that no altered transfer of the lactate or pyruvate ions between blood and tissue occurs Moreover it must be stressed that as long as the tissue concentrations of phosphocreatine and AMP are not significantly changed the oxidative metabolism of the cerebral tissue cannot be considered to be appreciably disturbed and such a condition could be compatible with normal values of globally measured CBF and $CMRO_2$

The results of the present series of experiments have a bearing on the mechanism regulating the cerebrovascular resistance at reduced perfusion pressures It has been assumed that the extracellular pH of the brain is one of the main factors responsible for the dilatation of the cerebral resistance vessels (see symposium ed. by Ingvar et al. 1968 and Paper III) In view of the present results it appears very improbable

that the extracellular pH could be an important factor regulating the CVR at moderate variations of the CPP Thus since the reduction of the CPP to e g 40 mm Hg in Paper V was associated with an increase in the intracellular lactate concentration of about 0 5 - 1 0 mmole per kg of intracellular water and since a corresponding increase could be expected in the extracellular lactate concentration the CSF bicarbonate should decrease by maximally 1 mEq/kg. At constant CO_2 tension such a decrease in bicarbonate should lead to a decrease in the extracellular pH of about 0 01 units It is thus probable that the dilatation of the cerebral vessels at reduced CPP values is due to other factors It is possible that the myogenic theory which has been proposed by many previous workers (Folkow 1962) can better explain cerebral autoregulation (Ekström-Jodal et al 1969)

IV GENERAL SUMMARY

The present thesis describes a series of experiments which were devised to study the effects of an increased cerebrospinal fluid pressure on the circulation and the oxidative metabolism of the brain. The rationale behind the experimental approach was that a generalized increase in the cerebrospinal fluid pressure will exert its effects on the tissue mainly by decreasing the effective perfusion pressure and that an adequate tissue blood flow can only be upheld at a lower perfusion pressure if the resistance vessels of the tissue dilate, i.e. if autoregulation is present. There had previously been a number of qualitative or semiquantitative studies indicating that the cerebral blood flow showed autoregulation if the cerebral perfusion pressure was decreased due to a decreased blood pressure or to an increased CSF pressure. However, there was very little information about the range of CSF pressures which can be induced without significant decreases in blood flow, and very little was known about the quantitative aspects of the reactive hyperemia which can be elicited by a decreased tissue perfusion. It was thus the intention to study the range of CSF pressures compatible with an efficient autoregulation, as well as the circulatory changes caused by a marked decrease in the perfusion pressure. When those results were obtained an attempt was made to define the effect of a markedly decreased perfusion pressure upon the oxidative metabolism of the tissue, as this is reflected in the tissue and CSF lactate and pyruvate concentrations, and in the tissue concentrations of phosphocreatine, ATP, ADP and AMP. These measurements gave an unexpected side result in that changes in the CSF lactate and pyruvate concentrations were observed even if the cerebral blood flow was not significantly lowered. This result prompted a separate investigation of possible metabolic changes in the tissue at perfusion pressures which had previously been found to be within the autoregulatory range.

The experiments were carried out on dogs and rats. The dogs were used to study the cerebral circulation with the tissue clearance method for a radioactive inert gas, to study changes in the CSF lactate, pyruvate and bicarbonate concentrations, and to study metabolic changes in the brain after a marked reduction in perfusion pressure. For the last study, and in order to facilitate experiments on a large series of animals, rats under nitrous oxide anesthesia were used.

The main results of the circulatory studies was the finding of an efficient

autoregulation over a wide range of CSF pressures and the finding of a very marked and often longlasting reactive hyperemia after a pronounced decrease in the cerebral perfusion pressure Thus the cerebral blood flow did not change appreciably if the CSF pressure was varied from about -15 to about 100 mm Hg i.e if the cerebral perfusion pressure varied between about 140 and about 30 - 50 mm Hg At these lower pressures the cerebral blood flow started to decrease and there occurred a concomitant increase in the mean arterial blood pressure ("Cushing effect") However the Cushing effect was not sufficient to restore the blood flow to normal. Control experiments showed that a lowering of the perfusion pressure to about 30 mm Hg did not give any marked decrease in the oxygen consumption of the tissue At these low pressures an increase in flow was induced by hypercapnia indicating that the dilatation of the vessels during autoregulation and during hypercapnia occurs by separate mechanisms

In general there was a fair correlation between the decrease in flow during a period of increased CSF pressure and the magnitude and duration of reactive hyperemia after the restoration of normal CSF pressure However the coupling was not obligatory in that a reactive hyperemia could be seen to follow upon an intracranial hypertension which was not accompanied by a decreased flow The peak flows during the period of reactive hyperemia could amount to 500 - 600 per cent of the control values and the hyperemia could last for 2 - 3 hours or more It is concluded that although a shortlasting hyperemia must be assumed to follow upon any reduction of the perfusion pressure however moderate it may be the longlasting and pronounced hyperemias must be assumed to be the result of a serious disturbance of the tissue metabolism

When the increase in the CSF pressure gave rise to decrease in flow there were marked and often very longlasting increases in the CSF lactate concentrations and in the lactate/pyruvate ratios and corresponding decreases in the CSF bicarbonate concentrations These changes were interpreted to reflect an increased anaerobic metabolism of the tissue and thus a disturbed oxidative metabolism In some instances after pronounced or repeated reductions of the perfusion pressure the lactacidosis of the CSF could persist for many hours However it was found that the cerebral blood flow could return to normal in spite of a persisting acidosis and it was thus not possible to relate the hyperemia to the extracellular acidosis

In order to study if the longlasting increases of the CSF lactate pyruvate and bicarbonate concentrations were due to a slow clearing of lactate from the relatively stagnant CSF pools or to a persisting lactacidosis in the tissue proper the tissue concentrations of lactate pyruvate bicarbonate phosphocreatine ATP ADP and AMP were measured at

various times during and after a 15 min decrease in the perfusion pressure to 30 – 35 mm Hg These experiments showed that the tissue lactate pyruvate and bicarbonate concentrations did not normalize until 60 – 90 min after that the perfusion pressure had been brought back to control values Further since the rephosphorylation of ATP and phosphocreatine was equally slow it was concluded that the circulatory alteration induced gave rise to a damage to the oxidative systems or to a state with persisting focal underperfusion

An unexpected finding was that decreases in the perfusion pressure to values still within the range of autoregulation could give rise to increases in the CSF lactate concentration and lactate/pyruvate ratio In order to study if these changes were the result of transient decreases in flow or if they represented persisting tissue events metabolic studies were done on rat brains after the CSF pressure had been increased to various levels It was found that the perfusion pressure could be decreased to about 30 mm Hg before the tissue concentrations of phosphocreatine ATP ADP and AMP were affected but that moderate lactate and pyruvate changes occurred at much higher perfusion pressures It was concluded that the results confirmed the previous findings that the oxidative metabolism of the tissue is not grossly changed until the perfusion pressure falls to values around 30 mm Hg but also that the lactate/ /pyruvate changes probably reflect minor degrees of tissue hypoxia possibly of focal nature If this interpretation is correct the results definitely indicate that the tissue lactate/pyruvate ratio is a more sensitive indicator of tissue hypoxia than e g the phosphocreatine or AMP concentrations

V LIST OF ABBREVIATIONS USED

ADP	adenosine diphosphate
AMP	adenosine monophosphate
ATP	adenosine triphosphate
CBF	cerebral blood flow
$CMRO_2$	cerebral metabolic rate of oxygen
CPP	cerebral perfusion pressure
CSF	cerebrospinal fluid
NAD^+	nicotine amide adenine dinucleotide (oxidated form)
NADH	nicotine amide adenine dinucleotide (*reduced form*)
P_{CSF}	cerebrospinal fluid pressure

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STUDENTLITTERATUR
LUND SWEDEN

ACTA PHYSIOLOGICA SCANDINAVICA
SUPPLEMENTUM 340

NEW ASPECTS OF THE FORMATION AND FUNCTION OF HISTAMINE, 5-HYDROXYTRYPTAMINE AND DOPAMINE IN GASTRIC MUCOSA

BY

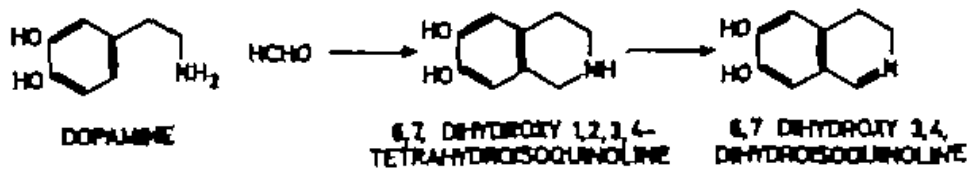
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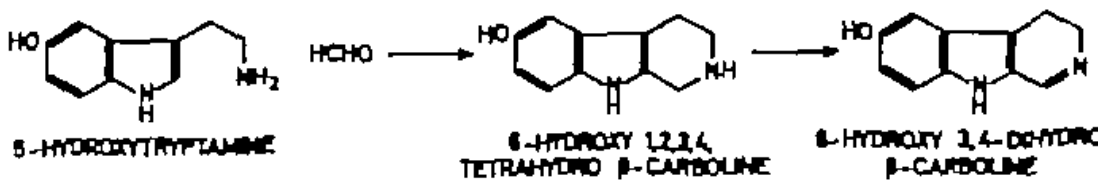
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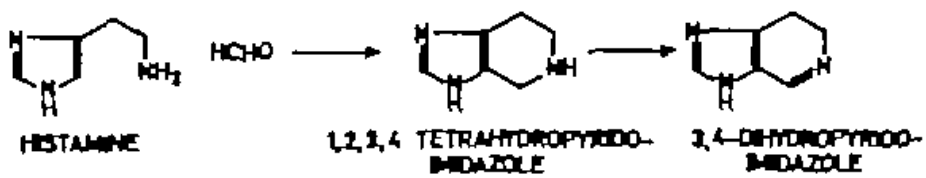
ERRATA

DISSERTATION:

p. 6 line 10 from below read amino acid decarboxylase
p. 8 line 1 from below read WHEELWRIGHT
p. 9 line 32 from above, read were interpreted
line 39 from above, read gastric acid secretion
p. 10 line 6 from below read exclude
p. 12 line 1 from below read: argyrophilic
p. 15 line 29 from above, read Catecholamines.
p. 24 line 10 from above, read SHEPHERD and MACKAY
line 14 from above read criticized
p. 28, Fig. 4, which is incorrect, should be replaced by the reproduction below







p 35 line 10 from below read stages
p 46 line 19 from above, read possibly
p 50 line 5 from above, read AURES HÅKANSON and SCHAUER, 1968.
p 54 line 5 from below read cells
p 58 line 9 from above, read: different
line 11 from below read: osmium-fixed
p 59 line 12 from below read: epithelium
p. 61 line 5 from above, read have been clearly
p 67 line 14 from above, read the thyroid C cells
line 20 from above, read pituitary hormones
p. 79 line 25 from above, read non-specific
p 81 line 20 from below read: HÅKANSON, 1966 b
p. 85 Footnote line 1 from above, read MATHEMATICAL
line 11 from above, read Michaelis and Menten
p 88 line 4 from below read DOPA decarboxylase

column headed
κk_m (k_m) line 4 from above read
$\times 10^{-3}$ ( ) 1.0×10^{-3} (s)

p. 91 Table 6 heading of column 5 read Hamster placenta

p. 93 line 10 from below read enzyme-activating

p. 99 Fig. 27 Conformations I and III are the images of pyridoxal 5-phosphate and L-α-amino acids Conformation II presented incorrectly as the image of a D-amino acid

p. 104 equation 3b read where $k_m = \frac{(E)(SH+)}{(ESH+)} = \frac{k_{-1} + k_{+2}}{k_{+1}}$

p. 133 lines 5-7 from above read UNGER, R.H. H. KETTERER, J. DUPRÉ and A.M. EISENTRAUT. The effects of secretin, pancreozymin and gastrin on insulin and glucagon secretion in anaesthetized dogs. *J. Clin. Invest.* 1967 46 630-645

PAPER 1 p. 491 line 6 from below read samples of tissue in order to avoid quenching of the fluorescence (cf. ref. 9). Known

PAPER 7 p. 1151 line 13 from below read preformed

PAPER 10 p. 103 Table 2 column 4 line 4 read 62 ± 30 (8)

PAPER 13 p. 189 line 7 from below read (i.e. non-specific aromatic L-amino acid decarboxylase)

p. 202 line 10 from below read almost non-measurable (<0.05 μg/g after removing the submucosa)

p. 219 Reference missing

McGuigan, J.E. Gastric mucosal intracellular localization of gastrin by immunofluorescence. Gastroenterology 55 315-327 (1968)

PAPER 15 line 1 from above read (Rosengren 1965)

PAPER 16 Reference missing

Ericsson, E. 1965 Histamine metabolism in the rat and non-pregnant hamster. Proc. Soc. Exp. Biol. 118 884-888

PAPER 22 on 6, read

$$\left[\frac{K}{(C)}\frac{K_m}{(S)} + \frac{K_m}{(S)} + 1\right]$$

ACTA PHYSIOLOGICA SCANDINAVICA

SUPPLEMENTUM 340

FROM THE DEPARTMENT OF PHARMACOLOGY AND THE INSTITUTE OF ANATOMY AND HISTOLOGY UNIVERSITY OF LUND LUND SWEDEN

NEW ASPECTS OF THE FORMATION AND FUNCTION OF HISTAMINE, 5-HYDROXYTRYPTAMINE AND DOPAMINE IN GASTRIC MUCOSA

I. Properties of enterochromaffin and enterochromaffin-like cells
II. Properties of mammalian histamine-forming enzymes

BY

ROLF HÅKANSON

LUND 1970

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This dissertation, which includes a critical survey of the literature is based on the following publications and some unpublished results

PART I

1 HÅKANSON R. and CH. OWMAN Distribution and properties of amino acid decarboxylases in gastric mucosa. *Biochem. Pharmacol.* 1966. *15* 489—499

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CONTENTS

1 INTRODUCTION

1.1 General remarks 7
1.2. Current concepts of the physiological role of histamine, 5-hydroxytryptamine and catecholamines 8
Histamine 8
5-Hydroxytryptamine and catecholamines 10
1.3 Presentation of the subject 11

2. METHODOLOGY

2.1 Chemical assay of amines and amino acid decarboxylases 14
Amines
Histamine 14
5-Hydroxytryptamine 15
Catecholamines 15
Amino acid decarboxylases
Aromatic L-amino acid decarboxylase 16
L-Histidine decarboxylase 17
Critical evaluation of methods for the determination of histidine decarboxylase activity 22

2.2. Assay of the gastric antipernicious principle (intrinsic factor) 25

2.3 Fluorescence microscopy of biogenic amines
5-Hydroxytryptamine and catecholamines 26
Histamine 27

2.4 Conventional light microscopy 31
Silver stains 31
Mast cell stain 31
Parietal cell stain 31
Mucous neck cell stain 31

PART I

3 PROPERTIES OF ENTEROCHROMAFFIN AND ENTROCHROMAFFIN LIKE CELLS

3 1 The concept of the enterochromaffin system 35
3 2. The typical enterochromaffin (argentaffin) cell 36
3.3 Distribution and properties of enterochromaffin cells in gastric mucosa 37
3 4 The typical enterochromaffin like (argyrophil, non-argentaffin) cell 38
3.5 Distribution and properties of enterochromaffin-like cells in gastric mucosa 43
3 6. Amine mechanisms in enterochromaffin and enterochromaffin-like cells of gastric mucosa 45
3 7 Prenatal and postnatal development of gastrointestinal enterochromaffin and enterochromaffin like cells 54
3.8. Ultrastructure of enterochromaffin and enterochromaffin like cells 56
3.9 Possible physiological role of gastric enterochromaffin and enterochromaffin-like cells 59
3 10 Possible function of amines in gastric enterochromaffin and enterochromaffin like cells 70

4 SHORT SUMMARY 74

PART II

5 PROPERTIES OF MAMMALIAN HISTAMINE FORMING ENZYMES

5 1 General characteristics of mammalian histamine forming enzyme 77
5 2. Properties of aromatic amingo acid decarboxylase 79
5.3 Properties of specific histidine decarboxylase 81
5 4 Cofactor requirements of mammalian histamine forming enzymes 93
5 5 Histamine forming enzymes in gastric mucosa 94
5 6. Molecular mechanism of enzyme-catalyzed histamine formation 98
General mechanism of amino acid decarboxylation 98
Molecular mechanism of histidine decarboxylation 101

6. SHORT SUMMARY 110

ACKNOWLEDGEMENTS 111

REFERENCES 113

INTRODUCTION

1.1 *General remarks*

The biogenic amines have, since their isolation and identification in the first two or three decades of this century attracted the attention of a great number of workers (see Gudernatsch, 1951). The remarkable pharmacological actions of some of these natural tissue constituents were soon recognized and led to the assumption that the biogenic amines were physiologically significant. For some of these compounds, such as acetylcholine and the catecholamines, their putative physiological significance was early linked with transmitter function, which as a consequence, for long has been the most prominent functional role ascribed to biogenic amines.

Amines are the result of decarboxylation of L-α-amino acids. All amino acid decarboxylases seem to be stereospecific, attacking only the L form of the substrate. It can be assumed that the number of biogenic amines is very large: every amino acid may give rise to at least one amine and in several cases the resulting primary amine undergoes secondary reactions. As a matter of fact, a great number of different amines were early isolated from biological material. Nervous tissue, to name one example, contains a wide variety of amines (see Perry Hansen Fourlas and Lido 1965; Seiler and Wiechmann 1965; Nakajima, Wolfram and Clark, 1967; Perry Hansen and MacDougall, 1967), most of which are still unidentified (see Seiler and Wiechmann 1965). In spite of this multiplicity interest was primarily focused upon a fairly small group of biogenic amines,* which included histamine, acetylcholine, 5-hydroxytryptamine (5-HT) and the catecholamines (adrenaline, noradrenaline and dopamine). This was a natural consequence of their powerful pharmacological effects, which permitted their quantitation through simple bioassay procedures. The interest in this particular group of compounds has led to an overwhelming wealth of intriguing information, which in turn has resulted in a number of provocative and attractive theories as to their functional roles.

Research on biogenic amines is based on certain fundamental assumptions. The first and most important of these assumptions pertains to the physiological significance of biogenic amines such as histamine, 5-HT and the catecholamines. It is generally assumed that these compounds play an important role in the regulation of both neuronal and extra-neuronal functions. The possibility that in some tissues they may be merely waste products is seldom, if ever, considered. Another perhaps even more questionable idea often intimated in the literature, can be formulated as follows: The physiological functions of biogenic amines are reflected in their pharmalogical effects, *i.e.* the p

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* Other biogenic amines have been disregarded to such extent that the term amines" now appears to be reserved for acetylcholine, histamine 5-hydroxytrypt catecholamines, a terminology which appears difficult to justify

cological effects of these compounds are sometimes regarded as extrapolations of their physiological actions. According to this view amines without strong pharmacological effects are less likely to be of physiological significance. This is not necessarily correct since there may not always be a relation between the pharmacological activity of biogenic amines and their physiological roles. A third assumption can be formulated as follows The observation of a high concentration of an amine in a tissue may be taken as an indication of a particularly important role for that amine in that particular tissue. This is a questionable assumption, since there may be no relation whatsoever between the concentration of a tissue component and its physiological importance. A low tissue level of a particular amine does not exclude its rapid formation and rapid turn-over neither does it exclude the possibility of a very important physiological role for that compound.

Although it is evident that the various assumptions in this field of research should not be uncritically accepted, there are good reasons to acknowledge the assertion that some biogenic amines do have physiologically important functions. The fundamental assumption that we have deliberately selected to form the basis of our investigation is that certain cells actively produce and store amines not only because they affect other cell systems but because the amine is somehow significant to the function of the cell itself

While earlier work emphasized the participation of biogenic amines in neuronal transmission, current studies tend to get more and more involved with the extra neuronal functions of these compounds (for a review see FALCK and OWMAN 1968) This re-orientation was greatly assisted by recent methodological advances in the histochemistry of biogenic amines. The fluorescence microscopic method developed by FALCK and HILLARP (FALCK and TORP 1961 FALCK, 1962 FALCK, HILLARP THIELE and TORP 1962) for the histochemical detection of 5-HT and the catecholamines has caused a major break through in research on biogenic amines in general and in our understanding of non-neuronal amine mechanisms in particular

This treatise attempts to survey recent observations on the cytochemistry of gastric amine-producing cells and to confront these observations with the vast amount of data available in the literature and in so doing try to put recent results in a proper perspective It has been our deliberate intention to integrate observations on the formation and functions of a variety of amines such as histamine dopamine and 5-hydroxytryptamine Since many of the biogenic amines appear to be functionally related—possibly also functionally interdependent—it is becoming increasingly evident that compartmentalization" in this area of research is to be avoided.

1.2. *Current concepts on the physiological role of histamine 5-HT and catecholamines*

Histamine In the mammalian organism, the basophils of the blood (GRAHAM LOWRY WHEELWRIGHT LEDUC and PARROTT, 1955 COOK and MITCHELL, 1957 ROBERMAN, 1962)

and the mast cells of the tissues (Riley 1953a and b Riley and West 1953 Carr, Riley West Head and Straub, 1954) regularly contain histamine. The distribution of histamine in most tissues parallels the distribution of mast cells, and it is now well established that the major portion of the amine is contained in these cells (Riley 1959) Agents such as Compound 48/80 which act as histamine-depleting drugs, do so through liberating mast-cell-histamine (Riley 1953a) However also non-mast-cell localizations of tissue histamine have been established For example, histamine is found in the brain predominantly in the hypothalamic region (Harris, Jacobsohn and Kahlson, 1952 Adam 1961 Garlick 1964 Adam and Hyde, 1966) where mast cells are scare, if present at all and the histamine content of the rat stomach is very high in spit of the fact that in this species gastric mast cells are few (Mota, Beraldo Ferri and Junqueira, 1956) Gastric histamine in the rat can thus be assumed to reside in another cell system. This assumption has received confirmation through the recent histochemical demonstration of histamine in an epithelial cell system in the oxyntic gland area of the rat and mouse (Håkanson and Owman 1967 Håkanson Owman and Sjöberg, 1967 Thureson, 1967) These histamine-containing cells are morphologically very similar to the enterochromaffin cells of the pyloric gland area, and have been referred to as enterochromaffin-like (Håkanson and Owman 1967 see also Håkanson, Lilja and Owman, 1967)

Very early mast cell histamine was proposed as one of the mediators of the allergic reaction (see review by Rocha e Silva, 1955) This concept initiated much experimental work directed towards alleviating the symptoms of allergy through antihistaminic agents. In many respects, this concept has since proved inadequate and histamine is now regarded as only one of many mediators of the allergic response (see Telford and West 1963)

During the last decade, interest has been focused on the role of non-mast-cell histamine mainly as a result of investigations by Schayer (1960a and b 196) and by Kahlson and co-workers (see reviews by Kahlson 1962 by Kahlson and Rosengren 1968) The latter authors showed that some tissues of the foetal rat and foetal mouse are very active in forming histamine and that the amine formed is rapidly eliminated from the foetus (Kahlson, Rosengren and White, 1960 Rosengren, 1963 see Rosengren, 1966) This and further observations of a similar nature on other rapidly growing tissues of the rat (Kahlson, Nilsson Rosengren and Zetterqvist 1960 Håkanson 1961 Kahlson and Rosengren, 1963) was interpreted as indicating growth-promoting function of histamine. Although this may be the case in the rat and mouse, it does not appear to be general phenomenon since in other species rapidly growing tissues fail to develop increased histidine decarboxylase activity (Mackay Reid and Shuttleworth 1961; Kahlswaran and West 1963 Mitchell, 1963 Håkanson, 1966b Rosengren, 1966)*

Kahlson and co-workers also studied the formation and turn-over of histamine in th rat stomach and suggested from their results that histamine is the final common mediator of gastric secretion (Kahlson, Rosengren, Svaetichin and Thunberg, 1964; Kahlson Rosengren and Thureson, 1967) Their main supporting evidence was the observation that injection of gastrin or the intake of food cause a loss of gastric histamine and a concomitant increase in gastric histidine decarboxylase activity This was interpreted as an indication of self-regulating feed-back mechanism with the reaction product, histamine acting as the enzyme repressor This concept will be further discussed in section 3 10.

Schayer has suggested role for histamine in the control of microcirculatory homeostasis (Schayer, 1962) He observed an activation of the histidine decarboxylase activity of tissues such as muscle and skin following treatment with a variety of stressful stimuli. These findings hav been used to develop theories on microcirculatory control function for newly formed histamine. It has been suggested that "intrinsic histamine is formed at rates commensurate with tissue requirements for blood and that histamine is the principal regulator of the microcirculatory system.

Histamine and other amines, such as 5-HT and the catecholamines, are also believed to play role in the acute inflammatory response and in tissu repair (Spector and Willoughby 1964 Lahiri and Sanyal, 1967)

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* Recent observations by Jänne (1967) Smith and Jänne (1967) and Russell and Snyder (1968, 1969) of remarkably high activity of ornithine decarboxylase in certain types of rapidly growing tissues suggest that, apart from histamine other amines may play part in the process of growth and tissue repair

In the light of the recent histochemical demonstration of histamine in the enterochromaffin-like cells of the rat stomach (Håkanson and Owman 1967 Håkanson, Owman and Sjöberg, 1967) the role of histamine in the function of these cells has attracted some interest. This particular aspect of the possible physiological function of histamine will be one of the main subjects of the present treatise.

Histamine in tissues may arise from two sources. One is by uptake of histamine absorbed from the intestines. The other is by *decarboxylation of histidine in the tissues. Histamine* formation in mammalian tissues may be catalyzed by at least two histamine-forming enzymes. One is believed to be identical with DOPA decarboxylase (EC 4 1 1.26)* also referred to as non-specific aromatic amino acid decarboxylase (Lovenberg, Weissbach and Udenfriend, 196) Another enzyme appears to be specific and is probably of greater physiological significance as a catalyst of histamine formation *in vivo* (Garbut Rosenquist and Rosenquist, 1961 Weissbach, Lovenberg and Udenfriend, 1961) The enzyme is generally referred to as mammalian histidine decarboxylase or specific* histidine decarboxylase (EC 4 1 1.22) Th specific histamine forming enzyme, which is identified by its very characteristic kinetic properties (Håkanson 1963 1964 1966b, 1967a and b) has been found in a limited number of tissues. Foetal rat liver hamster placenta and murine mastocytoma are rich sources of this enzyme (Garbut Rosenquist and Rosenquist 1961, B ichalier, 1962 Håkanson 1963 1967b Auerbach and Noll, 1966) Considerable activity has been demonstrated also in murine gastric mucosa, in red bone marrow and in various transplantable tumours of the rat (Håkanson, 1964 1966b Håkanson and Owman 1966b Auerbach, Håkanson and Schauer, 1968) In many tissues the enzyme appears to be adaptive since the enzyme activity is subject to considerable variation (for details see sections 3 10. and 5 5) The marked adaptability of mammalian histidine decarboxylase may be of great physiological importance.

In certain species, thus histamine may result from enzymatic formation or from a combination of enzymatic formation and intestinal absorption. I other species, where conclusive evidence is lacking for histidine decarboxylase activity histamine may be absorbed from the intestines in sufficient quantities to supply all tissue stores (Waton 1963 1964 Duncan and Waton, 1969)

5-HT and cat cholesterase. 5-HT has widespread occurrence in tissues of both vertebrates and invertebrates (see Garattini and Valzelli, 1965) Histochemical methods have been very successful in establishing the cellular localization of 5-HT and the catecholamines (cf *e.g.* Falck and Owman, 1968) Their occurrence and functions in the central nervous system has ttracted particular attention (Brodie and Costa, 1962 Hillarp Dahlström and Fuxe, 1966 Dewhurst 1968a and b) With the exception of the pineal nerves (Owman, 1964a) 5-HT has an extra-neuronal localization in peripheral tissues of mammals. The major portion of the 5-HT in the body is found in the intestines (Bertaccini, 1960), where it is located in the enterochromaffin cells (see Pearse, 1966 Vialli, 1966) In addition, 5-HT occurs in particularly high amounts in certain endocrine organs (Erspamer, 1966 Falck and Owman, 1968) such as the thyroid of the sheep (Falck, Larsson von Mecklenburg, Rosengren and Svensson, 1964) the pancreatic islet cells of the guinea-pig, cat, dog, horse, duck, pigeon and pig (see Grobbelaar, 1968) the pineal gland of the mouse, rat (Quay and Halevy, 1962 Quay 1963 Bertler, Falck and Owman, 1964) and pig (Owman, 1964b) There is reason to believe that in some of these endocrine cells 5-HT and dopamine can be present concomitantly (Grobbelaar, 1968) or the cells may contain catecholamine but not 5-HT (Grobbelaar, 1968) It should be pointed out that the absence of 5-HT (or catecholamines) i some of these endocrine cells does not immediately exclude the possibility that other amines are produced and stored in them, and that these amines are capable of performing the same intracellular function. It is therefore difficult to discuss one biogenic amine independent from the other amines. The physiological function of the peripheral extra-neuronal amines is largely unknown. The 5-HT containing enterochromaffin cells of the digestive tract are believed to hav an endocrine function, which involves the secretion of some still

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* In accordance with the nomenclature recommended by the Enzyme Commission f the International Union of Biochemistry these enzymes re referred to by the following systematic names DOPA decarboxylase 3,4-dihydroxy L-phenylalanine carboxy-lyase (EC 4 1 1.26) 5-HTP decarboxylase 5-hydroxy-L tryptophan carboxy lyase (EC 4 1 1 28) Histidine decarboxylase -histidine carboxy-lyase (EC 4 1 1.22)

unidentified polypeptide- or protein hormone(s). It has also been suggested that the gastric antipernicious principle (intrinsic factor) originates from these cells (Jacobsson 1939). Gastrointestinal 5-HT has also been associated with bowel motility (see Bülbring and Lin 1958, Haverback and Davidson 1958); 5-HT released from preganglionic nerve endings in the myenteric plexus as a result of vagal stimulation, is believed to cause relaxation of the smooth muscles of the stomach (Bülbring and Gershon, 1966, 1967). From their localization, usually in the basal part of the intestinal and pyloric glands, it appears more likely that the 5-HT-containing enterochromaffin cells are involved in secretory mechanisms. There is no histochemical evidence indicating the presence of 5-HT in the myenteric plexus (Jacobowitz, 1965; Gabella and Costa, 1967; Norberg, 1967; Read and Burnstock, 1969) and the participation of 5-HT in regulation of bowel motility does not appear to be a likely hypothesis (Gershon 1968; see also Boullin 1964).

Recently Hägerdal (1967) detected dopamine in human gastric juice, and Håkanson, Owman, Sjöberg and Stenström (1970d) reported the presence of dopamine-containing enterochromaffin cells in the gastric mucosa of the cat and rabbit. It should be noted that dopamine in the digestive tract may have a cellular storage site other than the enterochromaffin cells. Thus, large amounts of dopamine have been demonstrated in mast cells of the digestive tract of ruminants (Falck, Nyström, Rosengren and Stenflo 1964). The role of gastrointestinal 5-HT and dopamine is entirely unknown; for a discussion of their possible function in enterochromaffin cells see section 3.10.

5-HT and dopamine result from decarboxylation of 5-hydroxytryptophan (5-HTP) and 3,4-dihydroxyphenylalanine (DOPA), respectively. These reactions are probably catalyzed by one and the same enzyme, which is sometimes referred to as aromatic amino acid decarboxylase (Lovenberg, Weissbach and Udenfriend, 1962), sometimes as DOPA/5-HTP decarboxylase. The formation of 5-HT and dopamine, however, is preceded by hydroxylation of tryptophan to 5-HTP and of tyrosine to DOPA. The initial hydroxylation of tyrosine and tryptophan is considered to be the rate-limiting step in the biosynthesis of dopamine and 5-HT because the decarboxylating enzyme is available in high concentration in most tissues. It is very doubtful whether DOPA or 5-HTP are normally present in the blood. Available data suggest that the formation of these amino acids takes place in the same cell that converts them into amines. Thus, the hydroxylation is the critical step determining not only the rate of amine formation but also the identity of the amine which is formed in the cell.

1.3 *Presentation of the subject*

The presence in the intestines of a cell system, which can be visualized by the chromaffin reaction and which contained "enteramine" has been recognized for a considerable time (see Erspamer, 1954a); the cells are named enterochromaffin cells, and "enteramine" has been identified as 5-HT (see Erspamer and Asero 1952b; Vialli 1966). The histochemical fluorescence method of Falck and Hillarp (Falck and Torp 1961; Falck, 1962; Falck, Hillarp, Thieme and Torp 1962) in combination with microspectrofluorometry has directly confirmed that the chromaffinity of these cells is due to their high 5-HT content (Vialli and Erspamer, 1969; Håkanson *et al.* 1970d), except at certain locations (*e.g.* the gastric mucosa of the cat and rabbit) where some of the enterochromaffin cells have been found to contain dopamine* (Håkanson *et al.*, 1970d). The parietal cell region of

* The dopamine-containing epithelial cells of the gastric mucosa have not been identified as chromaffin by strict histochemical criteria. Nevertheless, they are referred to as enterochromaffin because of their morphological resemblance to the conventional 5-HT-containing enterochromaffin cells, and because cells which contain dopamine in extremely large amounts, are capable of giving the chromaffin reaction (Falck, Hillarp and Torp 1959). For definition of the enterochromaffin cell see section 3.2.

the rat and mouse is lacking in enterochromaffin cells, but has recently been found to contain cells morphologically similar to enterochromaffin cells and referred to as "enterochromaffin-like" (HÅKANSON and OWMAN 1966b HÅKANSON LILJA and OWMAN 1967) These enterochromaffin-like cells have been found to produce and store histamine (HÅKANSON and OWMAN 1967 HÅKANSON OWMAN and SJÖBERG 1967 THUNBERG 1967) Both the enterochromaffin and the enterochromaffin-like cells are agyrophil (HÅKANSON and OWMAN 1969) but only the enterochromaffin cells give a positive argentaffin reaction. Apart from mast cells, the cellular storage site of gastric histamine, dopamine and 5-HT is the argyrophil cell system (HÅKANSON *et al.*, 1970d) Thus, any physiological function of these amines in the gastrointestinal tract appears to be closely related to the function of the enterochromaffin (argyrophil, argentaffin) and the morphologically similar "enterochromaffin-like" (argyrophil, non argentaffin) cells. Since 1938 these cells have been referred to as endocrine on the basis of their morphology (FEYRTER, 1938) They have many of the ultrastructural characteristics common for polypeptide and protein-secreting endocrine cells (SOLCIA, VASSALLO and SAMPIETRO 1967) but so far it has not yet been possible to identify the hormones produced in the cells (for a discussion see AURIA and HÅKANSON 1968a AURIA, HÅKANSON and OWMAN 1970)

The histological classification of these cells is historically based on their "chromaffinity" and their reactivity towards various silver stains. The chromaffin reaction detects biogenic amines, such as 5-HT and the catacholamines, by converting them into pigments through oxidation, or by reduction of the bichromate with a concomitant cytoplasmic deposition of chromium (for a discussion see HALE, 1958) The fluorescence microscopic method of FALCK and HILLARP introduced for the histochemical detection of these amines, is much more sensitive and specific. The term enterochromaffin, however is accepted for historical reasons and for want of a better name. The argentaffin reaction is a consequence of the presence of a reducing substance such as 5-HT (or dopamine) in the enterochromaffin cells the non-argentaffin, argyrophil cells are morphologically indistinguishable from the argentaffin cells, but they are devoid of 5 HT and dopamine and lack the ability to reduce ammoniacal silver salts. The difference in reactivity towards silver stains is widely used to classify the various types of gastrointestinal endocrine cells although this may mean overemphasizing one particular cytochemical characteristic of these cells. Conceivably the conventional classification of these endocrine cells into two types on the basis of their 5-HT content (reflected in their argentaffinity) may be an arbitrary and unjustified distinction with little bearing on the endocrine function of these cells, which presumably involves the formation and secretion of some polypeptide or protein hormone (for a discussion see section 3 9)

It is now generally recognized that all known gastrointestinal endocrine cells, which by conventional argyhophilic stains are morphologically indistinguish-

able, in reality comprise several different cell populations, distinguishable on the basis of differences in their cytochemistry (amine producing enzymes, amine-storing mechanisms) (HÅKANSON and OWMAN 1966b HÅKANSON LILJA and OWMAN 1967 1969 HÅKANSON and OWMAN 1967 AURIS HÅKANSON OWMAN and SPORRONG 1968 HÅKANSON and OWMAN 1969 HÅKANSON *et al.* 1970d) as well as in their ultrastructure (SOLCIA and SAMPIETRO 1965 SOLCIA, VASSALLO and SAMPIETRO 1967 CARVALHEIRA, WELSCH and PEARSE, 1968 PEARSE, 1968a CAPELLA SOLCIA and VASSALLO 1969 VASSALLO SOLCIA and CAPELLA, 1969 FORSSMAN ORCI PICTET RENOLD and ROUILLER, 1969) The truly important differences between these various cell types are of course related to their function (of which we know very little) and not related to the identity and characteristics of the amine stored (of which we know somewhat more) It is therefore not surprising that at present, interest is focused on the functions of these amine-storing endocrine cells as such rather than on the role of the amines which they contain (cf *e.g.* CAPELLA, SOLCIA and VASSALLO 1969 FORSSMAN ORCI PICTET RENOLD and ROUILLER 1969 PEARSE, 1969 VASSALLO SOLCIA and CAPELLA, 1969) However the identification of the endocrine mechanisms operating in these amine-producing endocrine cells requires a detailed knowledge of their regional and topographical distribution as well as of their distinguishing cytochemical features.

2. METHODOLOGY

2.1 *Chemical assay of amines and amino acid decarboxylases*

Most of the techniques used for the chemical determination of the amines and of the amine forming enzymes are widely used and hardly deserving of a detailed description. Details will be given only when modifications have been introduced or when problems of specificity have been the subject of controversy. Thus particular emphasis will be given to certain aspects of the assay of histamine and of histidine decarboxylase.

Amines

Histamine In earlier days, histamine was quantitated by its contractile effect on the atropinized guinea-pig ileum. This technique has now largely been abandoned for other methods. In 1959 Shore, Burkhalter and Cohn introduced a sensitive fluorometric assay for the determination of histamine, using o-phthaldialdehyde (OPT) as the reagent. Prior to its determination, histamine is extracted from tissues according to the following general procedure (details are given by Hakanson 1963). The tissue is homogenized in a cold 5 per cent solution of trichloroacetic acid and precipitated proteins are spun down. Aliquots of the clear supernatant is extracted with a 3:2 mixture of n-butanol and chloroform after alkalinization with sodium hydroxide and saturation with sodium chloride. The organic phase, which now contains the histamine, is washed once with salt-saturated 0.1 N sodium hydroxide and then extracted with 0.1 N hydrochloric acid in the presence of heptane. By this procedure histamine is transferred to the aqueous phase. Aliquots of the hydrochloric acid extract can be taken for fluorometric analysis, which involves condensation of histamine with OPT under alkaline conditions and subsequent stabilization and enhancement of the fluorescence by acidification. The fluorescence intensity is determined in an Aminco-Bowman spectrophotofluorometer (excitation at 350 mμ, emission maximum at 450 mμ). The now well-known non-specificity of OPT as a reagent for histamine (see Cohn and Shore, 1961; Carlini and Green 1963a and b; Krumdieck and Pfeiffer, 1966; Shelley and Juhlin 1966; Maickel and Miller, 1966; Michaelson 1967; Aures, Fleming and Hakanson 1968; Tustner and Wightman 1968) makes special precautions necessary: 1) The activation and fluorescence spectra are registered routinely. This is useful in establishing inadvertent contamination with *e.g.* spermidine (Michaelson 1967) as well as with certain as yet unidentified OPT reactive tissue components (the emission max. of the OPT-spermidine fluorophore is at about 400 mμ). 2) Fluorescence is read also before the final acidification step. A lower fluorescence intensity at this stage eliminates the possibility that histidine, arginine and agmatine (which emit more intense OPT induced fluorescence at alkaline pH) cause the OPT induced fluorescence. 3) In some cases hista-

mine has been chromatographically identified by *e.g.* gel (Sephadex) chromatography ion exchange chromatography (Kreiznер and Wilson 1961 Håkanson 1963) or a combination of gel chromatography and ion exchange chromotography (Aures Håkanson and Spolter, 1968) and paper or thin-layer chromatography (Aures Fleming and Håkanson 1968) Levine, Sato and Sjoerdsma (1965) investigated the specificity of the fluorometric method in the assay of gastric histamine and reported, that provided small amounts of tissue were tested no other compound interfered with the assay

5-HT All determinations of 5-HT are made on fresh material since 5-HT is rapidly destroyed upon storage. The tissue to be analyzed is homogenized in 0.1 N hydrochloric acid and precipitated proteins are spun down. Aliquots of the supernatant are extracted from an alkaline environment with *n*-butanol (Udenfriend Weissbach and Clark, 1955) or *n* heptanol (Mirolli, 1966) The final aqueous extract is assayed for 5-HT by the fluorescence (activating wavelength 295 mμ fluorescence max. at 550 mμ) induced by strong hydrochloric acid (Bodanski Pletscher, Brodie and Udenfriend, 1956) The specificity of the fluorescence is secured by subsequent quenching of the specific fluorescence with hydrogen peroxide (Contractor, 1964) or with potassium ferricyanide followed by ultraviolet irradiation (Andén and Magnusson 1967) to produce a more correct tissue blank. The identity of 5-HT can also be established by paper or thin-layer chromatography where aqueous or gaseous formaldehyde is used as the detection reagent (Shepherd West and Erspamer, 1953 Bell and Somerville, 1966 Håkanson Lombard des Gouttes and Owman 1967 Aures Björklund and Håkanson 1968 Aures, Fleming and Håkanson 1968 Björklund Falck and Håkanson 1969 1970a and b) The intense yellow fluorescence resulting from the reaction of 5-HT with formaldehyde, can be readily detected by illumination in ultraviolet light.

Catecholamines Dopamine, noradrenaline and adrenaline are determined fluorometrically after isolation by cation exchange chromography (Bertler, Carlsson and Rosengren 1958 Carlsson and Waldeck, 1958) The identity of the various catecholamines can be confirmed by paper or thin-layer chromatography followed by treatment with various gaseous or aqueous formaldehyde reagents, which result in intensely fluorescent condensation products (Bell and Somerville, 1966 Aures Björklund and Håkanson 1968 Aures Fleming and Håkanson 1968 Björklund Falck and Håkanson 1969 1970a and b)

Amino acid decarboxylases

In principle the activity of amino acid decarboxylases can be determined in two different ways 1) Estimation of the carbon dioxide produced during reaction with the amino acid substrate 2) Estimation of the amount of amine produced during the reaction.

The classical method for the study of amino acid decarboxylases is the manometric determination of the amount of CO_2 released during the reaction. Determination of the amine product became possible when spectrophotometric and fluorometric methods were introduced (Boodanski *et al.*, 1956 Waalkes and Udenfriend 1957 Carlsson and Waldeck, 1958 Hess and Udenfriend 1959 Shore, Burkhalter and Cohn 1959) The growing interest in the formation and function of biogenic amines has since stimulated the development of simple, rapid and sensitive radiometric methods for measuring very low activities of amino acid decarboxylases (Schayer, 1956a and b Weissbach Kino Sjoerdsma and Udenfriend 1959 Kobayashi, 1963 Snyder and Axelrod 1964 McCaman McCaman Hunt and Smith 1965 Häkanson 1966a) During the last decade, such techniques have become available in great variety Most of them are superior to conventional spectrophotometric and fluorometric techniques in sensitivity sometimes also in simplicity and versatility The general principle for assays of amino acid decarboxylases applies also with radiometric techniques The enzyme activity can be estimated by determining the amount of radiolabelled amine produced or by determining the amount of $^{14}CO_2$ released from carboxy-labelled substrates.

Aromatic L-amino acid decarboxylase This enzyme is widely distributed in the mammalian organism and for the assay of this enzyme classical manometric techniques are quite often satisfactory In studies on the more precise distribution of this enzyme in *e g* discrete areas of the brain or in tissue sections, more sensitive and accurate methods have to be employed. Both fluorometric and radiometric techniques have been successfully applied to studies on minute amounts of tissue (Kuntzman Shore, Bogdanski and Brodie, 1961 McCaman *et al* 1965 Häkanson 1966a Robins, Robins Crontinger, Moses Spencer and Hudgins 1967 Aures Häkanson and Schauer, 1968)

Fluorometric techniques utilize either the fluorescence of the native amine or that of some conjugation or condensation product of the amine for its quantitative estimation. For this reason, the amount of amine produced can be measured fluorometrically only after adequate separation from the amino acid substrate which usually interferes with the assay This can be done by ion exchange chromatography or by selective extraction of the amine with organic solvents It should be pointed out that in studies on tissue homogenates or crude tissue extracts the presence of large amounts of preformed amines in the tissue sometimes makes accurate fluorometric estimation of newly formed amines difficult In this respect radiometric techniques are superior

All available radiometric techniques, based on the determination of the amount of amine produced, employ modifications of the conventional methods for separation of the product from its precursor (Weissbach *et al.* 1959 Snyder and Axelrod 1964 McCaman *et al* 1965 Häkanson 1966a) Both ion exchange chromatography and solvent extraction procedures have been adapted successfully for the special requirements of radiometry As a rule radiometric techniques make even greater demands on accurate separation of

product from precursor that the fluorometric techniques and the separation procedures have had to be modified accordingly The final radioassay can be performed in a gas flow-counter or by liquid scintillation.

One technique employed in the first part of the present investigation involved incubation of tissue extracts or small pieces of tissues with small amounts of ^{14}C-DL DOPA (3 μg) in a total volume of one ml (0 1 M phosphate buffer pH 7 0) Incubation was stopped after one hour by the addition of 4 ml 0 4 N perchloric acid containing 10 μg nonradioactive dopamine as carrier After homogenization, the precipitate was spun down, and the supernatant was neutralized with 2 N potassium carbonate to a final pH of about 6.5 The perchlorate was spun down and the supernatant was passed through a Dowex 50 Na^+ column. Dopamine was retained on the column whereas DOPA could be eliminated by washing the column with 35 ml 0 06 M phosphate buffer pH 6 6 and with 10 ml re-distilled water Dopamine was quantitatively eluted with 10 ml N hydrochloric acid. The eluate was evaporated to dryness on a steam bath during which procedure the heat-labile catechol amines were destroyed. The dry residue was washed several times with acidified acetone, which resulted in a quantitative extraction of the radioactivity The acetone extracts were transferred to standard planchettes, one inch in diameter and evaporated to dryness for measurement of radioactivity (HÅKANSON 1966) The method has been found useful for the assay of DOPA decarboxylase in a variety of tissues (HÅKANSON and MÖLLER, 1963 HÅKANSON MÖLLER and STORMBY 1965 HÅKANSON and OWMAN 1965 1966a and b HÅKANSON LOMBARD DES GOUTTES and OWMAN 1967)

In more recent studies (AURES HÅKANSON and SCHAUER, 1968 AURES and HÅKANSON 1968b AURES DAVIDSON and HÅKANSON 1969) tissue extracts were incubated with ^{14}C-carboxy-labelled DL DOPA (8×10^{-4}M) pyridoxal 5 -phosphate (10^{-5} or 2×10^{-5}M) and glutathione (5×10^{-4}M) in a total volume of 0.5 ml or 0 05 ml phosphate buffer pH 6.9 for one hour The amount of ^{14}CO liberated in the reaction was determined by liquid scintillation after trapping in a suitable absorbent medium (for details on this procedure see the section on measurement of histidine decarboxylase activity) This latter technique is superior in convenience and versatility and has a similar degree of sensitivity compared with the first method.

L-Histidine decarboxylase Histamine formation can be determined fluorometrically or radiometrically These methods seem to be superior to the bioassay procedure developed by WATON (1956a) In this procedure the histamine content of tissue extracts is determined on the atropinized guinea pig ileum after incubation in the presence or absence of histidine. The difference in histamine content at the end of incubation is the measure of histamine formation. Fluorometric techniques were developed as a result of the observation of SHORE, BURKHALTER and COHN (1959) that histamine condenses with o-phthaldialdehyde (OPT) to form a highly fluorescent conjugate.

Since histidine forms a similar fluorophore with OPT adequate separation of histamine from its precursor is essential. Isolation of histamine can be obtained through ion exchange chromatography (Kremzner and Wilson 1961 Gan rot Rosengren and Rosengren 1961 Lovenberg Weissbach and Udenfriend 1962 Häkanson 1963) or through extraction with organic solvents (Shore, Burkhalter and Cohn 1959 Burkhalter, 1962 Anton and Sayre, 1969) The most widely used method is the one in which the incubation mixture is deproteinized with acid, made alkaline with sodium hydroxide and extracted with a mixture of n-butanol and chloroform (Burkhalter, 1962 Häkanson 1963) After extraction histamine is in the organic phase but the bulk of histidine remains in the aqueous phase After one washing with dilute sodium hydroxide which removes residual histidine, the organic solvent mixture is extracted with dilute hydrochloric acid, by which procedure histamine is transferred into the aqueous phase for subsequent fluorometric assay after condensation with OPT This method is not sufficiently sensitive to detect the very small amounts of histamine produced by most mammalian tissues. The sensitivity of the fluorometric method can be greatly increased if the histamine produced is instead purified by ion exchange chromatography and then concentrated before the assay (Häkanson 1963) However it is necessary to exercise some caution in the interpretation of data from experiments on tissue homogenates or crude tissue extracts, In many tissues the histamine content is fairly high and the histidine decarboxylase activity comparatively low This makes it extremely difficult to assess accurately the amount of newly formed histamine against a high tissue blank. An additional difficulty is the non-specificity of the reaction with OPT (see above) A large variety of compounds, of which spermidine may be the quantitatively most important, are known to react with OPT to form highly fluorescent condensation products. It may therefore be difficult to establish beyond doubt that the newly formed OPT reactive compound is in fact histamine although spectrofluorometric analysis of th fluorophore may assist in establishing the identity

Thus the fluorometric assay for histidine decarboxylase is well suited for studies on highly active and highly purified preparations of the enzyme but is *not satisfactory for studies on crude extracts with a high histamine content.* For this purpose radiometric techniques are to be preferred. Several methods have been described Schayer, Davis and Smiley (1955) introduced a technique in which the histidine used is labelled with ^{14}C in the 2 position of the imidazole ring and in which the resulting radioactive histamine is diluted with non-radioactive carrier and converted to the picrate salt, crystallized and counted at infinite thickness in a gas-flow counter An alternative method is to prepare the dibenzenesulfonyl or the p-iodobenzenesulfonyl derivative (Schayer, 1957 see also Kahlson Rosengren and Thunberg 1963) with the latter method the radioactivity can be measured by the liquid scintillation technique, which is more efficient than assay in the gas-flow counter Methods have also been described in which the radioactive histamine formed is separated

from the substrate by paper chromatography before counting (HÅKANSON 1966a SKIDMORE and WHITEHOUSE, 1966b) One such technique (HÅKANSON 1966a) involved incubation of tissue extracts or small pieces of tissue with small amounts of ^{14}C-L-histidine (2 µg) in a total volume of one ml (0 1 M phosphate buffer) After incubation for one hour trichloroacetic acid was added to give a final concentration of 5 per cent and a total volume of 3 ml. Non radioactive histamine dihydrochloride, 20—40 µg was added as carrier After homogenization the resulting precipitate was spun down and discarded. The supernatant was washed twice with four volumes of diethylether to remove the trichloroacetic acid, and then extracted with a mixture of n-butanol and chloroform (3.2) according to the procedure described in detail elsewhere (HÅKANSON 1966a) Since histamine is not separated entirely from contaminating ^{14}C-histidine by organic extraction, a further purification of the amine by paper chromatography was found to be necessary The extract was evaporated to dryness on a steam bath and histamine was extracted quantitatively from the dry residue with alkaline ethanol and applied to a filter paper for chromatography The routine solvent system used separates histidine and histamine very effectively The histamine-containing spot was visualized with ninhydrin. This portion of the chromatogram was cut out and eluted overnight with 5 ml of ethanol containing a few drops of ammonia per 25 ml. The ethanol extract was evaporated to dryness on planchettes for measurement of the radioactivity in a gas flow-counter The reproducibility and sensitivity of this assay procedure was adequate for the determination of the histidine decarboxylase activity of a number of tissues (HÅKANSON 1964 1966a and b HÅKANSON and OWMAN 1966b) SKIDMORE and WHITEHOUSE (1966b) described a method in which extracts were incubated with ^{14}C-labelled histidine small aliquots of the incubation mixture were taken for paper chromatography at frequent intervals during the incubation the radioactivity of the histamine-containing portion of the paper chromatogram was determined by liquid scintillation. A similar procedure involving paper chromatography and/or electrophoresis has been described by SOMERVILLE (1964) for the assay of aromatic amino acid decarboxylase.

The simplest method for measuring histidine decarboxylase activity is that in which histidine labelled with ^{14}C in the carboxyl group is used as substrate. The ^{14}CO released is trapped in a suitable absorbent medium and its radioactivity is then determined directly by liquid scintillation. Several modifications of the original procedure of KOBAYASHI (1963) have been described (AURES and CLARK, 1964 LEVINE and WATTS 1966 1967 LEINWEBER and WALKER, 1967 AURES, DAVIDSON and HÅKANSON 1969) and the method has been adopted by a great number of workers (AURES and CLARK, 1964 LEVINE and WATTS 1966 LEINWEBER and WALKER, 1967 SMITH and CONE, 1967 FISCHER and SNYDER, 1965b JOHNSON 1967 MAUDSLEY RADWAN and WEST 1967 RADWAN and WEST 1967 POWELL and KUMAR, 1968 SNYDER and EPPS 1968 SCHWARTZ, RÖNNBERG COHEN and VALETTE, 1969 HÅKANSON and LIEDBERG 1970a and b) The method currently in use in our laboratory employs

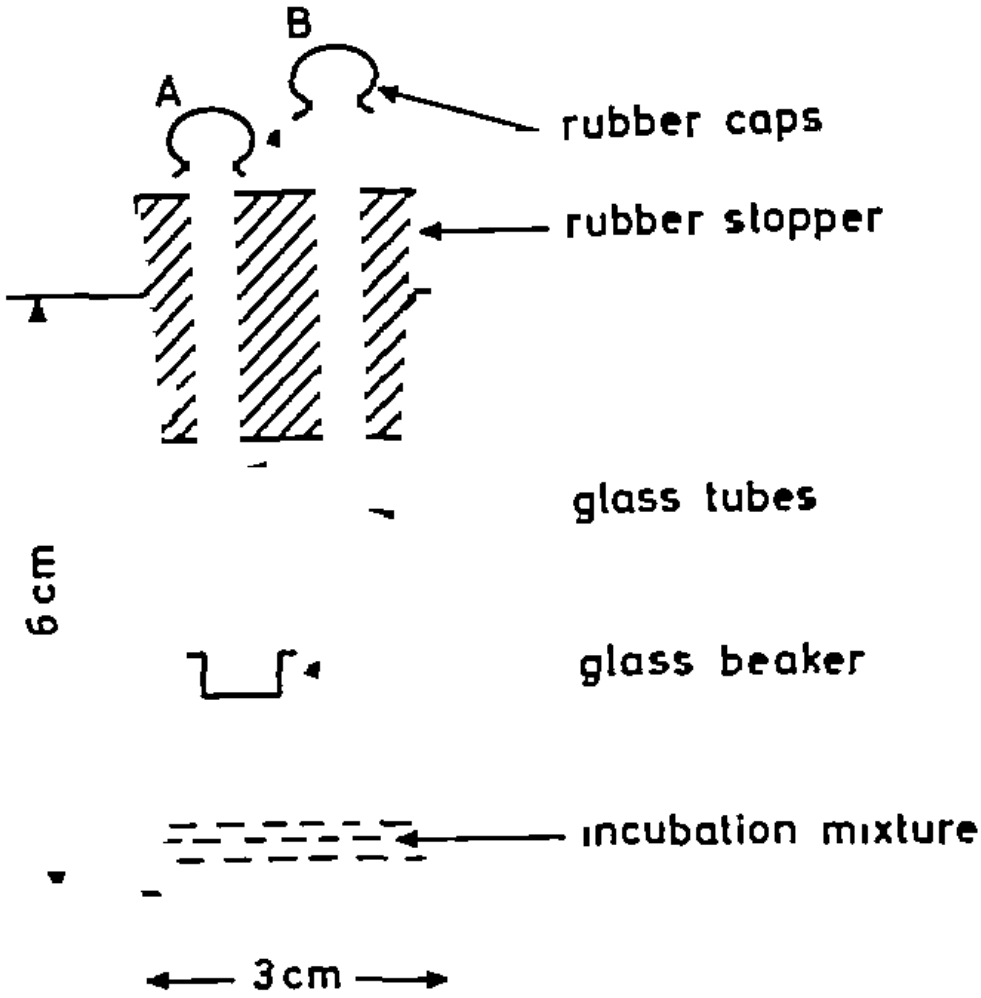


Fig. 1 Schematic drawing of the equipment used for radiometric determination of the amino acid decarboxylase activity

the technical set-up illustrated in fig. 1 Extracts are incubated with 1-^{14}C-L-histidine (4×10^{-4} or 4×10^{-5}M) pyridoxal 5 phosphate (10^{-5} or 2×10^{-5}M) and glutathione (5×10^{-4}M) at pH 6.9 in a total volume of 0.5 ml. The ^{14}CO produced in the reaction is released by acidification (0.5 ml 10 per cent trichloroacetic acid) and further expelled by the addition of sodium bicarbonate the samples are left in the mechanical shaker for another 30 min to permit all ^{14}CO to be trapped on a multifolded piece of filter paper (0.5×4 cm) previously moistened in NCS (Nuclear Chicago) or Digestin (New England Nuclear) The recovery of $^{14}CO_2$ released from ^{14}C-labelled sodium bicarbonate was between 95—100 % and the radioactivity registered was the same as when the isotope was added directly to the Bray (1960) liquid scintillation

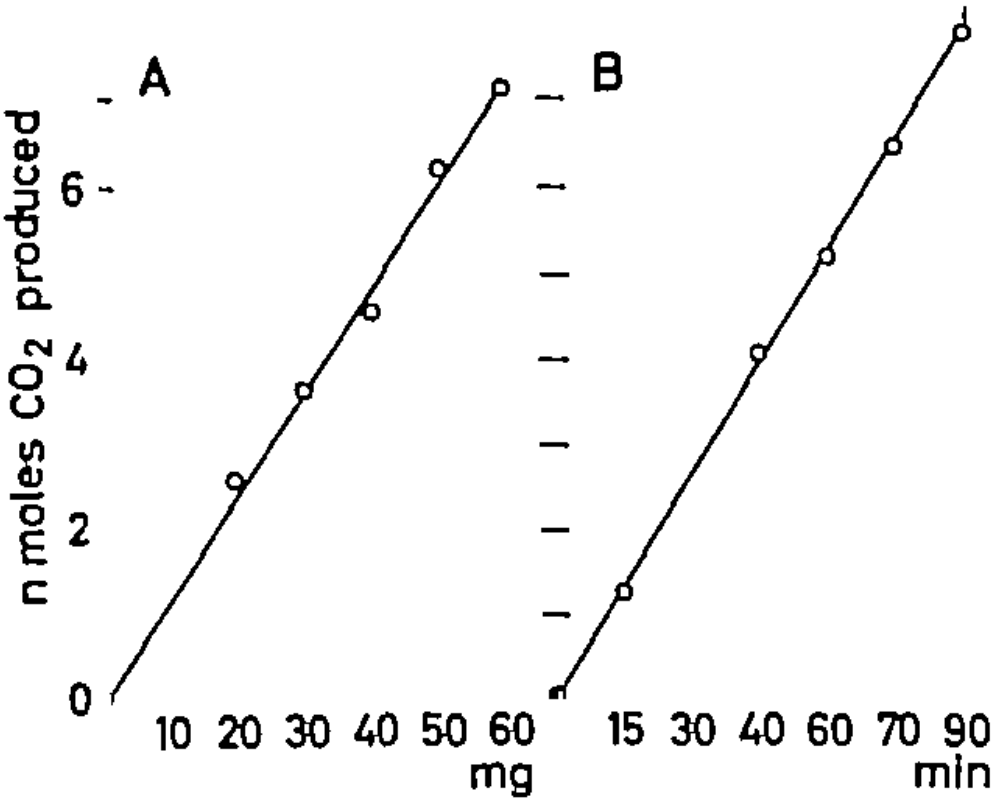


Fig. 2. Rate of histamine formation as dependent on enzyme concentration (A) and incubation time (B). The enzyme extract was incubated with 1-^{14}C-L-histidine (4×10^{-4}M 1,3 mc/mmol) pyridoxal-5-phosphate (10^{-5}M) and glutathione (5×10^{-4}M) in 0.1 M phosphate buffer at pH 6.9. Total incubation volume 0.5 ml. The extract was prepared by homogenization of the tissue (gastric mucosa of vagally denervated rats) high speed centrifugation and dialysis over-night (Håkanson and Liedberg, 1970). The concentration of the final extract corresponded to 200 mg tissue (wet weight) per ml. (A) Incubation time 60 min. (B) The amount of extract added corresponded to 50 mg tissue.

fluid.* The amount of ^{14}C-histidine routinely added to the incubation mixture corresponded to 250 000 cpm (instrumental values). Blanks were provided by incubating heated samples. Under these conditions the blank value never exceeded 100 cpm above background. The enzyme activity was rectilinear with time (up till 2 hr) and with the amount of enzyme (Fig. 2). This method has also been modified for assay of histidine decarboxylase (and DOPA decarboxylase) on a micro-scale (Fig. 3). This procedure permits the determination of enzyme activity in minute amounts of material such as histological sections. The incubation volume is approx. 50 µl (for details see Ahlman, Håkanson and Schauer, 1968; Ahlman, Davidson and Håkanson 1969).

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* The high counting efficiency of this procedure confirms earlier observations of Wang and Jones (1959), Nunez and Jacquemin (1961), Bousquet and Christian (1960) and Lupien, Hinde and Beelmouet (1968).

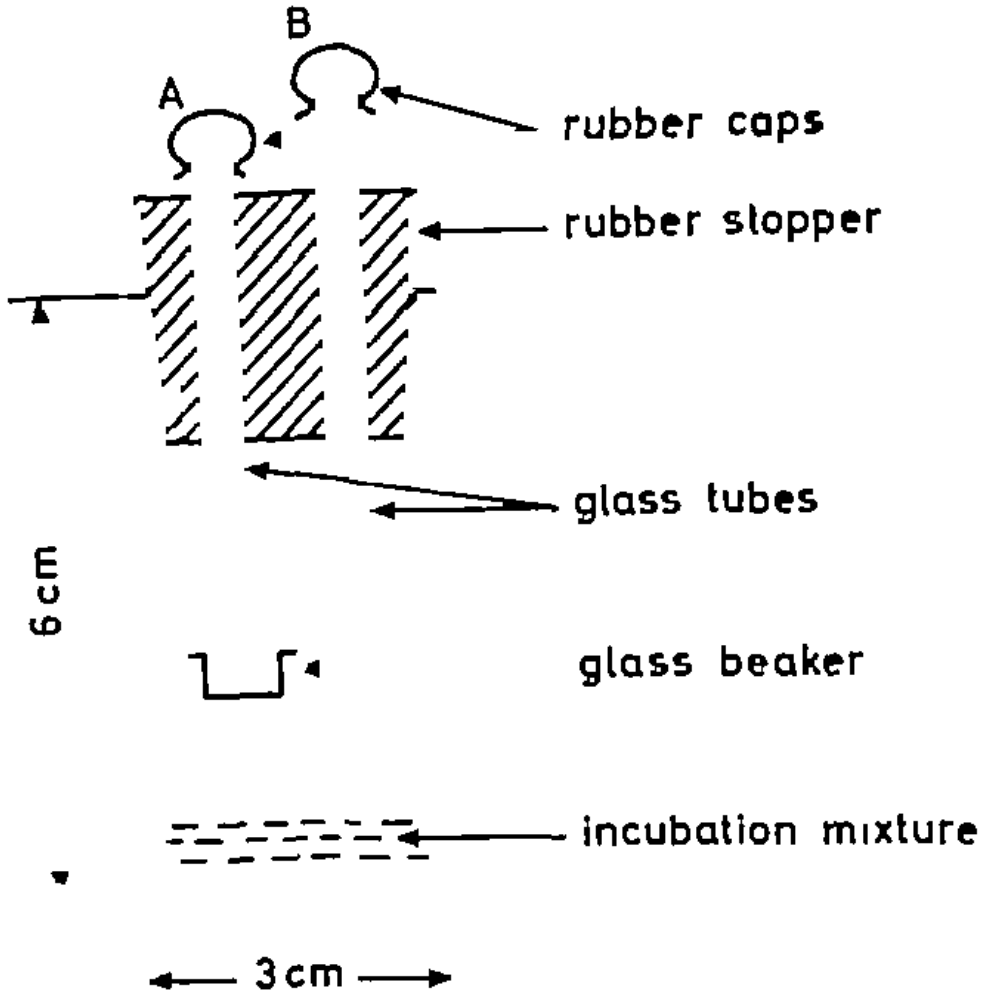


Fig. 1 Schematic drawing of the equipment used for radiometric determination of the amino acid decarboxylase activity

the technical set-up illustrated in fig 1 Extracts are incubated with 1-^{14}C-L-histidine (4×10^{-4} or 4×10^{-5}M) pyridoxal-5 phosphate (10^{-5} or 2×10^{-5}M) and glutathione (5×10^{-4}M) at pH 6.9 in a total volume of 0.5 ml. The $^{14}CO_2$ produced in the reaction is released by acidification (0.5 ml 10 per cent trichloroacetic acid) and further expelled by the addition of sodium bicarbonate the samples are left in the mechanical shaker for another 30 min to permit all $^{14}CO_2$ to be trapped on a multifolded piece of filter paper (0.5×4 cm) previously moistened in NCS (Nuclear Chicago) or Digestin (New England Nuclear) The recovery of $^{14}CO_2$ released from ^{14}C-labelled sodium bicarbonate was between 95—100 % and the radioactivity registered was the same as when the isotope was added directly to the BRAY (1960) liquid scintillation

1967 Graihn and Rosenoren 1968 Schayer, 1968 Levine and Noll, 1969) The method used for preparing the tissue specimen may be critical One widely used technique involves incubation of minced tissue samples. This makes impossible the exact assessment of enzyme activity since the enzyme is sensitive to changes in the concentration of various components in its environment (cf Burkhalter, 1965 Levine and Noll, 1969 for a general discussion see Reinter, 1959) The use of this method is defended on the grounds that in some cases one can detect decarboxylation of histidine in minced preparations or in cell suspensions but not in cell-free preparations (Schayer, 1956a) Generally however the initial step in preparing the tissue for assay is disruption of cells by homogenization. For studies on the kinetics of histamine formation further purification of the enzyme is essential since tissue homogenates may contain variable amounts of substrate, cofactor and endogenous inhibitors or activators. Levine and Watts (1966) recommended an initial purification also when studying physiological alterations of the enzyme activity in the rat stomach. Other workers have found this unnecessary (Kim, Ridley and Tuzel, 1968) Fluorometric methods for the study of amino acid decarboxylases are of limited usefulness. They are quite satisfactory with purified preparations from tissues rich in the enzyme but not very well suited for studies on crude tissue extracts which are rich in the amine and low in enzyme content. There are a number of instances where these methods have failed to detect histidine decarboxylase activity readily measured by radiometric methods. Radiometric techniques are superior since the tissue content of endogenous non-radioactive amine is of no consequence and since these methods have a very high sensitivity only limited by the specific radioactivity of the commercially available substrates. All radiometric techniques, in which the labelled amine product is measured, usually involves laborious separation procedures. Although as a rule these techniques have a high degree of sensitivity specificity and precision they all have the disadvantage of being somewhat tedious. The $^{14}CO_2$ techniques, on the other hand, have a similar sensitivity and offer the advantage of being very simple and versatile. Maudsley Radivan and West (1967) compared the method of Waton (1956a) with the radiometric techniques of Schayer (1956a and b 1957) and of Kobayashi (1963) The two radiometric methods were found to be more sensitive than the non radiometric one. In their opinion the method of Schayer was more sensitive than the $^{14}CO_2$ method because of a lower blank value. Graihn and Rosenoren (1968) criticized the ^{14}CO method on the grounds that addition of large amounts of adrenaline and ascorbic acid was found to cause the release of $^{14}CO_2$ from carboxy ^{14}C-labelled histidine. This reaction took place without a concurrent formation of histamine. From this observation they concluded that the ^{14}CO method "may not be trustworthy for the purpose of measuring the rate of histamine formation" The authors, furthermore, maintained that these decarboxylation reactions occur also when tissue is present in the incubation mixture, although in the only experiment they performed to test this assumption they found that

with the CO method adrenaline seemed to depress histamine formation in minced mouse and rat lungs" But even if spontaneous decarboxylation had occurred in the presence of tissue the error introduced by the lability of histidine (presumably not only carboxy ^{14}C-histidine) can be avoided by proper blanks. Moreover the problem is not that the $^{14}CO_2$ method gives too high values, but that it seems to give lower values for the histidine decarboxylase activity of most tissues (AURES DAVIDSON and HÅKANSON 1969) than those reported by KAHLSON and co-workers (cf KAHLSON *et al.* 1964) It should be noted that their results have been obtained using blanks provided by the addition of semicarbazide. MACKAY and SHEPHERD (1967) have pointed out that the use of carbonyl trapping agents such as semicarbazide may result in falsely low blank values. This may be critical in the assessment of enzyme activity in tissues with low enzyme content.

All ^{14}CO methods have quite correctly been criticized on the grounds that they may be non-specific, *i.e.* $^{14}CO_2$ may be released through break-down of the amino acid along other pathways than the one expected. It is thus advisable to verify results obtained with these methods by the parallel use of some other technique. It should be kept in mind, however that whereas the enzyme activity recorded with the $^{14}CO_2$-method may thus sometimes be too high, it is difficult to imagine a situation in which the enzyme activity registered is too low (unless carbon dioxide trapping systems exist in the extract, which can easily be tested by incubating the extract with known amounts of a ^{14}C-labelled carbonate salt) This is of importance in regard to *e.g.* histidine decarboxylase (EC 4 1 1.22) since its existence in many tissues has been disputed. A high histidine decarboxylase activity recorded with this technique may have to be verified by some other method, but there is no reason to doubt that unless CO fixation takes place in the extract the inability to register decarboxylase activity with the $^{14}CO_2$-method reflects a very low enzyme level. The use of carboxyl-labelled histidine moreover eliminates a potential error that is present in all methods in which histamine is detected as the product of the reaction, namely that some of the histamine formed may be destroyed or degraded during the incubation and thus escape detection by the assay procedure used.

The term HFC (abbreviation of histamine forming capacity) as a synonym for histidine decarboxylase activity has recently gained widespread use. HFC is defined as a new term to express the rate of histamine formation in minced tissue or cell suspensions incubated *in vitro* with small physiological concentrations of histidine at present measurable only isotopically (KAHLSON 1962) In such an assay the enzyme is not necessarily rate-limiting The HFC value depends on the amount of tissue, on the concentrations of endogenous histidine and pyridoxal-5 phosphate, and on the time of incubation. Therefore the quantitative assessment of HFC is possible only when the various conditions are strictly defined. But even so the HFC assay provides no information as to what type of catalyst is present in the tissue

being studied. For a detailed criticism of the conventional HFC assay see BURKHALTER (1965) and PEARLMAN and WATON (1966)

2.2. *Assay of the gastric antipernicious anaemia principle (intrinsic factor)*

The intrinsic factor (IF) is known to have a high vitamin B_{12}-binding capacity which can be quantitatively determined Tissue extracts were prepared by homogenization in 0 1 M sodium acetate-acetic acid buffer pH 3.5 After centrifugation in a refrigerated centrifuge at 10,000 x g for 10 min, small amounts of the supernatant were mixed with ^{57}Co-labelled cyanocobalamin (usually 20—40 000 cpm, instrumental values) Aliquots (0 05—0 2 ml) of the mixture were passed through a Sephadex G 25 column (length 200 mm, inner diameter 8 mm) The column was washed with 0 1 M phosphate buffer pH 7 0 Protein-bound vitamin B_{12} was excluded from the gel and appeared in the first 3 ml fraction after the void volume This fraction was collected and quantitated by gamma spectrometry Blank values were obtained by running a parallel separation of the same amount of free vitamin B_{12} and collecting the same fraction for quantitation. The blank value usually did not exceeded 100 cpm above background. Standards were provided by measuring the radioactivity of the aliquots before separation on the columns (HÅKANSON LINDSTRAND, NORDOREN and OWMAN 1969)

The capacity to bind vitamin B_{12} is a prerequisite for IF activity but IF may be only one of several vitamin B_{12}-binding proteins in the gastric mucosa. The possible existence of several binders was tested by column chromatography and paper electrophoresis. One ml of tissue extract was chromatographed on a Sephadex G 150 column (90 × 1.5 cm) equilibrated with 0.05 M sodium phosphate buffer pH 6 containing 0.2 M NaCl. The major vitamin B_{12}-containing fraction was further analyzed by chromatography on a diethylaminoethyl (DEAE) cellulose column (13 × 1.5 cm) Before application on the column the pooled vitamin B_{12}-containing protein fractions were concentrated in a dialysis bag against polyethyleneglycol. The concentrated fractions were then passed through a Sephadex G 25 column in order to change the buffer composition to 0.005 M phosphate buffer pH 7.5 which was desirable for the DEAE chromatography With the DEAE cellulose column, gradient elution was performed with phosphate buffer starting with a molarity of 0 005 and a pH of 7.5 whereafter the molarity was increased gradually to 0 05 and the pH shifted to 6.0. The fractions were analyzed for radioactivity

The major vitamin B_{12}-containing protein fractions obtained in the gel chromatography were also analyzed by paper electrophoresis in a 0 05 M Veronal buffer pH 8 6 (4 V/cm, 16 hrs.)

By definition IF is a protein which facilitates the intestinal absorption of vitamin B_{12}. The capacity of rat stomach vitamin B_{12}-binding proteins to perform this function was tested in rats in which the oxyntic gland area had been surgically removed. These animals failed to absorb ingested vitamin B_{12}. ^{58}Co-

labelled cyanocobalamin (with or without vitamin B_{12}-binding proteins) was given orally to the rats and the amount of radioactivity retained in the body was established by whole-body counting immediately after and 1 3 and 7 days after ingestion of the isotope (Hàkanson *et al* 1969)

2.3 *Fluorescence microscopy of biogenic amines*

5 HT and catecholamines The fluorescence of enterochromaffin cells in formalin fixed material (mammalian intestines) was described by Erős in 1932 the fact that the fluorescence was induced by the formalin was recognized and discussed by Feyrter in 1938. After the chromatographic identification of 5-HT extracted from intestinal mucosa (Erspamer and Asero 1952b) Shepherd West and Erspamer (1953) demonstrated in model experiments that formalin treatment of 5-HT on paper causes a strong yellow fluorescence in ultraviolet light. Barter and Pearse (1953) concluded that the fluorescence of the enterochromaffin cells seen after formalin fixation was due to the presence of 5-HT and caused by the condensation of 5-HT with formaldehyde. These authors further suggested that the fluorescent reaction product formed from 5-HT and formaldehyde was a β-carboline (Barter and Pearse, 1955)

The observation by Eränkö (1955) that noradrenaline-containing cells in the adrenal medulla showed a bright green fluorescence following fixation in aqueous formalin was the first report on the demonstration of catecholamines by fluorescence microscopy However because of diffusion of the amines from the cells into the fixation medium, the sensitivity of the technique was poor Thus, noradrenaline could not be demonstrated in adrenergic neurons, and it became necessary to reduce the diffusion artifacts.

Although many observations indicated that formaldehyde could react with certain biogenic amines forming highly fluorescent condensation products (see also Hess and Udenfriend, 1959) the procedure employing aqueous formaldehyde for the histochemical detection of 5-HT in enterochromaffin cells and catecholamines in adrenal medulla had a low sensitivity which in no way made it superior to other existing methods for the histochemical detection of 5-HT and catecholamines. Moreover the specificity of the formalin method was unexplored and the chemical mechanism underlying the reaction was poorly understood. At this point Falck and Hillarp developed the gaseous formaldehyde condensation technique which resulted in a break-through in the histochemistry of 5-HT and catecholamines (Falck and Torp 1961 Falck, 1962 Falck, Hillarp Thieme and Torp 1962) By adequate freeze-drying followed by treatment with formaldehyde gas they were able to devise a method, which would prove to have a sensitivity and reproducibility superior to all previous methods, and which for the first time permitted the demonstration of noradrenaline in nerve terminals (for a description of the procedure as currently used see Falck and Owman 1965 Corrodi and Jonsson 1967) The chemical mechanism of the reaction between formaldehyde and these biogenic monoami-

nes was elucidated by Corrodi, Hillarp and Jonsson in a series of communications (Corrodi and Hillarp 1963 1964 Corrodi Hillarp and Jonsson 1964 Corrodi and Jonsson 1965a and b 1966 Jonsson 1967a, b and c) which gave a solid theoretical basis for the practical application of the histochemical technique. Their studies provided a starting point for further investigations on the complex condensation reaction (Axelsson Björklund and Håkanson 1970) and also for perfecting the method to permit the histochemical detection of previously non-detectable amines, such as tryptamine (Björklund Falck and Håkanson 1968)

It could be established that the formation of the fluorophores takes place as a sequence of reactions, which involves an initial Pictet-Spengler condensation, followed by secondary dehydrogenation reactions (Fig 4) In the first step, the catecholamines and indolamines are transformed into tetrahydroisoquinolines and tetrahydro-β-carbolines, respectively these derivatives are believed to be non-fluorescent or only weakly fluorescent. In a second step the condensation products are oxidized (dehydrogenated) to strongly fluorescent end products, the catecholamine fluorophores emitting a green fluorescence (emission max. 480 mμ) and the 5-HT fluorophore a yellow fluorescence (emission max. 535 mμ) The dehydrogenation reactions are catalyzed by protein (see Jonsson 1967b) and seem to proceed particularly fast if the monoamines are enclosed in a solid matrix *e.g.* in freeze-dried tissues or in a protein film. This second step can be controlled to some extent by manipulation of the oxidizing capacity of the reaction milieu and this has been utilized to give higher sensitivity and specificity to the reaction (Björklund Falck and Håkanson 1968 1969) Many aspects of the various reactions involved are still poorly understood. Recently silica gel thin layers have been introduced as a model system for the condensation reaction (Aures, Björklund and Håkanson 1968 Aures Fleming and Håkanson 1968 Björklund, Falck and Håkanson 1970a) and this has aided in the further elucidation of the reaction sequence. With this technique, it has been established that the fluorophores resulting from formaldehyde condensation of the various indolamines and catecholamines are heterogenous and consist of a number of fluorophores with different fluorescence colours and fluorescence intensities (Axelsson Björklund and Håkanson 1970)

The histochemical method of Falck and Hillarp has enormously increased our knowledge of adrenergic and tryptaminergic structures, and it has also been used for the cytochemical and cytopharmacological characterization of numerous nervous and endocrine cell systems (see *e.g.* review by Hillarp Dahlström and Fuxe, 1966 Falck and Owman 1968)

Histamine Schauer and Werle (1959) were first to suggest a technique for the histochemical detection of histamine. This technique was based on the ability of histamine to form a precipitate with Reinecke salt. The histamine-Reineckat in the tissue could then be stained with diazotized sulfanilic acid. The specificity of the method has not been fully established but its sensitivity

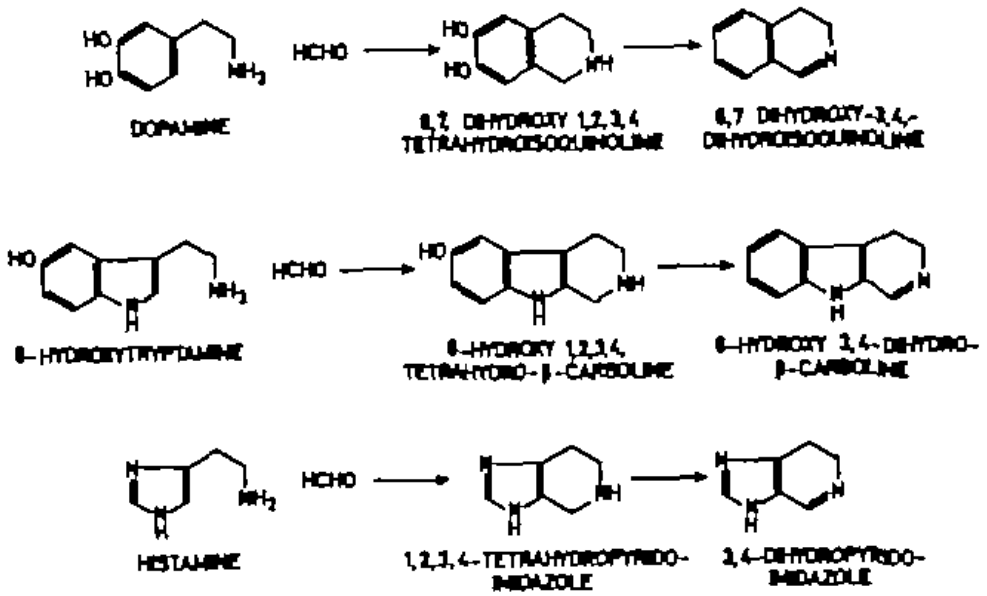


Fig 4 Proposed reaction scheme for the condensation of dopamine, 5-hydroxytryptamine and histamine with formaldehyde, and for the subsequent dehydrogenation of the condensation products. For dopamine and 5-hydroxytryptamine this sequence of reactions leads to highly fluorescent products. Histamine, however does not seem to yield fluorophores with formaldehyde, although it may condense along the route indicated.

appears to be adequate for the detection of mast cell histamine in spreads of rat mesentery The technique has not been widely used.

The treatment of freeze-dried tissues with paraformaldehyde vapour was suggested by Lagunoff Phillips and Benditt (1961) for the demonstration of mast cell histamine by fluorescence microscopy From observations on the fluorescence of solid histamine (pure crystals) after formaldehyde treatment, Lagunoff Phillips and Benditt suggested that the fluorescence exhibited by rat mast cells was due to histamine and caused by the formation of tetrahydropyridoimidazole (Fig 4) Also Van Orden (1966) studied the condensation of histamine with gaseous formaldehyde and claimed that fluorescence of fairly high intensity was observed after this reaction. Characterization of the fluorophore by microspectrofluorometry revealed excitation max. at 365 mμ and emission max. at 440 mμ the fluorophore was believed to be dihydropyridoimidazole (Fig 4) These results have not been confirmed in our laboratory It is possible that formaldehyde condenses with histamine (see Dale and Dudley 1921) but the resulting condensate seems to emit fluorescence of low intensity only (Björklund Håkanson and Sporrong unpublished observation) Moreover there is no evidence of formaldehyde induced fluorophore formation in tissues, which can be attributed to histamine-storing cells. The fluorescence observed by Lagunoff Phillips and Benditt (1961) in rat mast cells was probably not due to histamine but to 5 HT Dog mast cells which contain histamine but are devoid of 5-HT gave no fluorescence.

In 1966 JUHLIN and SHELLEY introduced another technique for the histochemical detection of histamine. They applied *o*-phthaldialdehyde (OPT) dissolved in xylene onto fresh cryostat sections, and, as a result, were able to demonstrate intense fluorescence in mast cells. The OPT method, as described by JUHLIN and SHELLEY did not reveal with certainty any other histamine-containing cell system besides mast cells. Independently two other groups had been working on the application of OPT as a histochemical detection reagent for histamine. By adequate freeze-drying, strictly anhydrous procedures and the use of gaseous OPT it could be shown that OPT induces fluorescence in mast cells (EHINGER and THUNBERG 1967 AUREN *et al.*, 1968 HÅKANSON OWMAN and SPORRONG 1968a HÅKANSON LILJA and OWMAN 1969 HÅKANSON OWMAN SJÖBERG and SPORRONG 1969) rabbit thrombocytes, basophil leucocytes (EHINGER and THUNBERG 1967) and also in several epithelial cell systems, such as the enterochromaffin-like cells of the rat and mouse stomach (EHINGER and THUNBERG, 1967 HÅKANSON and OWMAN 1967 HÅKANSON OWMAN and SJÖBERG 1967 THUNBERG 1967) rat pinealocytes (HÅKANSON LINDSTRAND OWMAN and TVERRING 1970c) pancreatic islet cells in the mouse, and certain unidentified cells in the pituitary (EHINGER, HÅKANSON OWMAN and SPORRONG 1968) * Unfortunately for unknown reasons the OPT method has been found unsatisfactory (high background fluorescence) in studies on brain (HÅKANSON OWMAN and SPORRONG unpublished observation) and the cellular localization of hypothalamic histamine is still unknown.

The enterochromaffin-like cells of murine gastric mucosa, on the other hand, have been extensively studied. The histochemical detection of histamine in epithelial cell systems is possible only with thoroughly dried tissue specimens (EHINGER and THUNBERG 1967 HÅKANSON and OWMAN 1967) Condensation with OPT however requires slight hydration, which unless carefully controlled, may cause diffusion of the fluorophore. Thus, to avoid diffusion artifacts either of the following two procedures is applied 1) Freeze-dried cryostat sections cut at temperatures below —30 C are exposed to OPT gas at room temperature for 1—2 min. This treatment is followed by gentle hydration under controlled conditions. As a consequence, an intense fluorescence appears in mast cells as well as in some epithelial cell systems (EHINGER and THUNBERG 1967 HÅKANSON and OWMAN 1967 THUNBERG, 1967) 2) Sections from freeze-dried, paraffin-embedded specimens are deparaffinized and then treated as under 1) with identical results (EHINGER, *et al.*, 1968) However since under certain conditions OPT reacts with a number of biogenic compounds, forming fluorophores with several, it may be argued that the specificity of this histochemical reagent is poor When properly

* In at least one of these cell systems (rat pineal) the fluorogenic compound has been shown to be different from histamine and from all other known OPT reactive compounds (Håkanson, Lindstrand, Owman and Tverring, 1970; see also the report by Ohman and Shelley 1968, on the OPT induced fluorescence in the retina)

performed, however the OPT method may well have a specificity similar to that of the formaldehyde method. With both these histochemical techniques the primary requisite is that the compound to be demonstrated should occur accumulated in sufficiently high concentrations at specific cellular sites. This requirement is sometimes fulfilled by biogenic amines, which often occur in special cells of limited distribution and often are stored in granular storage sites.

From model experiments it may be concluded that apart from histamine and histidine, other known OPT reactive biogenic compounds give poor fluorescence under the conditions used (Ehinger and Thunberg 1967) * Moreover those compounds that give an OPT induced fluorescence can be distinguished from histamine by the spectral characteristics of the resulting fluorophores (Ehinger and Thunberg 1967 Håkanson Juhlin Owman and Sporrong 1970a)

The specificity of the OPT technique as a method for detecting cellular histamine has to be verified chemically pharmacologically or otherwise in each individual case (see Ehinger and Thunberg 1967 Håkanson and Owman 1967 Håkanson Owman and Sjöberg 1967 Thunberg 1967 Håkanson *et al.* 1970a) One disadvantage here is that also the conventional chemical assay of histamine involves OPT as reagent which limits its usefulness as a means for identification of the OPT reactive tissue component. Another difficulty is the finding that the OPT induced fluorescence in tissues sections may appear in either a blue or yellow form (Ehinger and Thunberg 1967 Håkanson *et al.*, 1970a) Mast cells are usually yellow whereas fluorescing epithelial cells are blue. This is not due to inadvertent variations in experimental conditions, since the two flurophores may appear in one and the same tissue section. In recent studies using histamine on silica gel thin-layers and in protein droplets as models, it could be established that the OPT induced fluorescence changes in colour from blue to yellow as the concentration of histamine was increased This concentration-dependent red-shift has been studied by microspectrofluorometry (Håkanson *et al.*, 1970a) The blue fluorophore was maximally excited at 36 mμ and had its emission max. at 420—430 mμ the corresponding values for the yellow fluorophore was 405 mμ and 540—550 mμ (see also Ehinger and Thunberg 1967) Microspectrofluorometric analysis of OPT reactive gastric epithelial cells showed that the properties of their fluorophore were similar to those of the blue fluorophore in the model systems whereas the OPT-induced fluorophore of mast cells usually resembled the yellow fluorophore (Håkanson *et al.* 1970a) The difference in fluorescence colour between mast

* Indole compounds which under aqueous conditions and at extremely acid pH are known to form fluorophores with OPT (Maickel and Miller 1966) do not give any fluorescence under the conditions of the histochemical OPT technique. Spermidine, moreover which occurs in high concentrations in the central nervous system (Michaelson, 1967) does not seem to interfere with the histochemical detection of histamine in peripheral tissues (Ehinger and Thunberg, 1967 Håkanson and Owman, unpublished observations)

cells and certain gastric epithelial cells may reflect markedly different histamine concentrations in the two cell systems in analogy with the observations in the model experiments. From this assumption a new principle for the quantitation of intracellular histamine has been suggested according to which the emission maximum (and/or excitation maximum) can be used as a parameter of the histamine concentration (HÅKANSON *et al* 1970a)

2.4 *Conventional light microscopy*

Silver stains Silver impregnation has proved useful for the demonstration of cytoplasmic granules in certain epithelial cells. Such techniques can be divided in two main groups those in which no reducing agents are added (*e g* the FONTANA and MASSON HAMPERL techniques, cf PEARSE, 1960) and in which consequently endogenous compounds in the cytoplasm reduce the silver in an ammoniacal silver salt solution and those in which the silver salt is reduced by the addition of a reducing agent (BONIAN GRIMELIUS) the metallic silver forms precipitates, selectively impregnating the granules. In the first case the reacting cells are referred to as argentaffin in the second as argyrophil, although strictly both words have the same meaning To add to the confusion the term argentophil is sometimes used as a synonym for argyrophil There is a fundamental difference between the argentaffin and argyrophil reactions in that the former is brought about by the reducing capacity of a tissue component (such as 5-HT and catecholamines) and the latter by the addition of an extraneous reducer Argentaffin cells are usually also argyrophil, but argyrophil cells may very well be—and often are—non-argentaffin.

The argyrophil reaction, *i.e* the black to brown cytoplasmic granules seen on light microscopy corresponds on the ultrastructural level to secretory granules, to which the silver particles are localized. What chemical or physico-chemical factor that give rise to this formation of silver precipitates is unknown. All these techniques have been developed empirically

Mast cell stain Mast cells are recognized by their metachromasia after staining with toluidine blue in ethanol solution (cf ROMEIS 1948)

Parietal cell stain. Parietal cells and chief cells were stained by haemalun-Congo red stain (cf *ROMEIS 1948*)

Mucous neck cell stain. Mucoid cells were recognized by the PAS-stain (cf. PEARSE, 1960)

3 PROPERTIES OF ENTEROCHROMAFFIN AND ENTEROCHROMAFFIN LIKE CELLS

3 1 *The concept of the enterochromaffin system*

In 1870 HEIDENHAIN described small cells in the gastric mucosa of dogs and rabbits which became stained after fixation with solutions containing dichromate. Such cells were demonstrated by TOLDT (1880) and NICOLAS (1891) also in other species. CIACCIO (1906) reported that the staining properties of these chromaffin cells of the gut were similar to those of the chromaffin cells of the adrenal medulla and introduced the term enterochromaffin cells.

Enterochromaffin cells have also been referred to as the KULTSCHITZKY SCHMIDT CIACCIO or MASSON cells (see *e g* PENTTILÄ, 1966) Because of their characteristic granular cytoplasm, KAUFMANN WOLF (1911) introduced the term basal granular cells ("basalgekörnte Zellen") Mostly however the cells were named after their staining properties. KULL (1925) used the term chromaffin cells, and CORDIER (1926) referred to this cell type as chromoargentaffin cells. Silver reactions were soon found to be important for the demonstration of this type of cells. MASSON (1914) was first to use the term argentaffin to characterize those cells which reduced ammoniacal silver salts. Later HAMPERL (193? 1952) and ERSPAMER (1937) introduced the terms argyrophil and argentophil respectively for epithelial cells which accumulated cytoplasmic silver deposits in the presence of reducing agents. According to the initial definition of ERSPAMER (ERSPAMER, 1937a and b see also VIALLI, 1966) the enterochromaffin cell system consists of all cells hypothetically derived from the primary gut and giving the chromaffin reaction, and also of cells showing the same morphology and reactivity to argyrophilic stains. The argentaffin and/or argyrophilic character of these cells is believed to be a particularly important distinction. With the aid of these two types of silver reactions, ERSPAMER (1937a 1938) and VIALLI and ERSPAMER (1939) divided the cells of the enterochromaffin system into four classes 1) pre-enterochromaffin, non-argyrophil, ?) pre-enterochromaffin, argyrophil, 3) enterochromaffin cell proper argentaffin 4) empty enterochromaffin, argyrophil or non-argyrophil cell. This classification claimed to represent the various successive tages in the maturation (or in the functional cycle) of the enterochromaffin cells. The existence of types 1 and 4 has not been established unequivocally

The peculiar cytochemical characteristic which is reflected in the chromaffinity and argentaffinity of some of these cells resulted in many theories about the chemical identity of the reacting substance in their cytoplasm. ERSPAMER and co-workers (ERSPAMER and BORETTI, 1950 1951 ERSPAMER and ASERO, 1952a, b ERSPAMER, 1953 1954a, b 1958) presented evidence indicating that 5-HT was the specific reducing agent in the enterochromaffin cells. This hypothesis has been confirmed in subsequent investigations (see section 3.2.)

True enterochromaffin cells can be stained with a number of staining reactions, such as the argyrophil, argentaffin, ferric ferricyanide, diazo coupling and indophenol reactions. All the latter four stains detect 5-HT when present in sufficient amounts. The true enterochromaffin cell (type 3) is the only member of the enterochromaffin system which contains 5-HT in quantities that can be revealed by histochemistry.

The concept of the enterochromaffin system was later modified by ERSPAMER (1954a, 1958, 1961). He included in the enterochromaffin system all cells which, after formalin fixation, give a chromaffin and argentaffin reaction, and which are capable of coupling with diazonium salts and of showing a formalin-induced fluorescence. According to this definition, the basic criterion deciding whether a cell belongs in the system or not is the histochemical demonstration of 5-HT (or similar reducing compounds) in the cell. Consequently ERSPAMER (1961) included also the mast cells of the rat and mouse, which are rich in 5-HT (BENDITT, WOOD, ARASE and ROEPER, 1955; BENDITT, 1958), while excluding the argyrophil, non-argentaffin cells, which are morphologically indistinguishable from the argentaffin (enterochromaffin) cells, but devoid of 5-HT. From a physiological point of view this seems unfounded. The argyrophil, argentaffin and the argyrophil, non-argentaffin cells of the gastrointestinal mucosa may all have similar endocrine functions (see section 3.9).

3.2. *The typical enterochromaffin (argentaffin) cell*

Enterochromaffin cells are not readily recognized by conventional staining methods such as haematoxylin-eosin: the cytoplasm is clear and only slightly stainable; the dimension and shape of the nucleus are not different from the nuclei of adjacent cells.

The typical "true" enterochromaffin (argentaffin) cell, i.e. type 3 according to the classification of ERSPAMER (1937a, 1938), is identified by the presence of granules of particular shape and by all those histochemical characteristics which ERSPAMER described in 1954. The shape of the cells is somewhat variable; in general they are flask-shaped, being larger at the base, where the nucleus is situated. At times the base protrudes somewhat with respect to neighbouring cells, modifying the arrangement of the basement membrane; sometimes processes extend sideways from the basal portion of the cell. Towards the apical extremity the cell always becomes more narrow and in many cases exhibits a thin process which may extend towards the glandular lumen in between neighbouring epithelial cells. The granules are the most prominent characteristic of the enterochromaffin cell. From a morphological point of view they are perfectly distinguishable from the granules present in other cell types, such as PANETH's cells and the mucoid cells of the gastric epithelium (see section 3.8 on ultrastructure). In the light microscope, the granules (revealed by the silver precipitates) are minute and stand out more or less clearly against the cytoplasm. Normally they cluster more densely in the infranuclear portion of the cell; towards the apex they may or may not be present.

The enterochromaffin granules give strong chromaffin and argentaffin reactions, which gave the first clue towards identification of the enterochromaffin substance. The classical chromaffin reaction, first described by HENLE (1865) in the adrenal medulla, is—like the argentaffin reaction—believed to reflect a high concentration of reducing agents, such as *e.g.* catecholamines and 5-HT. However numerous other substances besides these amines may react with chromium solutions to form brownish pigments (see COUPLAND 1954, HALE, 1958, PEARSE, 1960). In 1940 ERSPAMER observed that acetone extracts of the digestive tract were biologically active; he called the active substance enteramine. In 1952 and 1953 enteramine was identified as 5-HT (ERSPAMER and ASERO, 1952b; DALGLIESH, TOH and WORK, 1953) on the basis of its pharmacological actions and its chromatographic properties. Since 5-HT is capable of giving a positive argentaffin and chromaffin reaction, this finding suggested that 5-HT was the specific reducing substance of the enterochromaffin cells.

The formalin-induced fluorescence exhibited after excitation with ultraviolet light is one of the characteristics of the enterochromaffin cell. An interpretation of the chemical mechanism behind this fluorescence was formulated by BAETER and PEARSE (1953, 1955) and by PEARSE (1956, 1960). The fluorescence is due to a β-carbolin resulting from the condensation of 5-HT with formaldehyde. According to VIALLI and TRAVERSO (1960) the β-carbolin compound resulting from formalin treatment of 5-HT is not an end-product but may be further converted by oxidation (see section 2.3). This assumption has since been confirmed (CORRODI and JONSSON 1965b; BJÖRKLUND, FALCK and HÅKANSON 1968; see also HESS and UDENFRIEND 1959). CASELLA and REGOLIANI (1946, 1949) were first to analyze the formalin-induced fluorophor of the enterochromaffin cells and gave the emission max. at 535 mμ. Using the FALCK-HILLARP technique, NOSEKBO (1964) described cells with an intense yellow fluorescence, typical of 5-HT, in the intestinal mucosa of the rat and cat. These cells were found to be identical with the 5-HT-containing enterochromaffin cells (PENTTILÄ 1966). The yellow formaldehyde-induced fluorophore in the gastrointestinal enterochromaffin cells has been analyzed by microspectrofluorometry which revealed an excitation maximum at 405 mμ and an emission maximum at 535 mμ (VIALLI and PRENNA, 1969; HÅKANSON *et al* 1970d). The spectral properties of the fluorophore agreed very well with those of authentic 5-HT in various model systems after treatment with gaseous formaldehyde (cf. *e.g.* AURES, BJÖRKLUND and HÅKANSON 1968; BJÖRKLUND, FALCK and HÅKANSON 1968; BJÖRKLUND, FALCK and HÅKANSON 1970a). *In the present study all gastric epithelial cells exhibiting a formaldehyde-induced fluorescence have been classified as enterochromaffin.*

3.3 *Distribution and properties of enterochromaffin cells in gastric mucosa*

Enterochromaffin cells are widely distributed in the epithelium of organs and ducts derived from the primitive gut: the trachea, gastrointestinal tract, biliary tract, pancreatic ducts, prostate and vagina (VIALLI, 1966; FALCK and OWMAN

1968) After the observation of the association between 5-HT and enterochromaffin cells, Erspamer (1953 1954a, b) and others (Feldberg and Toh, 1953 Rennick and Gray 1961 Jacobsson and Stacey 1962 Kalinoman Kardaman and Haber, 1964 Schofield Ho and Southwell, 1967) charted the distribution of 5-HT in the digestive tract of a number of species. The distribution between various parts of the gastrointestinal tract differed widely among the species, but there was regularly a striking parallelism between the distribution of 5-HT and of the enterochromaffin cells. (The rat and mouse were exceptional in that a quite considerable portion of the bowel 5-HT was found to be stored in mast cells.) Most of the data on enterochromaffin cells in the digestive tract concern the intestine only The investigations summarized in the present treatise deal specifically with their distribution in gastric mucosa (see Håkanson *et al* 1970d) In most species (*e.g* mouse, rat hamster dog, pig and man) enterochromaffin cells are present predominantly in the antropyloric mucosa. However there are species, *e.g* guinea pig and the cat, where they have a more uniform distribution in all regions of the stomach, and also species such as the rabbit where they are conspicuously more numerous in the oxyntic gland area (Fig 5) It should be noted that in the cat and rabbit enterochromaffin cells with a green formaldehyde-induced fluorescence (see definition of the enterochromaffin cell, section 3.2) have been recognized in addition to the classical type of yellow-fluorescent, 5-HT-containing enterochromaffin cells (Håkanson *et al*., 1970d) Microspectrofluorometric analysis according to the principle of Björklund Ehinger and Falck (1968) indicated that the green fluorescence in these cells was due to dopamine (Håkanson *et al*. 1970d) But also among conventional 5-HT-containing enterochromaffin cells sub-types have been observed Their sensitivity to the amine-depleting effect of reserpine is one of the factors which permit a distinction between various types of enterochromaffin cells. In the rabbit, one population of 5-HT-containing enterochromaffin cells is markedly affected by reserpine treatment, whereas another one responds not at all (Zbinden Pletscher and Studer, 1957 Pletscher, 1958 Håkanson *et al* 1970d)

3.4 *The typical enterochromaffin-like (argyrophil, non-argentaffin) cell*

In 1966 Håkanson and Owman observed by fluorescence microscopy (Falck Hillarp technique) a large population of green-fluorescent epithelial cells in the oxyntic gland area of rats, injected with L DOPA (Fig 6) * These cells

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The rat stomach is made up of one glandular portion and one non-glandular portion, the rumen. The glandular portion is made up of two histologically different parts the pyloric gland rea (antrum) and the oxyntic gland area (sometimes referred to as the fundus, although this term is inadequate from strictly anatomical point of iew) In ou first publications in this fi ld (see Håkanson, 1965 Håkanson and Owman 1966b) w adopted the terminology of Schayer (1956 1957) in which the gland lar portion is referred to as the pyloric part and rumen is called the fundus. I our subsequent publications, histological rather than anatomical criteria have been used t characterize the various regions

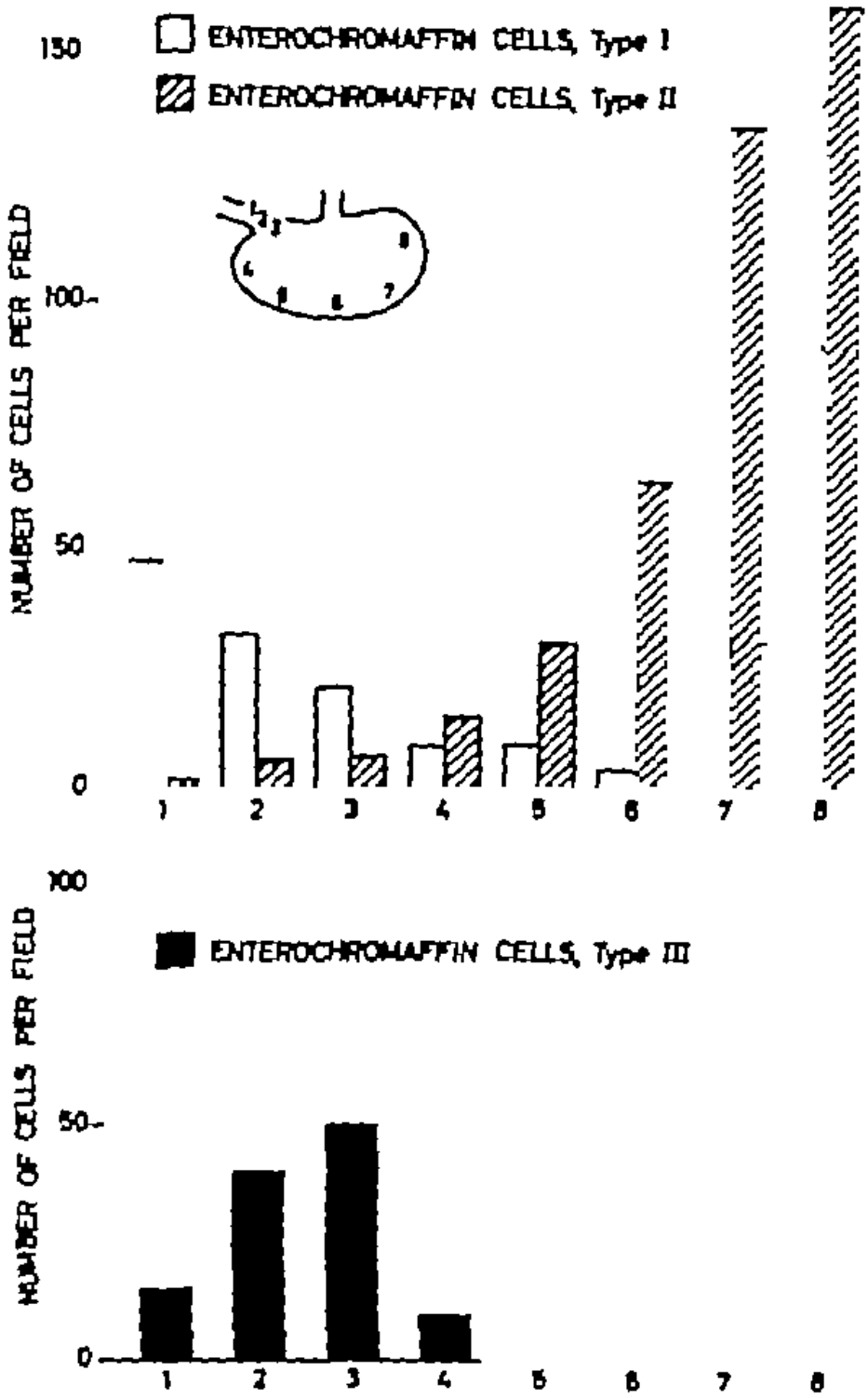


Fig. 5 Regional distribution of various types of enterochromaffin cells in the rabbit stomach. Treatment with reserpine revealed one reserpine-sensitive (Type I) and one reserpine-resistant (Type II) yellow-fluorescent enterochromaffin cell type. In addition, a third type of weakly greenish yellow enterochromaffin cells (Type III) was recognized. These cells may be identical with the enterochromaffin-like cells, which became intensely green-fluorescent after injection of DOPA (100—200 mg/kg) (For details see Håkanson et al. 1970d) Finally dopamine-containing, green-fluorescent enterochromaffin cells (Type IV) have also been demonstrated in gastric mucosa of th non-treated rabbit although few in number and with scatterred and irregular distribution.

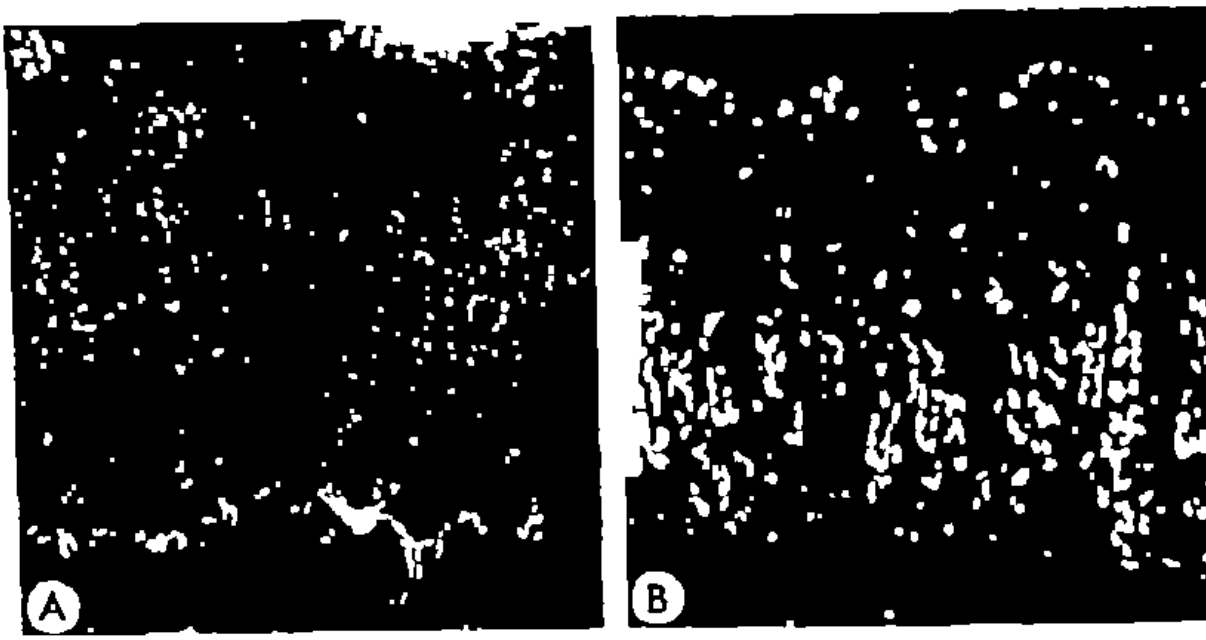


Fig. 6. Transverse sections, 100 x. Formaldehyde reaction (A) N pretreatment. Fl orescent adrenergic nerves around essel in submucosa. Mast cell with specific yellow fluorescence scattered in the submucosa, the muscle coat and t the surface of the mucosa. The glandular epithelium contains large amounts f min te granules with autofluorescence. (B) Pretreatment with L DOPA (intraperitoneal i jection, 100 mg/kg the animal was sacrificed 2 hr after injection) After this treatment numerous green-fluorescent enterochromaffin-like cells are visible in the basal part of the mucosa. Mast cell now with green fluorescence, are seen near the mucosal surface

were morphologically very similar to the yellow fluorescent 5 HT-containing enterochromaffin cells of the pyloric gland area and they were thus referred to as enterochromaffin like. It was found that the injection of L-DOPA resulted in the simultaneous accumulation of dopamine in the oxyntic gland area of the stomach (HÅKANSON 1965 HÅKANSON and OWMAN 1966b) This indicated that the green fluorescence observed in the enterochromaffin-like cells reflected the presence of dopamine rather than of DOPA, an assumption which was confirmed in subsequent studies (HÅKANSON LILJA and OWMAN 1967) It could be shown that pretreatment with DOPA decarboxylase inhibitors marked! reduced the fluorescence intensity of the enterochromaffin-like cells. Moreover administration of large amounts of dopamine failed to induce any fluorescence in these cells, indicating that the dopamine, which accumulates after L DOPA treatment, results from decarboxylation *in situ* In control rats, no fluorogenic amine could be demonstrated by the formaldehyde technique in the enterochromaffin-like cells, and dopamine was not chemically detectable. After this initial observation in the rat, a similar cell system was observed in the gastric mucosa of the mouse, hamster guinea pig, rabbit, cat, dog, pig and man (see HÅKANSON *et al.* 1970d) and it could be established that these cell were argyrophil (Fig 7) but not argentaffin in contrast to the enterochromaffin cells, which were both argyrophil and argentaffin.

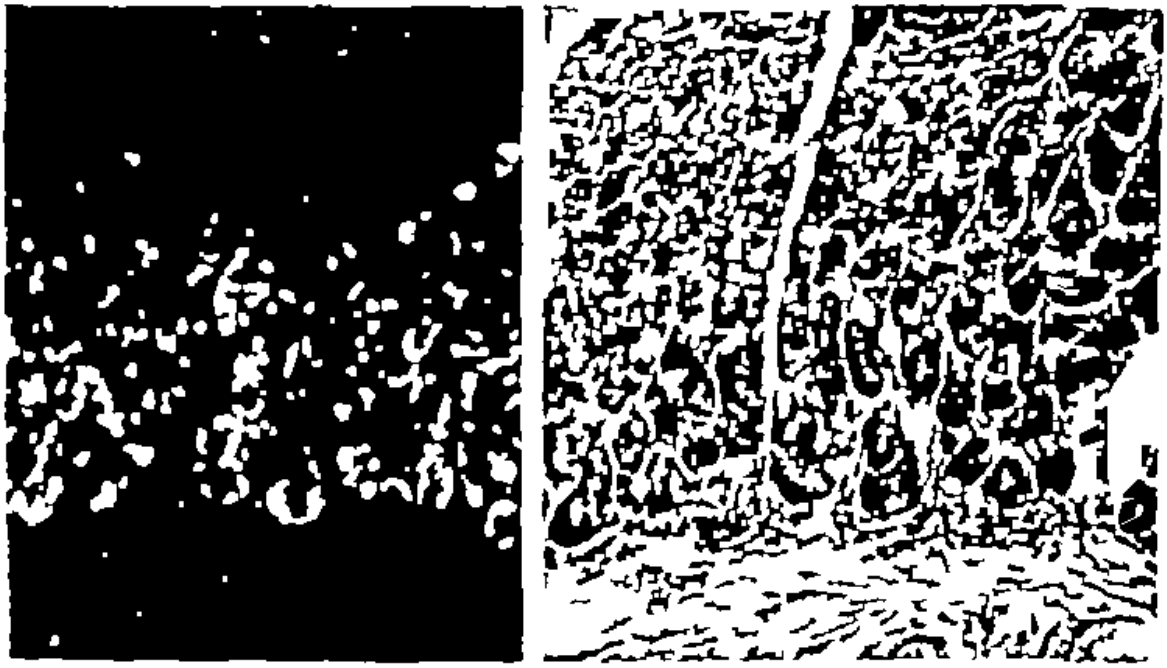


Fig. 7 Photomicrographs of rat stomach wall oxyntic gland area. Transverse section, 115 x. Pretreatment with L DOPA. Formaldehyde condensation (left) Fluorescent mast cells near the mucosal surface and in the submucosa. In the basal part of the mucosa an extensive system of fluorescent enterochromaffin-like cells. The section was subsequently silver-impregnated (right) to show the agreement between the enterochromaffin-like cells and the argyrophil cells.

The *enterochromaffin-like cell* may be defined as follows *It is an epithelial argyrophil but non-argentaffin cell morphologically indistinguishable from the usual type of 5-HT-containing enterochromaffin (argentaffin) cell but devoid of 5-HT and catecholamines and consequently not detectable by the fluorescence microscopic procedure of* FALCK *and* HILLARP *after treatment of the animal with* L *DOPA however these cells which now contain dopamine can be readily detected by their green fluorescence* Usually the yellow fluorescent, 5-HT-containing enterochromaffin cells retain their yellow fluorescence also after the administration of L DOPA (HÅKANSON *et al* 1970d) indicating that their 5-HT stores are unaffected by this treatment. In addition, the enterochromaffin-like cells in the oxyntic gland area of the rat and mouse contain histamine (HÅKANSON and OWMAN 1967 HÅKANSON OWMAN and SJÖBERG 1967) which has been established by means of the histochemical OPT technique (Fig 8) In no other species has histamine been detected in the enterochromaffin-like cells (AURES HÅKANSON OWMAN and SPORRONG, 1968 HÅKANSON LILJA and OWMAN 1969 HÅKANSON *et al.* 1970d) Recent observations on the enterochromaffin-like cells in the gastric mucosa of the rabbit suggest that they contain an amine, which reacts with formaldehyde, giving a very weak greenish-yellow fluorescence. The amine responsible for this fluorescence has not yet been identified (HÅKANSON *et al* 1970d)

The observation that the argyrophil, non-argentaffin cells are capable of tak

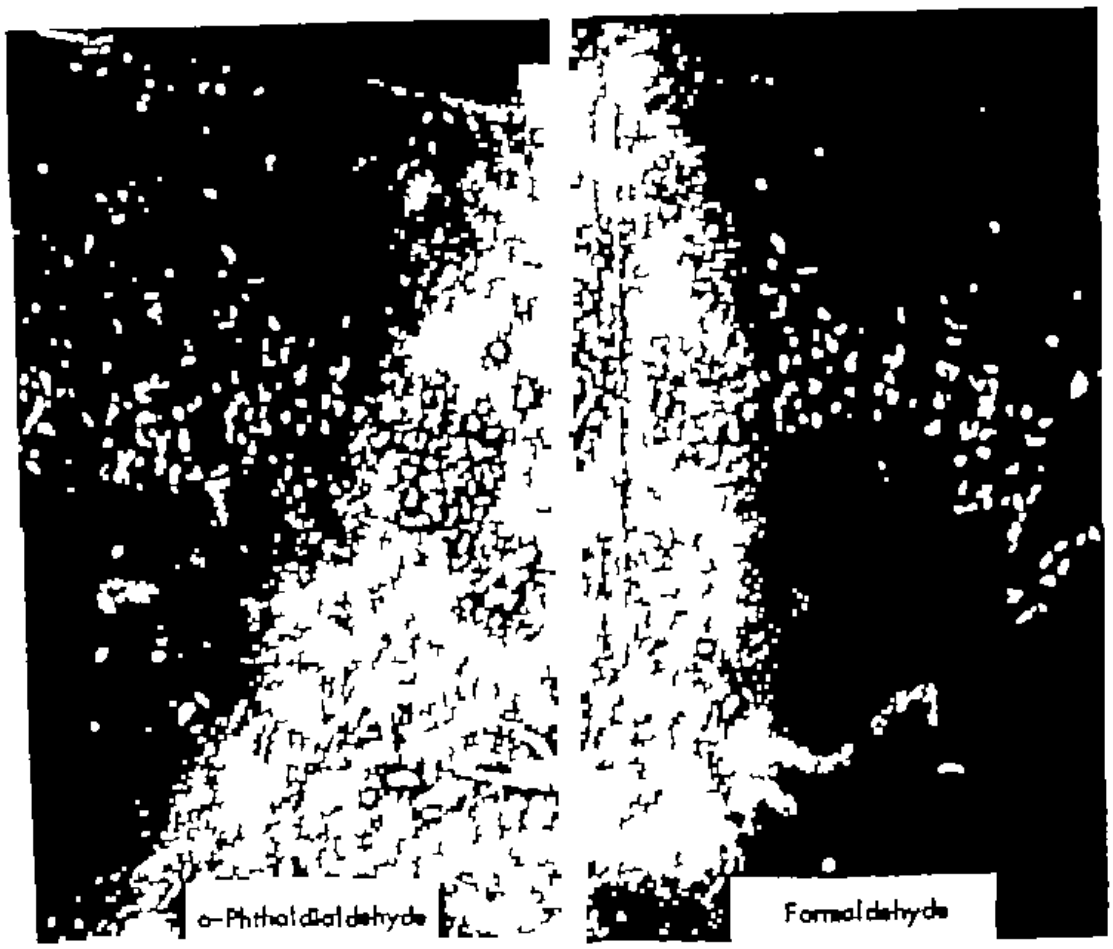


Fig. 8. Fluorescence photomicrograph of rat stomach wall, oxyntic gland area. Transverse section, 1.5 μ. Pretreatment with L-DOPA. Specimens were sectioned in a cryostat (−36° C). The same section was first treated with gaseous o-phthaldialdehyde to demonstrate the histamine-containing enterochromaffin-like cells. Most cells at the surface of the mucosa and the submucosa were found to contain both histamine and 5-hydroxytryptamine. The histamine-containing cells in the gastric epithelium (left) were found to be identical with the enterochromaffin-like cells (right). The formaldehyde treatment abolished the o-phthaldialdehyde induced fluorescence as was seen when sections from control rats (not given L-DOPA) were subjected to the same histochemical procedures.

ing up and decarboxylating certain aromatic amino acids was novel and introduced the idea that amine mechanisms may operate also in those enterochromaffin-like cells where amines have not yet been demonstrated. It was soon realized (Håkanson and Owman 1969) that the capacity to produce and store amines such as 5-HT and dopamine was characteristic of the cells earlier described in gastric mucosa by Dawson (1944, 1945, 1948) and designated as argentophil (i.e. argyrophil) cells. The granules of these cells reacted readily with the Bodian protargol method. They could not be detected with the Masson Hamperl silver method, which selectively stains argentaffin cells.

The argyrophil but non argentaffin cell of gastric mucosa has the same morphological appearance as the typical enterochromaffin cell, but it lacks many of its histochemical characteristics. The problem of the relationship between

the argentaffin and the argyrophil non-argentaffin cell types is still unsolved. Erspamer (1938) and Hamperl (1952) maintained that the argyrophil cell is a potential enterochromaffin (argentaffin) cell and may remain argyrophil or develop into a true enterochromaffin cell. Clara (1957) however believed that only a proportion of the argyrophil cells could be regarded as precursors of the enterochromaffin cells. This view is supported by recent observations by Håkanson and Owman (1969) (see section 3.5)

3.5 *Distribution and properties of enterochromaffin-like cells in gastric mucosa*

In the rat argyrophil enterochromaffin-like cells were found to be numerous in the mucosa of the oxyntic gland area and were associated chiefly with the deeper halves of the gastric glands (Fig 9) Scattered cells were also present in the pylorus and a few cells were seen in the cardiac region (Håkanson Lilja and Owman 1967) When the Masson Hamperl technique was applied to the gastric mucosa of the rat it was found that only the cells in the cardiac and pyloric zones were stained. This indicated that the numerous cells of the oxyntic gland area were not argentaffin in nature but argyrophil (Håkanson and Owman 1969) Thus, argentaffin cells occur in relatively small numbers in the rat stomach and are limited primarily to the pylorus.

The striking differences in the distribution of these two classes of cells in mammals (see Table 1 and Fig 11) are in discord with the concept of a secretory cycle from the argyrophil to the argentaffin stage, with the implicit belief that the argentaffin stage is the functionally significant one. Available evidence

TABLE 1

Regional distribution of enterochromaffin and enterochromaffin-like cells in the gastric mucosa of various mammals.

Species	Enterochromaffin cells		Enterochromaffin-like cells	
	Pyloric gland area	Oxyntic gland area	Pyloric gland area	Oxyntic gland area
Mouse	+	0	(+)	+++
Rat	++	0	0	++++
Guinea-pig	+++	++	(+)	+
Hamster	++	(+)	(+)	++
Rabbit	+(+)	++++	+	++
Cat	+	(+)	+(+)	+(+)
Dog	++	(+)	++	(+)
Pig	+++	++	+++++	++
Monkey	+	+	+	+(+)
Man	++	(+)	++	(+)

The tissue specimens analyzed were taken from the greater curvature as indicated in Fig. 5 The amount of enterochromaffin and enterochromaffin-like cells in the two stomach areas is expressed arbitrarily from 0 to +++++ as a measure of the number of such cells in the mucosa. It should be realized that the distribution of the enterochromaffin and enterochromaffin-like cells usually does not agree with the conventional histological division of the gastric mucosa into oxyntic, pyloric and cardiac gland areas, and so that these cells usually are not uniformly distributed in the region of predominance.

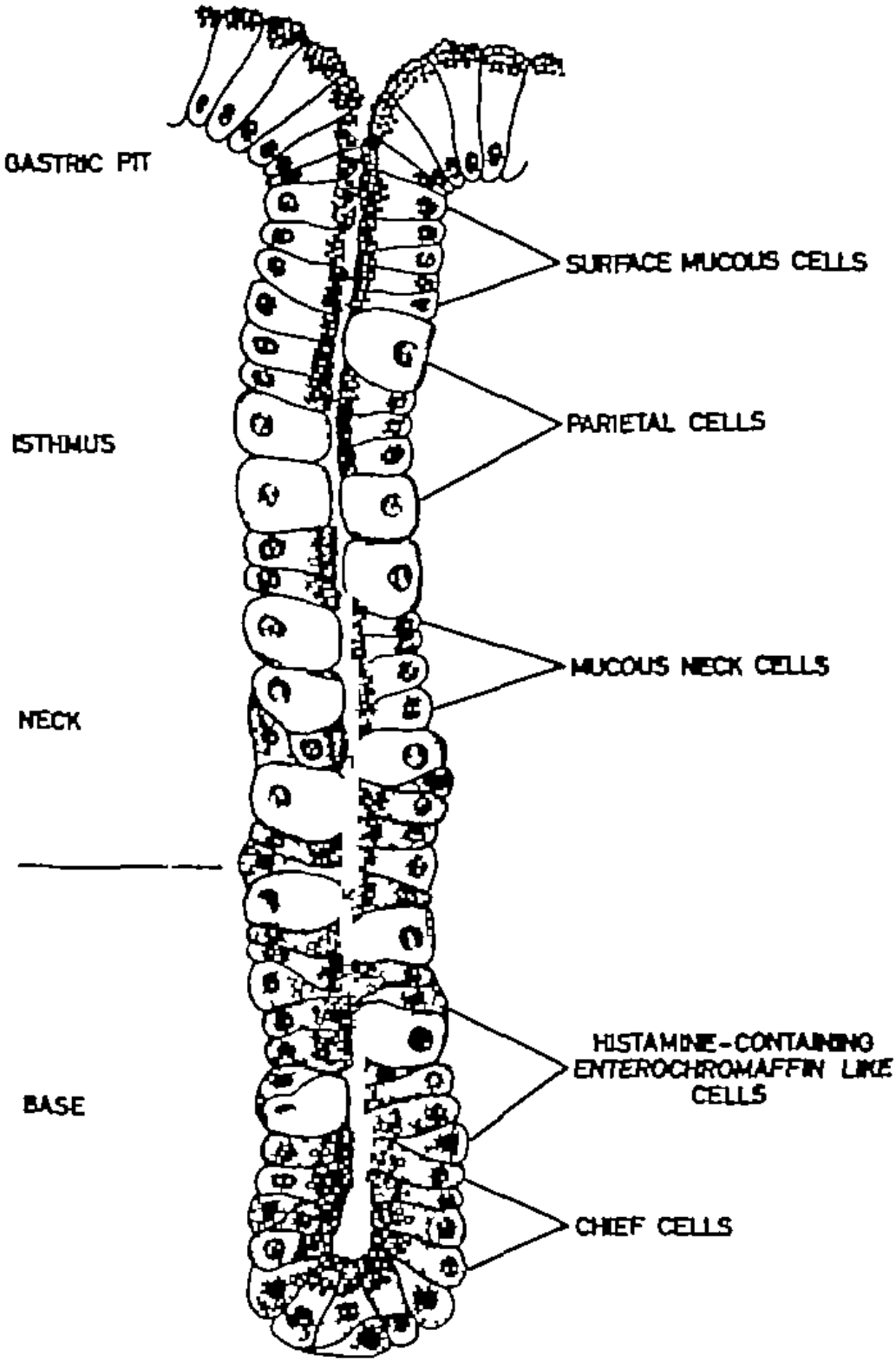


Fig. 9 Topography of an oxyntic gland in the rat stomach (modified from Ito and Winchester 1963)

strongly suggests that at least some argyrophil cells exist throughout their life history without ever reaching an argentaffin stage (Håkanson and Owman

1969 HÅKANSON *et al* 1970d) This may be the case either in restricted regions of the digestive tract, such as in the stomach, or perhaps along the entire intestinal tract. Therefore the argyrophil cell may not always represent a stage in the secretory cycle of the argentaffin cell. On the contrary it may have a physiological role of its own, similar to but distinct from that of the argentaffin cell.

It is apparent that the conventional terminology is unsatisfactory in several respects. The terms enterochromaffin, enterochromaffin-like, argentaffin and argyrophil refer to purely histological or histochemical characteristics. The subdivision of cells of the enterochromaffin system into argentaffin and argyrophil is generally considered fundamental, and a hard line of distinction is drawn between them by most workers. However classifying the cells according to their reactions to different silver stains appears arbitrary and conceivably unrelated to the physiological roles of the cells (for a discussion see section 3.9)

3.6 *Amine mechanisms in enterochromaffin and enterochromaffin-like cells of gastric mucosa*

The classical system of enterochromaffin (argentaffin) cells is the major storage site for gastrointestinal 5-HT (cf VIALLI 1966) In addition to the conventional, 5-HT-containing enterochromaffin cells, which upon formaldehyde treatment exhibits a yellow fluorescence, morphologically similar cells in the gastric mucosa of the cat and rabbit have been found emitting a green formaldehyde-induced fluorescence suggesting the presence of a catecholamine. Chemical and microspectrofluorometric analysis revealed that the compound was in all probability identical with dopamine (HÅKANSON *et al*., 1970d) These cells have also been classified as enterochromaffin (see definition, section 3.2.) The pyloric gland area of the rabbit contains another (argyrophil) cell type with a very weak formaldehyde-induced, greenish yellow fluorescense Fig. 5 Because of this fluorescence also these cells are referred to as enterochromaffin (see section 3 2) although the fluorogenic compound in the cytoplasm has not yet been identified (HÅKANSON *et al* 1970d)

Another *cell system morphologically similar to the enterochromaffin cells* and designated enterochromaffin-like, can be demonstrated by fluorescence microscopy only after the administration of certain aromatic amino acids, which give a formaldehyde-induced fluorescence in themselves or after conversion to their amine product (HÅKANSON and OWMAN 1966 HÅKANSON LILJA and OWMAN 1967 1969 ADRES *et al*., 1968 HÅKANSON *et al*., 1970d see also ROSELL, SEDVALL and ULLBERG 1963 RITZÉN HAMMARSTRÖM and ULLBERG 1965 HAMMARSTRÖM, RITZÉN and ULLBERG 1966) These enterochromaffin like cells (see section 3 4) which normally store neither 5-HT nor catecholamines—at least not in amounts detectable by fluorescense microscopy—can take up amino acids such as DOPA and 5-HTP by an active mechanism that can be blocked with cocaine

(HÅKANSON LILJA and OWMAN 1967) DOPA decarboxylase within the cell converts the amino acid to the corresponding amine, which is retained in the cytoplasm by a fairly reserpine resistant storage mechanism. After pretreatment with a DOPA decarboxylase inhibitor the administration of DOPA induced only a very weak fluorescence in the enterochromaffin like cells this may reflect the presence of non-converted L DOPA in the cytoplasm. Thus, the intense green cellular fluorescence observed after DOPA administration alone seems to reflect the presence of dopamine predominantly The conversion of L DOPA to dopamine occurs within the enterochromaffin-like cells since exogenous dopamine even in massive doses, produced only a very slight green fluorescence in this cell system (HÅKANSON LILJA and OWMAN 1967) These findings strongly suggest tthat DOPA decarboxylase is present in the enterochromaffin like cells. Apart from DOPA and 5-HTP a number of aromatic amino acids gave rise to a formaldehyde induced fluorescence in the enterochromaffin-like cells, possibly as a result of intracellular decarboxylation (AURES HÅKANSON and SCHAUER, 1968 HÅKANSON and OWMAN 1968) Both chemical and histochemical evidence supported the assumption that *e g* tryptophan was decarboxylated in these cells. This may possible be the ca als ith some of the fluorogenic aromatic amino acids tested, such as methyl *m* tyrosine, α-methyl DOPA 3 4-dihydroxyphenylser hydroxyphenylalanine, 3-methoxy-4-hydroxyphenylanine and 5 h han (AURES HÅKANSON and SCHAUER, 1968) Pretreatment wi mine oxidase inhibitors such as nialamide or pargyline markedly incr s fluorescence intensity in the enterochromaffin like cells following ration of L-DOPA (HÅKANSON LILJA and OWMAN 1967) This be presence of monoamine oxidase within these cells an assu h received further support by the histochemical demonstration of oxidase in the basal portion of the mucosa (HÅKANSON LILJA and OWMAN 1967)

By th se of the histochemical OPT method gastric mucosal histamine in the rat and mouse was found to have primarily a non mast-cell localization, being stored in a specific epithelial cell system in the basal portion of the oxyntic gland area (HÅKANSON and OWMAN 1967 THUNBERG 1967) In addition, histamine was found in gastric mast cells, which were few in the rat and somewhat more numerous in the mouse. In studies on rats, treated with L DOPA it could be shown that the histamine-containing epithelial cells, detectable with the OPT method were identical with the enterochromaffin-like cells, subsequently demonstrated in the same section by their green formaldehyde-induced dopamine fluorescence (HÅKANSON and OWMAN 1967) A high histamine forming capacity had previously been demonstrated in the gastric mucosa of the rat (SCHAYER, 1956b 1957 WELSBACH, LOVENBERG and UDENFRIEND 1961 KAHLSON *et al.*, 1964) indicating that gastric histamine was produced locally

The following observations support the view that the OPT induced fluores-

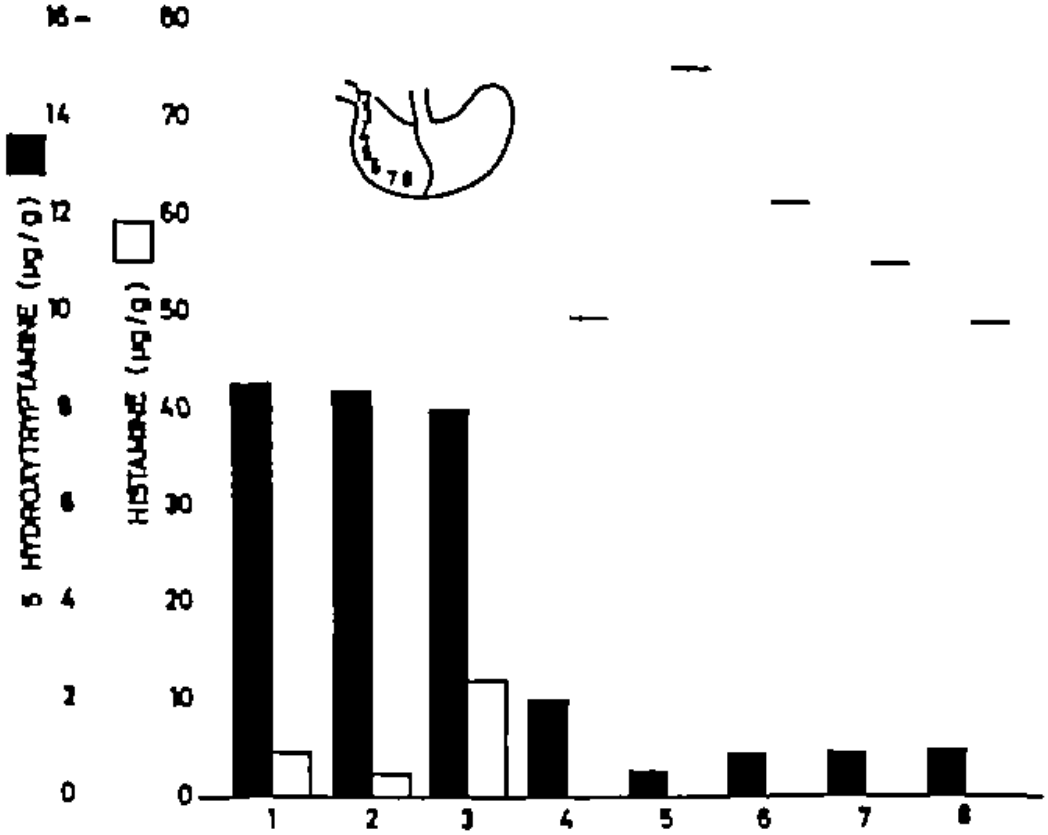


Fig. 10. Regional distribution of 5-hydroxytryptamine and histamine in the rat stomach. Small tissue samples were taken from the wall of the greater curvature as indicated in the drawing and assayed for amine content as described in section 2.1. The numbers on the abscissa refer to the locations shown in the inserted drawing. The distribution of 5-hydroxytryptamine corresponds well with that of the enterochromaffin cells; histamine, on the other hand, has a very different distribution, closely resembling that of the enterochromaffin-like cells.

cence of the enterochromaffin-like cells reflects the presence of histamine. 1) The regional and topographical distribution of gastric histamine agreed very well with that of the OPT-reactive cells (Thunberg 1967, Aures Håkanson and Schauer, 1968; see also Fig. 10). 2) The regional and topographical distribution of gastric histidine decarboxylase likewise agreed very well with that of the OPT-reactive cells (Thunberg 1967, Aures Håkanson and Schauer, 1968). 3) The quantitative development of gastric histamine in the new-born and young rat coincided with development of the OPT-induced fluorescence in the enterochromaffin-like cells (Håkanson, Owman and Sjöberg, 1967). 4) Microspectrofluorometric analysis of the OPT-induced fluorophore in the enterochromaffin-like cells showed a good agreement with the properties of the OPT-histamine fluorophore in models (Håkanson *et al.*, 1970a). 5) Reserpine, which reduces the level of gastric histamine by about 50 per cent (Kim and Shorr, 1963; Kowalewski, 1965; Håkanson, Lindstrand, Nordgren and Owman, 1970b), was recently found to abolish the OPT-induced fluorescence in approximately 50 per cent of the enterochromaffin-like cells (Håkanson *et al.*, 1970b).

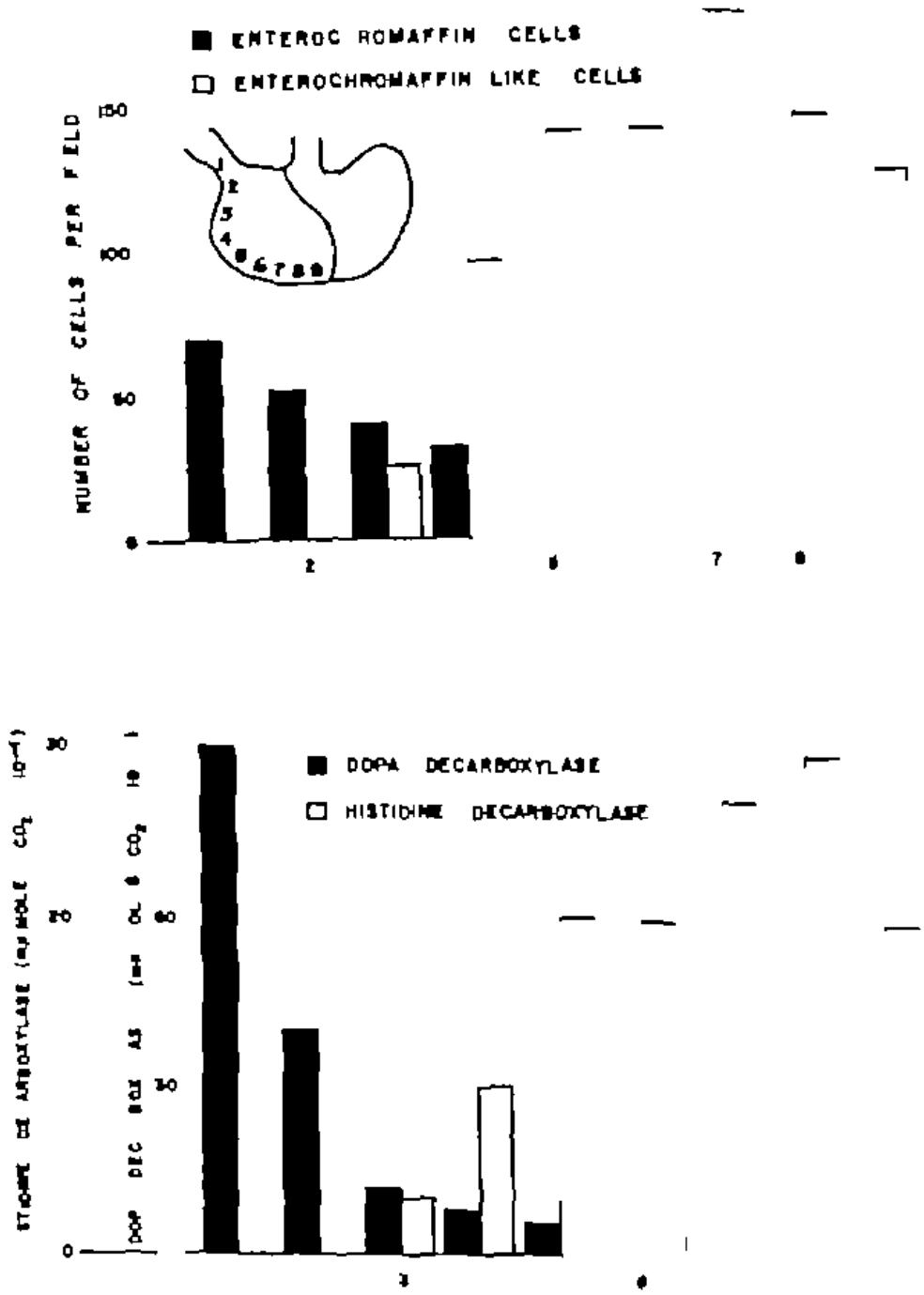
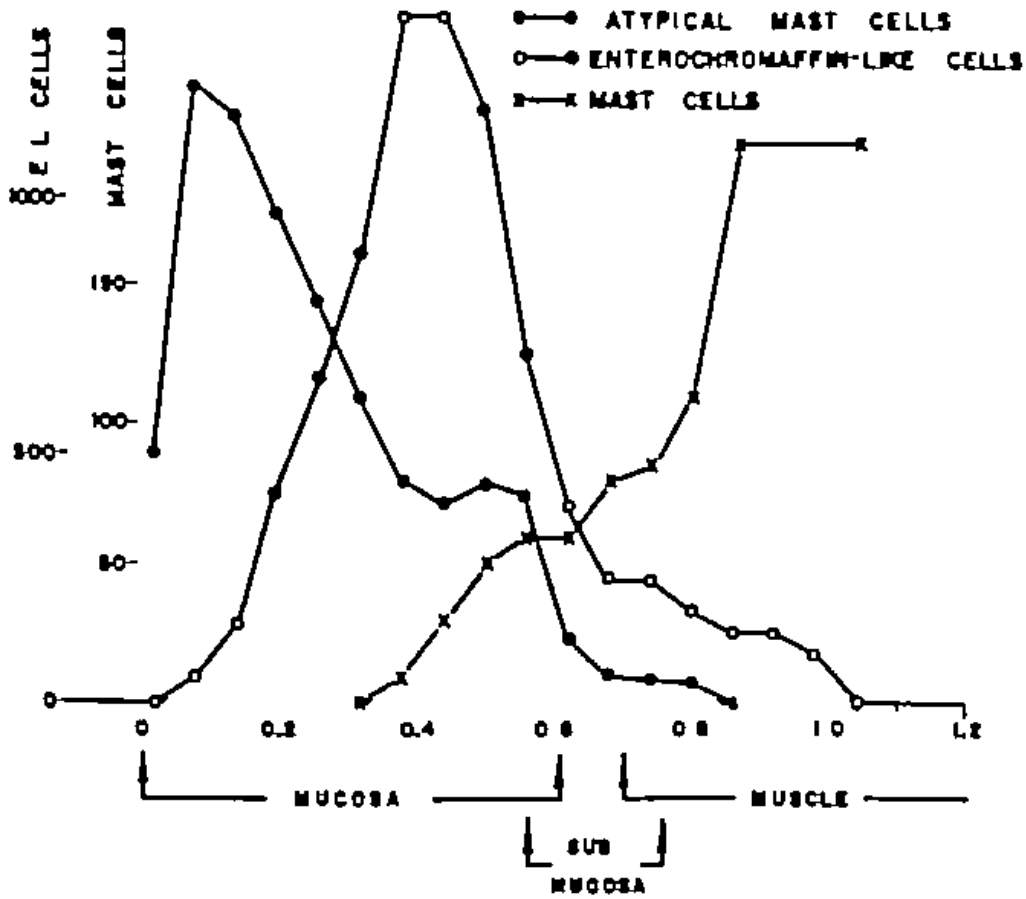


Fig. 11. Regional distribution of enterochromaffin and enterochromaffin-like cells in the rat stomach compared with the distribution of histidine decarboxylase and DOPA decarboxylase. Enterochromaffin cells were recognized by their yellow formaldehyde-induced fluorescence, enterochromaffin-like cells by their green fluorescence 1— hr after intraperitoneal injection of L-DOPA (100—200 mg/kg). The cells were counted in transverse sections at 160 × magnification. Enzyme assays were performed on small pieces of tissue (10—20 mg). (For details see Aures Håkanson and Schauer 1968)

Histamine formation in mammalian tissues may be catalyzed by at least two enzymes—one is specific and referred to as histidine decarboxylase (EC 4.1.1.22), the other one is non-specific and presumably identical with DOPA



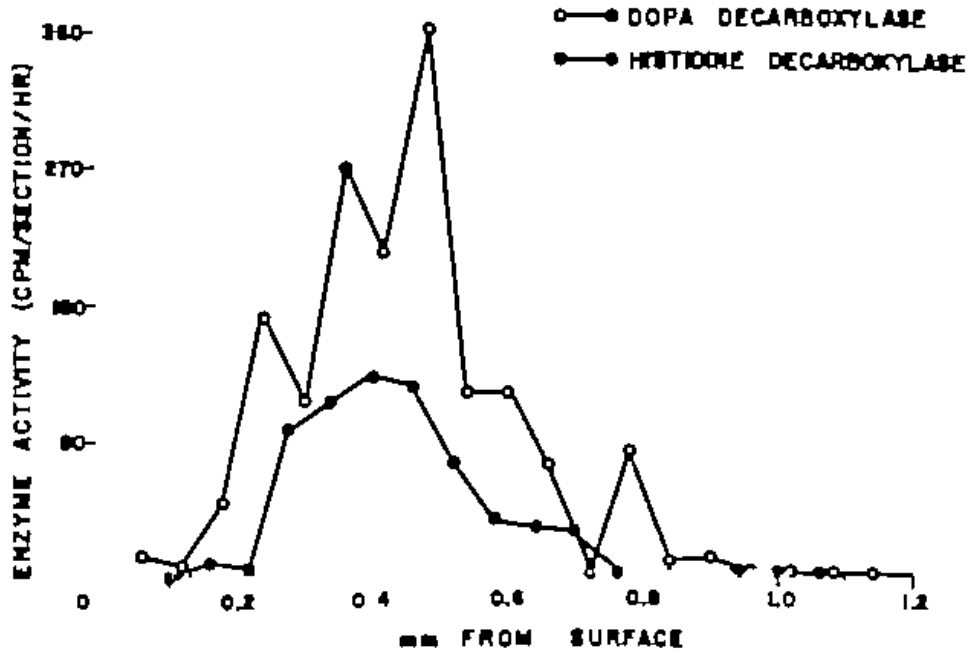


Fig. 12. The topographical distribution of histidin decarboxylase and DOPA decarboxy lase in the oxyntic gland area of the rat stomach. The localization of both enzymes agrees with that of the enterochromaffin-like cell system. There is no correlation between the enzymes and gastric mast cells. (For details see Aures, Håkanson and Schauer, 1968) Some mixing of cell populations from different levels of the mucosa occurred in individual experiments. Furthermore, th height of the mucosa varied somewhat from one experiment to another It may thus falsely appear as if enterochromaffin-like cells sometimes occurred in the submucosa th enterochromaffin-like cells, however were strictly confined to the lower half of the mucosa with the greatest number in the depth of the glands.

decarboxylase (EC 4 1 1 26) The classification of the gastric histamine-forming enzyme was unknown (Schayer, 1956 1957 Lovenberg, Weissbach and Udenfriend 1961) until the enzyme of rat and mouse stomach was identified as "specific" histidine decarboxylase by kinetic analysis (Håkanson and Owman 1966b Håkanson 1967a Aures Håkanson and Schayer, 1968)

In the rat, the system of enterochromaffin-like cells is known to contain the major portion of gastric histamine (Håkanson and Owman 1967 Håkanson Owman and Sjöberg 1967 Thunberg, 1967) and presumably also histidine decarboxylase (Thunberg 1967 Aures Håkanson and Schauer, 1968) This hypothesis was supported by the investigation illustrated in Fig 11 Samples were taken at frequent intervals along the major curvature of the stomach wall and assayed for histidine decarboxylase and DOPA decarboxylase. In parallel histochemical studies enterochromaffin and enterochromaffin-like cells were counted in sections from the same areas. There appeared to be a very close relationship between histidine decarboxylase and the enterochromaffin-like cells. The distribution of DOPA decarboxylase was well correlated to that of the enterochromaffin cells (Aures Håkanson and Schauer, 1968 see also Håkanson *et al.*, 1970d) There is no doubt, however that DOPA decarboxylase also occurs in the enterochromaffin-like cells (see Håkanson Lilja and Owman 1967) Although the enterochromaffin cells appear considerably richer in DOPA decarboxylase, whether viewed as a system or in terms of individual cells (Aures Håkanson and Schauer, 1968) Further support for the assumption that in the rat both histidine decarboxylase and DOPA decarboxylase occur in the system of enterochromaffin-like cells was obtained from experiments (Aures Håkanson and Schauer, 1968) in which pieces of the stomach wall were sectioned parallel to the mucosal surface (Fig 12) (see also Thunberg 1967) In mucosa taken from the oxyntic gland area, the topographical distribution profile of histidine decarboxylase and DOPA decarboxylase was similar to that of the enterochromaffin-like cell system, which was found exclusively in the basal layer of the mucosa. In the pyloric gland area, which was poor in histidine decarboxylase, the topographical distribution of DOPA decarboxylase and enterochromaffin cells was very similar (Fig 13)

Also the gastric mucosa of the dog is rich in histamine (see Easterlin and Kahlson 1944 Douglas Feldberg Paton and Schachter, 1951) Fluorescence microscopic analysis revealed that in this species, however gastric histamine is located exclusively in mast cells of a peculiar atypical variety (Aures *et al.*, 1968) characterized by a marked resistance to the histamine-releasing action of Compound 48/80 (see Enerbäck, 1966) After treatment with DOPA, enterochromaffin-like cells could be demonstrated in the pyloric gland area and in the adjacent portion of the oxyntic gland area (Fig 14) Under these conditions, the enterochromaffin-like cells exhibited a green formaldehyde-induced fluorescence. The pyloric region was also rich in the usual type of 5-HT-containing enterochro-

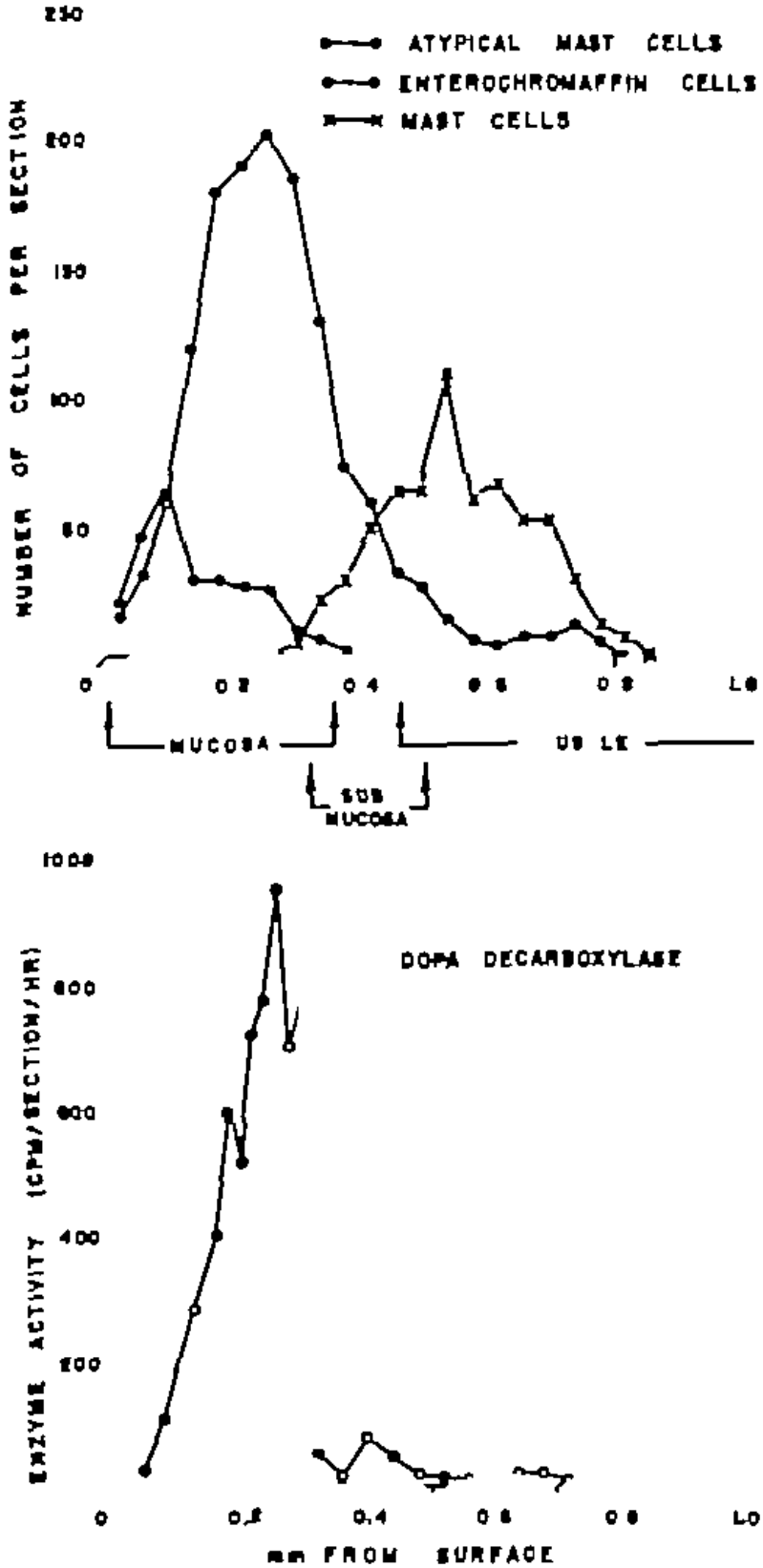


Fig. 13. The topographical distribution of DOPA decarboxylase in the pyloric gland area of the rat stomach (under the experimental conditions used histidine decarboxylase is non-measurable in this region). The enzyme activity is well correlated with the distribution of the enterochromaffin cells. (For details see Auren, Håkanson and Schaner 1968)

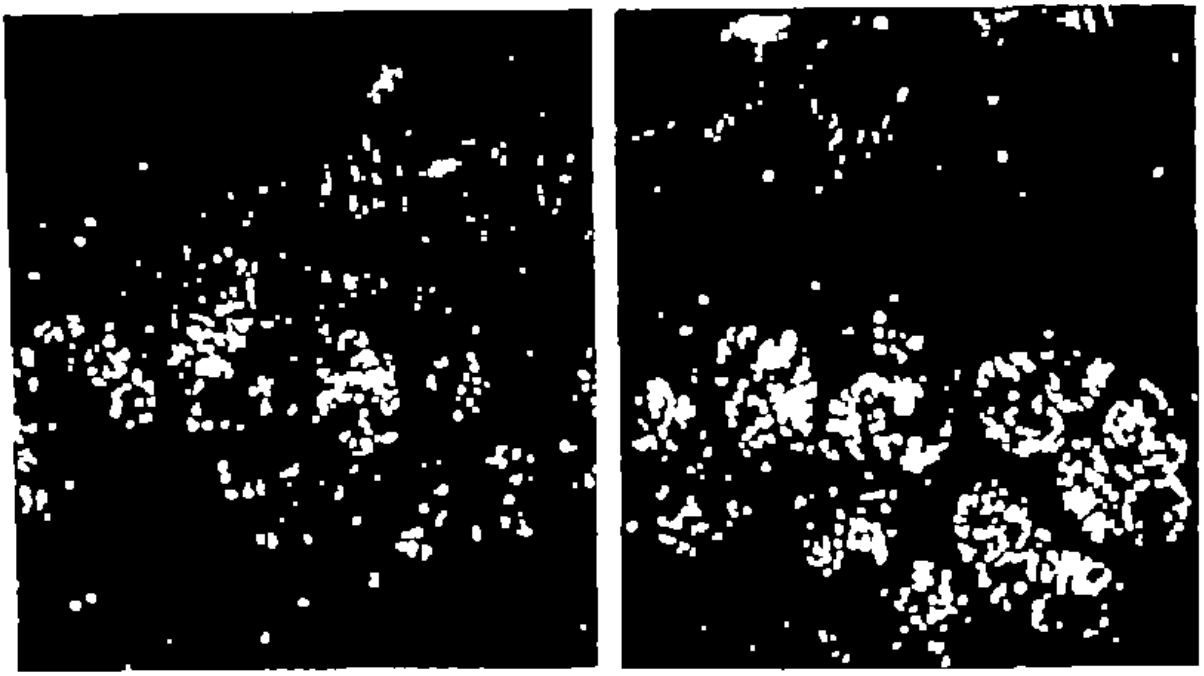


Fig. 14 Fluorescence photomicrographs of dog stomach wall. Transversally sectioned antrum region, 100 x. Formaldehyd reaction. (Left) Untreated animal: large number of intensely yellow fluorescent 5-hydroxytryptamine-containing cells in the middle and basal portions of the mucosa. Weakly fluorescent mast cells higher up in th lamina propia (Right) Specimen incubated with L-DOPA (20 μg/ml) in Tyrodes solution In addition to the yellow-fluorescent enterochromaffin cells another system of morphologically similar cells is now visible, particularly basally in the mucosa, with an intense green fluorescence.

maffin cells. These cells retained their yellow fluorescence also after treatment with DOPA, which thus permitted the simultaneous and selective detection of both enterochromaffin and enterochromaffin like cells, also when they occurred in the same region of the stomach as was the case in the dog Gastric mucosa of the dog was found to be rich in DOPA decarboxylase but the histidine decarboxylase activity was non-measurable (Aures *et al.* 1968 Aures Davidson and Håkanson 1969 Håkanson *et al* 1970d) thus markedly contrasts with the high level of gastric histamine. The origin of gastric histamine in this species is unknown. An active uptake of preformed, circulating histamine rather than a local biosynthesis is a distinct possibility (see Waton 1963 Dunpan and Waton 1969)

Also in the human stomach, gastric histamine was located exclusively in mast cells (Håkanson Lilja and Owman 1969) Both enterochromaffin and enterochromaffin-like cells were recognized in the mucosa, the enterochromaffin cells by their yellow 5-HT fluorescence, the enterochromaffin-like cells by their green fluorescence upon L-DOPA administration. Both cell types were found predominantly in the pyloric gland area and in the adjacent region of the oxyntic gland area. Histidine decarboxylase did not occur in measurable amounts in any region of the gastric mucosa. These results have recently been confirmed in experiments where the patients have been pretreated with pen-

tagastrin in order to stimulate the enzyme activity (see sections 3 10 and 5.5 in spite of this no histidine decarboxylase activity could be measured (HÅKANSON and LIEDBERG 1970b)

In a systematic study of the enterochromaffin and enterochromaffin-like cells in the gastric mucosa of a number of mammals it could be established that the rat and mouse were the only species in which histamine had a non-mast-cell localization, being present in enterochromaffin like cells (HÅKANSON *et al.*, 1970d) In the other species studied gastric histamine was found in mast cells exclusively although enterochromaffin like cells could be demonstrated in all species. The gastric DOPA decarboxylase activity was high in all species—particularly in the guinea pig—but histidine decarboxylase was measurable only in the stomach of the mouse, rat and hamster (AUREN DAVIDSON and HÅKANSON 1969) This was the case also when the enzyme assay was performed on specimens taken from pentagastrin-injected or newly fed animals.

As discussed above, there is much evidence that both the enterochromaffin and the enterochromaffin-like cells contain L-DOPA decarboxylase which is a non-specific enzyme and has been referred to as aromatic L amino acid decarboxylase (LOVENBERG WEISSBACH and UDENFRIEND 1962) It appears that the non-specific DOPA decarboxylase, although indispensable for the formation of 5-HT and catecholamines, is of no consequence in determining the identity of the amine occurring in the enterochromaffin and enterochromaffin-like cells. Since the capacity to decarboxylate aromatic amino acids and to store the resulting amine is common to both cell types, it is conceivable that the 5-HT content of the yellow fluorescent enterochromaffin cells is due to the presence of tryptophan-5-hydroxylase in these cells (see COOPER and MELCER, 1961) although it has not been possible to confirm this chemically (RENSON WEISSBACH and UDENFRIEND 1962 HÅKANSON and HOFFMAN 1967) A small number of dopamine-containing enterochromaffin cells, exhibiting a green formaldehyde-induced fluorescence, were regularly found scattered in the mucosa of the stomach, sometimes also in the stomach of the rabbit (HÅKANSON *et al.* 1970d) In analogy with the above reasoning, it may be suggested that the dopamine content in these cells reflects the presence of tyrosine hydroxylase.

The histamine demonstrated in the enterochromaffin-like cells of the rat and mouse stomach is probably produced within these cells for the following reasons (1) Treatment with large doses of histamine does not affect the gastric histamine content in general, nor the histamine content of the enterochromaffin like cells (HÅKANSON unpublished observation) (2) Injection of large amounts of L histidine does not affect the gastric histamine level. If ^{14}C-labelled or ^{3}H-labelled histidine is used, however radioactive histamine can be demonstrated in the oxyntic gland area of the stomach (SCHAYER and IVY 1958 AUREN HÅKANSON and SCHAYER, 1968) This supports the concept of a local histamine biosynthesis in the rat stomach (see also HIGH, SHEPHERD

and Woodcock, 1965 Shepherd and Woodcock, 1968) (3) The regional and topographical distribution of histidine decarboxylase agrees very well with that of the histamine-containing enterochromaffin-like cells (Thunberg 1967 Aures Håkanson and Schauer, 1968) (4) The development of gastric histidine decarboxylase activity in the new-born rat precedes the appearance of gastric histamine and of OPT reactive enterochromaffin-like cells (Håkanson Owman and Sjöberg 1967 Aures and Håkanson 1968)

The specific histidine decarboxylase is probably more important than the non-specific aromatic amino acid decarboxylase for the formation of histamine *in vivo* (Ganrot Rosenqren and Rosenqren 1961 Weissbach, Lovenberg and Udenfriend 1961 Levine, Sato and Sjoerdsma 1965) Since histidine decarboxylase is rate limiting, the enzyme activity should have a very direct bearing on the actual rate of histamine formation in the enterochromaffin-like cells of the rat stomach

In conclusion it may be pointed out that the amine mechanisms demonstrated in those types of the enterochromaffin like cells, which are devoid of fluorogenic amines, may perhaps reflect the formation and intracellular storage of some as yet unidentified amines, which cannot be detected by the conventional histochemical fluorescence methods, *i.e* the formaldehyde and OPT techniques (for a discussion see Aures Håkanson and Owman 1969 Håkanson *et al* 1970d)

3 7 *Prenatal and postnatal development of gastrointestinal enterochromaffin and enterochromaffin-like cells*

The embryological derivation of these two cell types is unknown. It has been suggested that they all develop from the primary gut. Alternatively the neural crest has been proposed as the original source of these cells (Pearse,1969b) Kull (1925) reported that in the intestines of the chick embryo, enterochromaffin cells appeared on the 15th day of embryonic life. According to Cordier (1926) they could be detected at between the 16th and 18th day However Simard and van Campenhout (1932) and Ghidini (1940) claimed that the first argentaffin cells were found in the appendix of the chick as early as on the 10—11th day of embryonic life. Monesi (1960a and b) on the other hand, reported that argyrophil cells appear at the 14-day stage and argentaffin, enterochromaffin cells approx. two days later At the 14-day stage the cells would perhaps more correctly be referred to as pre-argentaffin or pre-enterochromaffin, since at least part of the cell population later develops into "true" enterochromaffin (argentaffin) cells. Dawson and Mover (1948) observed an even earlier appearance (8th day of incubation) of argyrophil cells in the glandular portion of the chick embryo stomach. Enemar, Falck and Håkanson (1965) described the appearance of duodenal enterochromaffin cells with a yellow formaldehyde-induced fluorescence in the 14-day embryo At this stage only the basal portion of the cells contained fluorescent material These cells were numerous

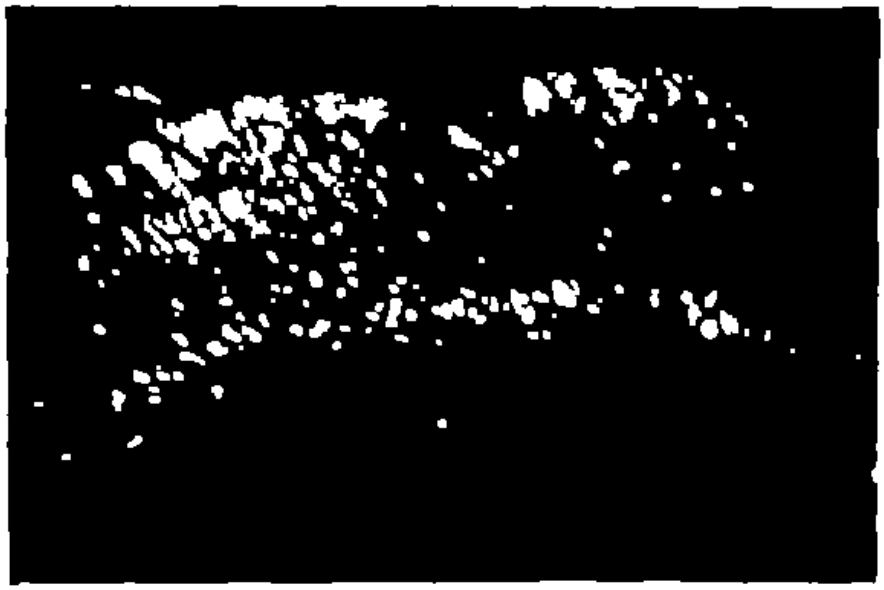


Fig. 15. Fluorescence photomicrograph of rat stomach wall, oxyntic gland area. Transverse section, 100 x. Young rat, 7 days old, injected with L-DOPA. Green-fluorescent enterochromaffin-like cells characteristically concentrated to the most basal part of the mucosa. In addition their number is considerably lower than in adult rats. Fluorescent mast cells are seen scattered in the rest of the mucosa, the submucosa, and the muscular coat.

in the 16-day embryo and at this stage they were richly supplied with fluorescent material throughout the cytoplasm. Using the same method, PENTTILA and MUSTAKALLIO (1968) and PENTTILÄ (1968) observed duodenal enterochromaffin cells in the 14-day-old chick embryo. In their studies, the identification of the cells as enterochromaffin was confirmed by argentaffin staining of adjacent sections. At this early stage the yellow formaldehyde-induced fluorescence of the enterochromaffin cells was very weak.

For human embryos, the data of CORONIA (1926) PATZELT (1931) and CLARA (1934) place the time of appearance of the enterochromaffin cells at the 12th week. CLARA (1934) reported that the appearance of the goblet cell (9th week) preceeds that of the enterochromaffin cell while the appearance of Paneth's cell follows it (17th week). FALCK, HÄKANSON, OWMAN and SJÖBERG (1967) described enterochromaffin and enterochromaffin-like cells in the gastric and intestinal mucosa (duodenum and ileum) of human embryos at between the 12th and 13th week (the embryos studied measured from 11 cm total length and more).

In many other mammals, the enterochromaffin cells have been found to appear at a corresponding embryonic age, *e.g.* in the rat on the 15—17th day (SHARPLES 1945) and in some other mammals towards the end of the gestation. The first yellow fluorescent enterochromaffin cell in the rat pyloric gland area, duodenum and ileum could be detected on the 18th day (HÄKANSON, OWMAN and SJÖBERG 1969). Gastric enterochromaffin-like cells were first observed at the time of birth. At this stage, however their number was low. Subsequently their number increased progressively (Fig. 15) and reached adult

levels at about 14 days after birth. The enterochromaffin like cells did not store histochemically detectable amounts of histamine until 8—9 days after birth (HÅKANSON OWMAN and SJÖBERG 1967)

Gastric DOPA decarboxylase could be demonstrated very early in the foetal development of both rat and man (FALCK *et al.* 1967 AURELL and HÅKANSON 1968 HÅKANSON OWMAN and SJÖBERG 1969) This seems to agree fairly well with the early appearance of the enterochromaffin cells.

Gastric histidine decarboxylase activity which was very high during certain stages of foetal development, was non measurable at time of birth the activity increased slowly but progressively during the subsequent development to reach adult or near-adult levels 21 days after birth (HÅKANSON OWMAN and SJÖBERG 1967 AURELL and HÅKANSON 1968) In the adult rat, the activity of gastric histidine decarboxylase appears to be regulated by an antral factor presumably gastrin (see AURELL HÅKANSON and SCHAUER, 1968 JOHNSON JONES, AURELL and HÅKANSON 1969 AURELL JOHNSON WAY and CLARK, 1969 HÅKANSON and LIEDBERG 1970a) so that fasting (diminished gastrin release) reduces the enzyme activity and feeding (stimulated gastrin release) or injection of gastrin causes an increase in the enzyme activity This control mechanism is not established in the young rat until 14 days of age Before this stage, neither fasting nor feeding affects the enzyme activity (AURELL and HÅKANSON 1968)

From the results of MONESI (1960a and b) it appears that initially the enterochromaffin cell is devoid of 5 HT and hence can be detected by agryrophil stains only—not by the argentaffin reaction. This pre-enterochromaffin cell then matures to its final enterochromaffin state, with a full store of 5 HT fairly early in the prenatal development. The histamine-containing (enterochromaffin-like) cell of the rat gastric mucosa which presumably remains argyrophil and non argentaffin throughout its life cycle (*i.e.* it remains enterochromaffin-like) seems to appear much later in development and to mature much slower than the enterochromaffin cell (HÅKANSON OWMAN and SJÖBERG 1967 AURELL and HÅKANSON 1968 HÅKANSON OWMAN and SJÖBERG 1969) For a diagrammatic presentation of its development see Fig 16.

3.8 *Ultrastructure of enterochromaffin and enterochromaffin-like cells*

The first electron microscopic description of a gastrointestinal argentaffin cell is that presented by CHRISTIE (1955) Subsequently KUROSUMI SHIBASAKI UCHIDA and TANAKA (1958) BULLION RAMIREZ, LANDER and SCHULTZ (1960) LUSE and LACY (1960) TAYLOR and HAYES (1961) HELANDER (1961 62 64) WETZSTEIN and DOERFLER (1962) WETZSTEIN DOERFLER and SCHWINK (1962) ITO and WINCHESTER (1963) and RATZENHOFER and LEB (1965) reported on the argentaffin and argyrophil cells of the gastrointestinal mucosa. The species studied were the rabbit, the guinea-pig, th mouse and the bat The terms argentaffin (enterochromaffin) and argyrophil seem to have been used arbitrarily by most of these authors usually the hi tochemical criteria necessary

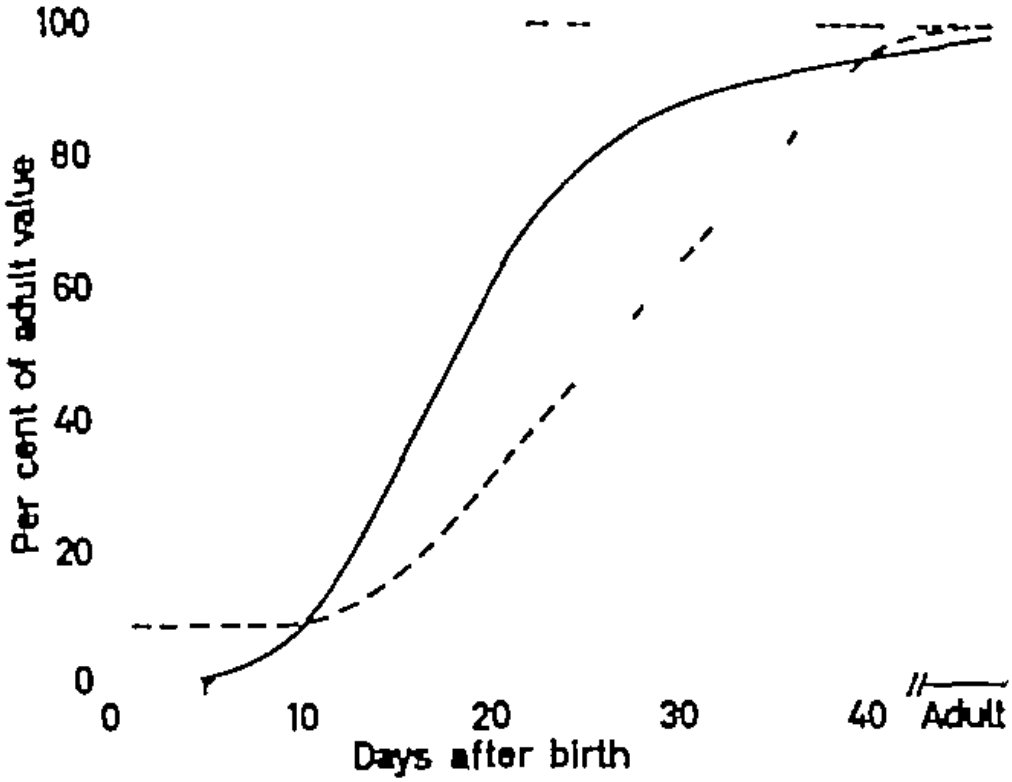


Fig. 16. Development of gastric histamine content (— — —) gastric histidine decarboxylase activity (————————) and of enterochromaffin-like cells ( ) in the new-born and young rat. The data are presented as per cent of adult values. The quantitative development of gastric histamine corresponded very well with the gradual appearance of o-phthaldialdehyde—induced fluorescence in the enterochromaffin-like cells. From results presented by Håkanson, Owman and Sjöberg (1967) Aures and Håkanson (1968) and Håkanson *et al.* (1969)

for distinguishing between argentaffin and purely argyrophil cells have not been applied. Most of these authors, nevertheless, seemed to agree that all these cells are endocrine, on the basis of their apparent lack of contact with the glandular lumen, being situated in close proximity to the basement membrane the absence of any sort of secretory capillary in their cytoplasm and the presence of secretory granules which are the primary ultrastructural characteristic of these cells. By electron microscopy as well as by light microscopy several categories of endocrine-like cells have been observed in the mammalian gastrointestinal mucosa (cf. Capella, Solcia and Vassallo 1969 Forssmann *et al.*, 1969 Pearse, 1969 Vassallo Solcia and Capella, 1969 Håkanson *et al.* 1970d Håkanson Owman and Sporrong 1970) The characteristics of these various types of cells have been extensively studied by Pearse and colleagues (Carvalheira, Welsch and Pearse, 1968 Pearse, 1969b) by Solcia and co-workers (Solcia, Vassallo and Sampietro 1967 Vassallo Solcia and Capella, 1969 Solcia, Vassallo and Capella, 1969 Capella, Solcia and Vassallo 1969) and by Forssman Orci and co-workers (Forssmann Orci, Pictet and Rouiller, 1967 Forssmann Orci and Rouiller, 1968 a and b Forssmann *et al.*, 1969 Orci, Forssmann

Forssmann and Rouiller, 1968a Orci Pictet Forssmann Renold and Rouiller, 1968b) Their observations will be only briefly summarized and discussed here. The various types of endocrine cells can be distinguished on the basis of variations in the size and shape of their secretory granules. Forssmann *et al.* (1969) thus described five different types of endocrine cells in the gastrointestinal mucosa of the rat. Also Vassallo Solcia and Capella (1969) described five types of endocrine cells in the digestive tract of the cat. More recently they reported the presence of six different types of endocrine cells in the bowel of the rabbit (Capella, Solcia and Vassallo 1969) The existence of several ultrastructurally different populations of endocrine cells in the gastrointestinal mucosa have now been amply confirmed in a number of laboratories including our own (Håkanson Owman and Sporrong 1970) It is still obscure, however whether some of the morphological distinctions described are representative of distinct cell types or merely of different functional stages of one and the same cell (see also Forssmann and Orci 1969) One type is reminiscent of the pancreatic A-cells in that they contain uniformly rounded granules varying in size (500—700 mμ) and electron density with a narrow pale area characteristically discernible between the granular membrane and its enclosed content. Another cell type contains granules (150—250 mμ) similar to those of the pancreatic D-cells. A third type contains granules of medium size (300—500 mμ) and of varying density A fourth type has granules similar to those of the chromaffin cells of the sympathetic chain. The fifth type, finally is the predominant endocrine cell type in the bowel mucosa. It is characterized by polymorphous granules of high electron density Generally the clear cytoplasm of all these endocrine cell types, provided with small secretory granules of varying density upon osmium fixation, sharply contrasts with the cytoplasm of the mucoid cells and of the parietal and peptic cells. Usually in the electron microscope these cells, which have a very characteristic proximity to the basement membrane, appear to be without contact with the glandular lumen. Sometimes, however the endocrine cell can be seen to reach the luminal surface with its upper narrow end (Luse and Lacy 1961 Ito and Winchester, 1963 Solcia, Vassallo and Capella, 1969 Forssmann and Orci, 1969)

It should be noted that by electron microscopy of osmium-fied material it is difficult to identify the different endocrine cells with the various types of enterochromaffin and enterochromaffin-like cells detectable by fluorescence microscopy or light microscopy One type of these endocrine cells has been claimed to be identical with the enterochromaffin cells because of its frequency and its regional and topographical distribution in the gastrointestinal tract. As yet, the electron-microscopic identity of the histamine-containing endocrine cell in the oxyntic gland area of the rat is unknown on the basis of combined fluorescence histochemical and electron microscopic findings it can be assumed that most endocrine cells in this region store histamine (Håkanson and Owman, 1967 1969 Håkanson Owman and Sporrong 1970)

The ultrastructure of the enterochromaffin and enterochromaffin-like cells is very similar to that of certain other endocrine cell systems. Thus, the granules of the gastrointestinal endocrine cells are similar to those described in the adrenal medulla (Sjostrand and Wetzstein 1956 Coupland 1965) in catecholamine-containing cells in the heart of cyclostomes (Bloom, Östlund, von Euler, Lishajko, Ritzén and Adams Ray 1961) in thyroid parafollicular cells (C-cells) (Azzali 1962 Luciano and Reale, 1964 Pearse, 1966a and b) in the carotid body (Lever, Lewis and Boyd 1959 Ross 1959 de Kock and Dunn 1964) in the endocrine pancreas (Munger, Carabia and Lacy 1963) and the pars intermedia of the pituitary (Kobayashi, 1965) A common characteristic of all these cells is that they have the capacity to produce and store polypeptide hormones.

3.9 *Possible physiological role of gastric enterochromaffin and enterochromaffin-like cells*

The system of argyrophil (enterochromaffin and enterochromaffin-like) cells constitute a quantitatively significant portion of the gastric mucosal cell population in all species studied. Some of these cells contain 5-HT (or dopamine) and can, as previously discussed (sections 3 1 and 3.2.) be expected to give a positive chromaffin or argentaffin reaction. The remainder of the argyrophil cells (enterochromaffin-like cells) are devoid of amines detectable by the Falck Hillarp formaldehyde technique, although they are capable of decarboxylating various exogenous aromatic amino acids and of storing the resulting amine intracellularly (Håkanson and Owman 1966b Håkanson Lilja and Owman 1967 Aures Håkanson and Schauer, 1968 Håkanson and Owman 1968 Håkanson *et al.*, 1970d) In the rat and mouse gastric enterochromaffin-like cells are rich in histamine and histidine decarboxylase (Håkanson and Owman 1967 Håkanson Owman and Sjöberg 1967 Thunberg 1967 Aures, Håkanson and Schauer, 1968) In all species enterochromaffin and enterochromaffin-like cells have the same general appearance and are characteristically interpersed among the other glandular cells in the mucosal epitheilium. It may well be that the classification of these cells into enterochromaffin and enterochromaffin-like, or into argentaffin and argyrophil is unjustified since the terms imply a distinction between these two cell types which may be functionally irrelevant. The endocrine pancreas may offer a useful analogy In some species, pancreatic islet cells, which in many respects resemble the gastric enterochromaffin and enterochromaffin-like cells, have been shown to contain 5-HT or dopamine, or varying proportions of both (Falck and Hellman 1963 1964 Crobeil, Falck and Hellman 1964 Crobeil, 1968) whereas in some other species the islet cells are devoid of histochemically detectable amines. Furthermore, pronounced variations in the concentration of the pancreatic amines with age and pigmentation have been documented (Crobeil, 1967a and b Crobeil,

1968) There is no evidence, however that the varying amine content of the islet cells from one species to another or according to age and pigmentation in one and the same species, causes the islet cells to differ in their functional characteristics.

It was very early suggested (see Feyrter, 1938) that the argentaffin and non-argentaffin, argyrophil cells of the digestive tract might have an endocrine function. This assumption was based on their apparent lack of contact with the glandular lumen and their resemblance to other cells with a well-known endocrine function (such as the pancreatic islet cells) The concept of an endocrine function of the endocrine like cells of the gastrointestinal mucosa has since received ultrastructural confirmation (Kurosumi *et al.* 1958 Helander, 1961 Ratzenhofer and Lee, 1965 Solcia and Sampietro 1965 Solcia *et al* 1967 Orci *et al.* 1968a and b Carvalheira *et al* 1969 Forssmann *et al.* 1969 Solcia Vassallo and Capella, 1969 Vassallo *et al.*, 1969) The endocrine function of these cells has not yet been defined. It has been suggested that they are active in the formation of gastrointestinal polypeptide hormones. Waddell, Coppinger and Loughry (1968) have referred to the portion of the gut immediately above and below the ampulla Vateri, including antrum, duodenum and pancreas, as the abdominal endocrine organ. The hormones of this area are concerned with the regulation of digestion, absorption, and utilization of food. All of the endocrine cells of this region are believed to arise from a limited area of the primitive foregut. The concept of an "enteroinsular axis" whereby insulin-releasing agents from the gut control the insulin release, emphasize the close functional relationship of this particular region (Unger, Ketterer, Dupré and Eisentraut 1967 Pfeiffer and Raptis 1968 Unger, Ohneda, Valverde, Eisentraut and Exton 1968 Singleton 1969) The chemical similarity between many of the polypeptide hormones of this region (see Jorpes 1968) also supports the concept of a hormone-producing locus, comprising several endocrine glands, whose functions are closely coordinated, and which perhaps are developed from the same primordial origin. Among the well-known gastrointestinal polypeptide hormones of this area are gastrin, secretin and pancreozymin-cholecystokinin. Other putative gastrointestinal hormones and related substances include enterogastrone, duocrinin, villikinin, enterocrinin, antral chalone, urogastrone, gastrone enteroglucagon, and possibly other insulin releasing hormones (see review by Grossman 1968)

Using selective histochemical and histological methods in combination with electron microscopic studies, it has been possible to distinguish between several types of endocrine cells in gastric mucosa (Carvalheira *et al.*, 1968 Cappella *et al* 1969 Forssmann *et al* 1969 Vassallo *et al.*, 1969 Håkanson Owman and Sporrong 1970) It has been suggested that one of these of cells contain gastrin (Solcia *et al.*, 1969) and another enteroglucagon (Orci *et al.*, 1968b Forssmann *et al* 1969) It is interesting, in view of the finding of high concentrations of "glucagon-like immunoreactivity" in the

gastric mucosa (Unger *et al.*, 1968) that cells which are histochemically and ultrastructurally similar to the pancreatic A-cells are found in the gastric mucosa (Munger, Caramia and Lacy, 1965) In addition to insulin -secreting B-cells and glucagon-secreting A-cells (A_2-cells) other endocrine cells has been clearly identified in the pancreatic islets (Meyer and Bencosme, 1965 Munger, Caramia and Lacy 1965) One of these cells fails to give the staining reactions characteristic of either B- or A_2-cells. It has been identified (Munger, Caramia and Lacy 1965) as the D-cell first described by Bloom (1931) It has also been designated A (Peterson 1966) The ultrastructure of the D-cell is clearly different from that of the B- and A_2-cells (Munger, Caramia and Lacy 1965) The secretory product of the D-cell has not been identified but its ultrastructure indicates an endocrine function. Histochemically identical cells have been found in the antro-pyloric area (Solcia and Sampietro 1965) Several authors have suggested that D-cells in both pancreatic islets and gastric mucosa secrete gastrin (see Solcia and Sampietro 1965) Support for this view is furnished by studies on Zollinger Ellison tumours. This tumour which secretes gastrin, has been found to consist wholly or in part of D-cells (Greider and Elliot 1964 Cavallero Solcia and Sampietro 1967 Toner, 1967) All suggestions offerred concerning the functions of the various gastric endocrine cells are thus based on their resemblance to those pancreatic islet cells, which are believed to contain glucagon (A_2-cells) or gastrin (D-cells, Zollinger Ellison tumours) These views are also based on the agreement between the localization of the gastric cells, supposed to store the hormones, and the general distribution of gastrin and glucagon in the gastrointestinal tract (Solcia and Sampietro 1965 Solcia *et al.*, 1969) It must be emphasized that most of this is mere conjecture and that differences in opinion exist. For instance, Forssmann and co-workers believe the F-cell (see Munger, Caramia and Lacy 1965) to store gastrin. They recently showed that certain gastric endocrine cells, similar to the pancreatic F-cell, were degranulated as a result of feeding and of other gastrin-releasing stimuli (Forssmann and Orci, 1969) Using an immunofluorescent technique, McGuigan (1968) recently demonstrated a gastrin-containing cell in the pyloric gland area of man and the pig the cell was non-argentaffin and characterized as enterochromaffin-like. No attempt has yet been made to correlate his findings with electron microscopy

Also the gastric antipernicious principle (the intrinsic factor IF) has been brought into the discussion concerning the functional significance of the enterochromaffin cell (Erös 1933 Jacobson 1939 Jacobson and Simpson 1946 Jacobson 1948) This hypothesis fell into disrepute because of a poor correlation between the distribution of these cells and of IF in many species (Gillman 1942 Landboe-Christensen 1950 1952) In the rat, the chief cell has been proposed to be the storage site for IF (Keuning Arends, Mandema and Nieweg 1959 Boass and Wilson 1964) It is quite evident from the literature that in several other species the chief cell is not the cellular storage

site for IF and both the oxyntic cell and the pyloric gland cell have been advocated (Hollemaekers, Abels, Wachters Arends and Nieweg 1964 1966 Hollemaekers, 1965) Current opinion appears to locate IF to different cells in different species (Chanarin 1968) From pure speculation it appeared probable that a compound with such a specific function as IF should be produced and stored in the same type of cell in all species. A series of investigations was initiated to search for a common cellular storage site for IF

It was observed that in the rat the regional and topographical distribution (Fig 17 and 18) of vitamin B_{12}-binding proteins, associated with IF activity coincided remarkably well with the distribution of the histamine-containing enterochromaffin-like cells (Håkanson Lindstrand Norddoren and Owman 1969) It should be noted, however that the distribution of the chief cells is very similar to that of the enterochromaffin-like cells and that consequently in the rat, studies on distribution and topography are insufficient in excluding either of the cell systems. However the hypothesis that IF is contained in the enterochromaffin-like cells was further supported by evidence of a correlation between the maturation of this cell system and the appearance of gastrin vitamin B binders in the neonatal rat (Håkanson *et al.* 1969)

The capacity to bind vitamin B is a necessary prerequisite for IF activity but gastric mucosa has been shown to contain vitamin B_{12}-binding proteins other than IF *i.e.* proteins that bind but do not facilitate the intestinal absorption of vitamin B_{12} (Grasbeck, Simons and Sinkkonen 1962 1965 Glass, Uchino and Schwartz, 1962 Glass 1963 Ellenbogen and Highley 1967a and b Flodh, Berglund and Olén 1968) The vitamin B_{12}-binding capacity is thus not by itself a measure of the IF content the estimation of the IF level must be based on the capacity of gastric vitamin B_{12}-binders to facilitate the intestinal absorption of cyanocobalamin. Evaluation of the IF activity of the vitamin B_{12}-binding proteins demonstrated in the oxyntic gland area of the rat was carried out using partially gastrectomized rats (the oxyntic gland area was removed) Such rats had a markedly diminished capacity to absorb vitamin B_{12}. The absorption of vitamin B_{12} was normalized if vitamin B_{12}-binding proteins from the basal mucosa of the oxyntic gland area were added. Colum chromatographic and electrophoretic analysis of the vitamin B_{12}-binders from the oxyntic gland area revealed only one major protein fraction, presumably containing the IF (Håkanson *et al* 1969) These findings together suggested the possibility that the enterochromaffin-like cells may be the common storage site of IF in all species. Subsequently the regional and topographical distribution of gastric vitamin B_{12}-binding proteins was compared with that of the enterochromaffin and enterochromaffin-like cells in the mouse, hamster guinea pig and rabbit (Håkanson Liedberg and Lindstrand, 1970 see also Fig 19 and 20) In the first two species the regional distribution of the vitamin B_{12}-binders followed that of the enterochromaffin-like cells very closely In these species enterochromaffin cells are almost exclusively found in the pyloric gland area, whereas the enterochromaffin like cells occur mainly in the oxyntic gland

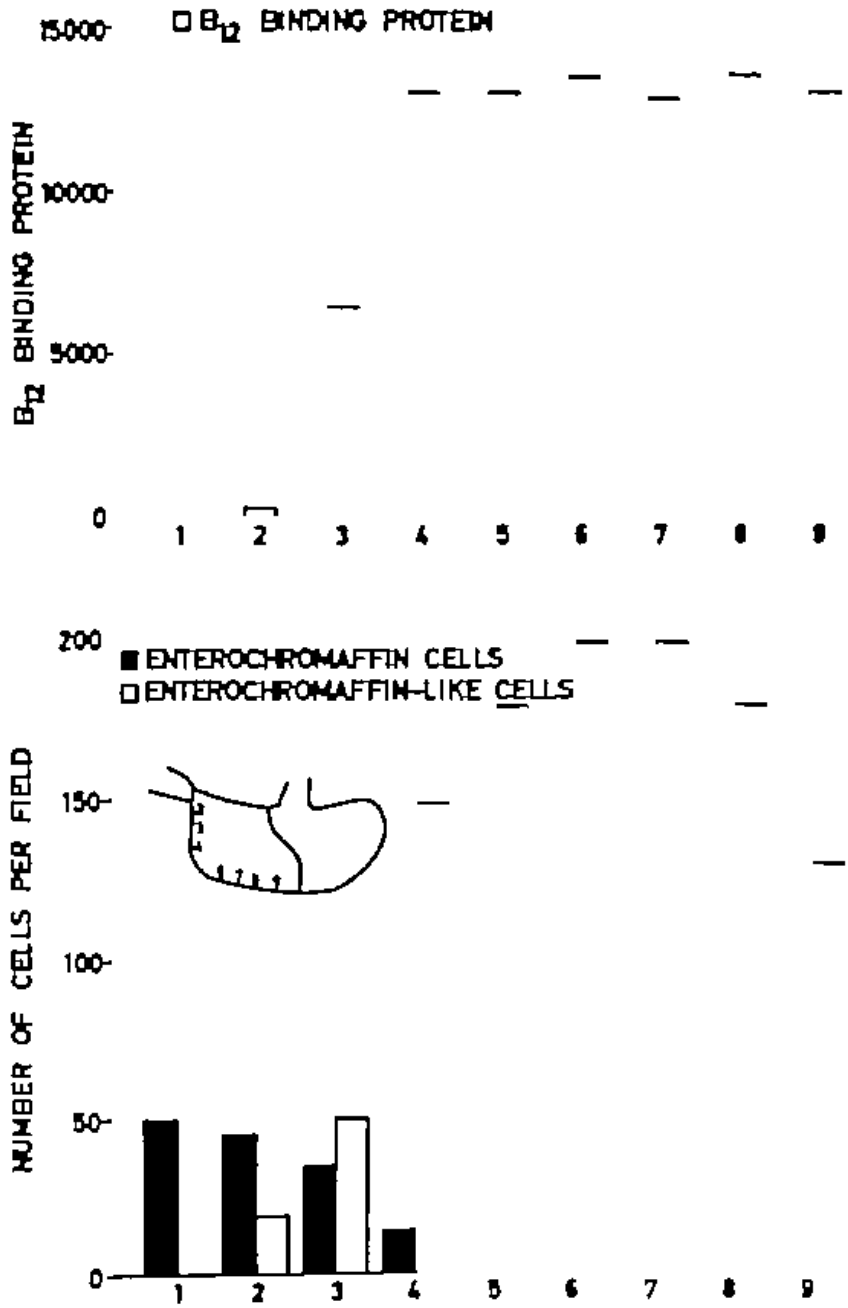


Fig. 17 Regional distribution of vitamin B binding proteins (abov) and of enterochromaffin and enterochromaffin-like cells (below) in the stomach wall of the rat. Vitamin B_{12} binding proteins are expressed as the amount of ^{57}Co-cyanocobalamin (cpm) bound per mg tissue (wet weight) The enterochromaffin-like cells were counted in the fluorescence microscope (6 μ transverse sections. 125 × magnification) after condensation with formaldehyde (L-DOPA-injected rats) or with o-phthaldialdehyde (non-injected rats)

area. Also in the guinea pig, the distribution of the vitamin B_{12}-binding proteins was well correlated with that of the enterochromaffin-like cells, which occurred in the pyloric gland area and in the adjacent portion of the oxyntic gland area. There was no correlation between vitamin B_{12}-binders and chief cells in this

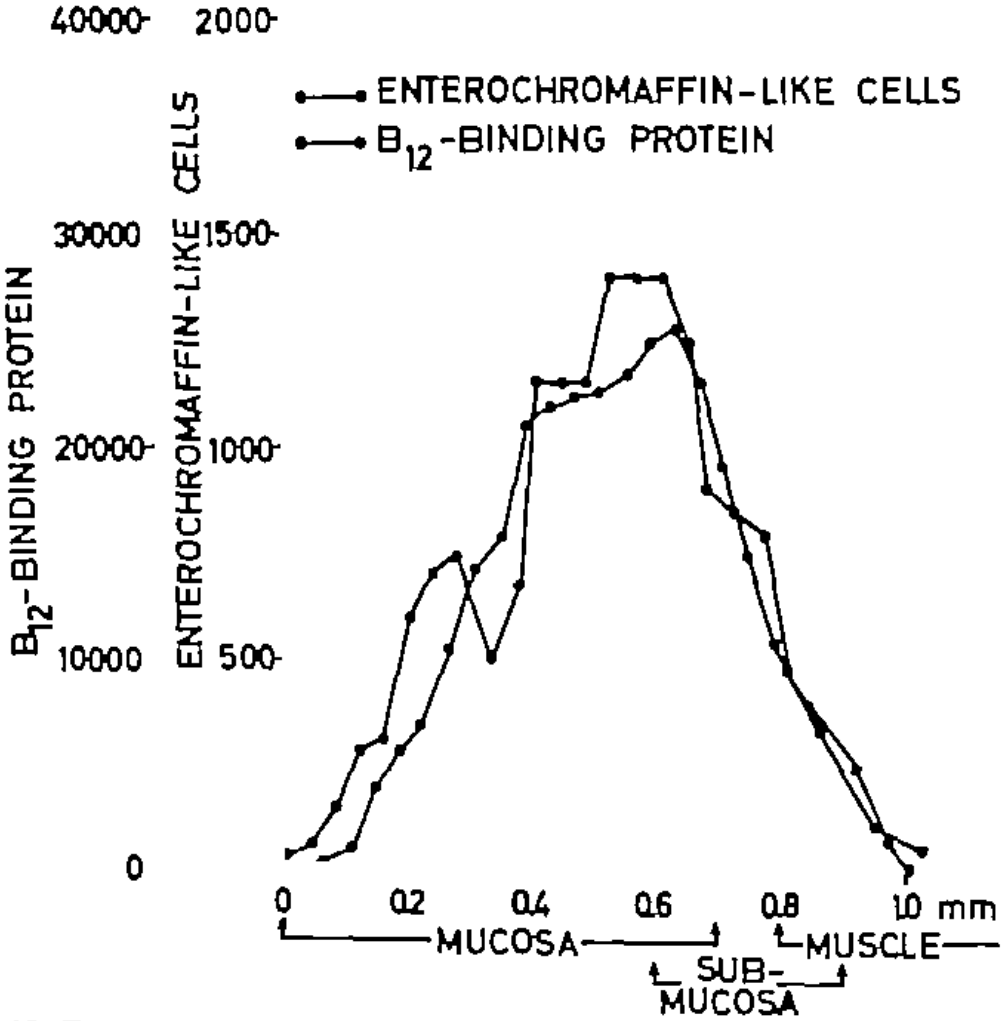


Fig. 18 Topographical distribution of enterochromaffin-like cell and of vitamin B -binding protein in the oxyntic gland area of th rat stomach. The number of cells is given per section (20 μ thick, 3—4 mm square) The amount of vitamin B -binding protein is expressed as cpm per section.

species. The rabbit was exceptional in that the distribution of the vitamin B $_{12}$-binding proteins closely agreed with that of a special type of enterochromaffin cells, which are found exclusively in the oxyntic gland area. It could be shown that in this region the vitamin B_{12}-binding proteins were restricted to the basal layer of the mucosa, a topographical distribution which corresponds closely to that of the enterochromaffin cells. This observation is not necessarily in disagreement with the assumption that IF is contained in one and the same type of cell in all species, but it rather emphasizes the very distinct possibility that the argyrophil cells of gastric mucosa (whether enterochromaffin or enterochromaffin-like) are functionally very closely related.

From these results it is evident that gastric vitamin B $_{12}$-binding proteins are not regularly associated with one particular region of the stomach and that

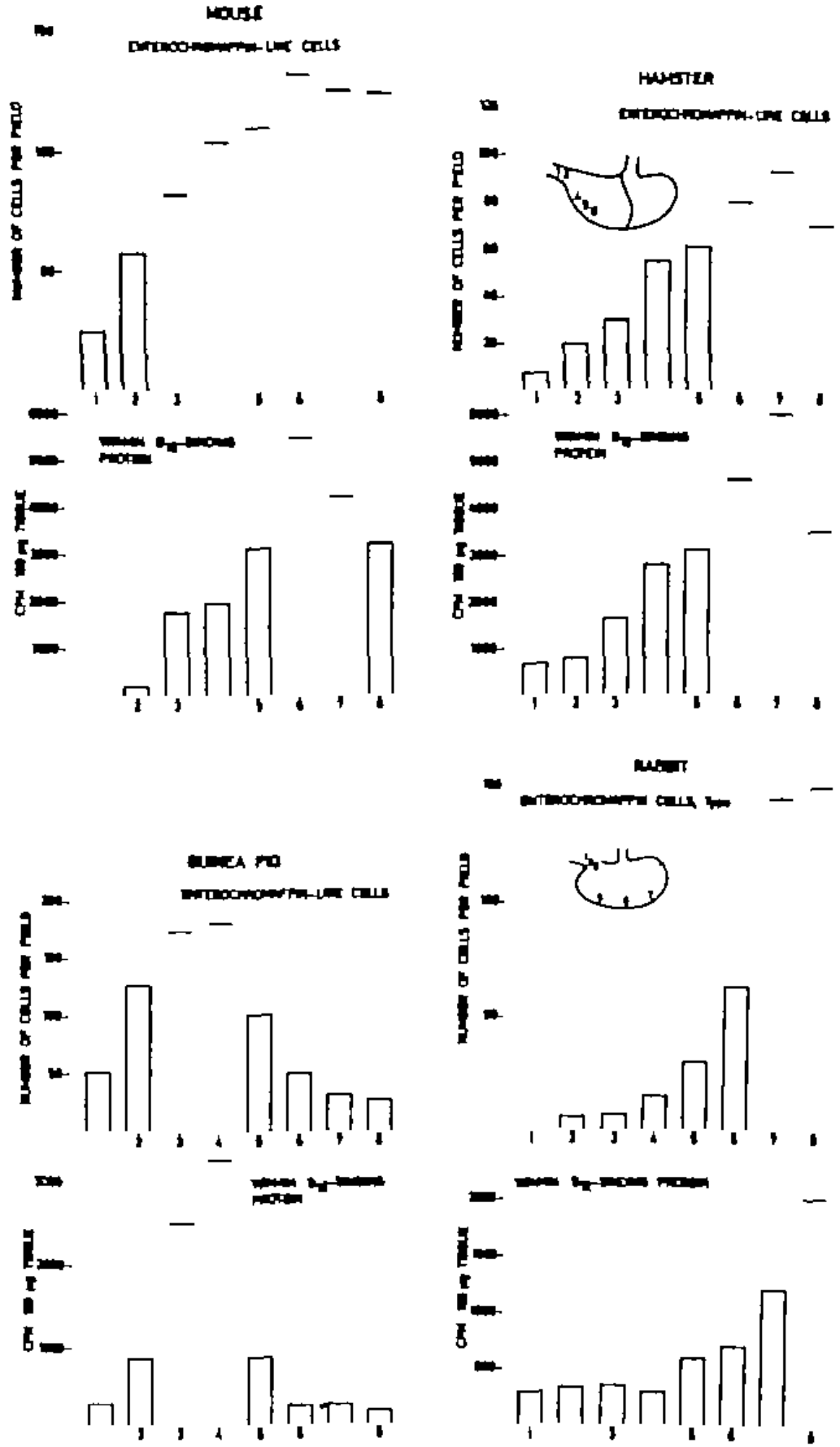


Fig. 19 Regional distribution of vitamin B -binders and gastric endocrine cells in the mouse, hamster guinea-pig and rabbit. For details see Hakanson, Lindberg and Lindstrand (1970)

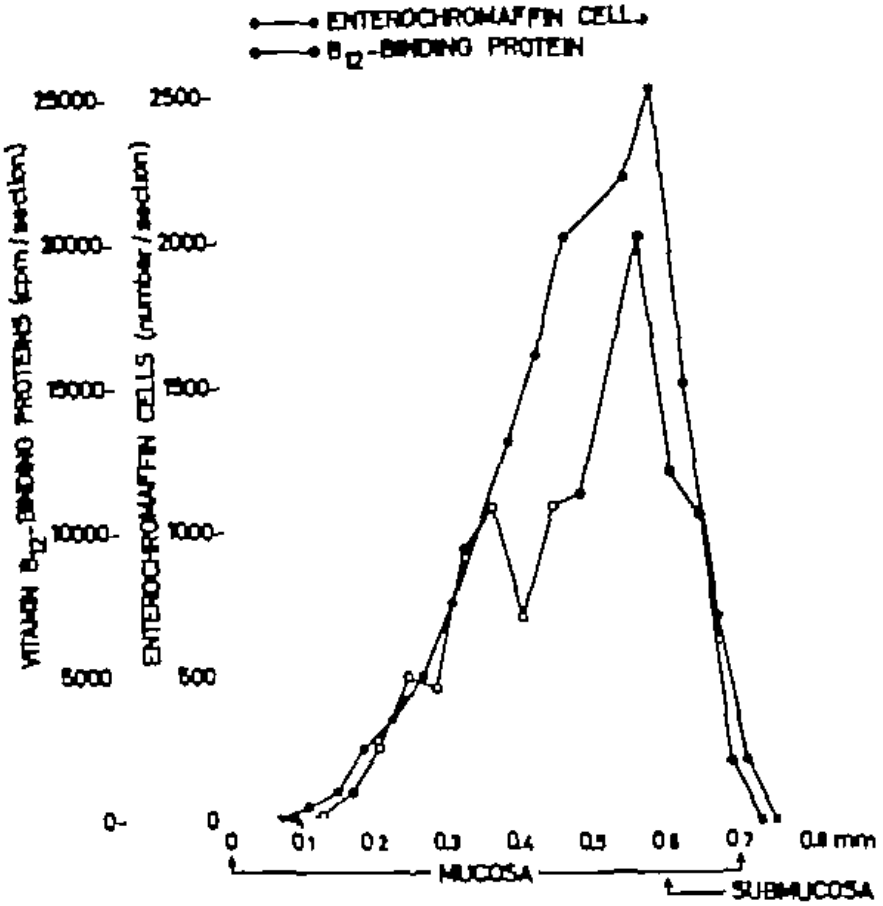


Fig 20 Topographical distribution of vitamin B_{12}-binders and enterochromaffin cells in the oxyntic gland area of the rabbit. For details see Håkanson, Liedberg and Lindstrand (1970)

no previously proposed cell type can be the single cellular storage site for such proteins. It is suggested that the enterochromaffin-like cells in the stomach of the mouse rat, hamster and guinea-pig and the enterochromaffin cells of the rabbit contain gastric vitamin B_{12}-binding proteins, which may include the IF Recently reserpine, which is known to mobilize histamine from enterochromaffin-like cells of the rat stomach, has been found to induce a simultaneous depletion of the IF stores (Håkanson *et al* 1970b) This observation provides further support for the belief that IF in the rat is contained in the histamine-storing epithelial cells of the oxyntic gland area.

Feyrter (1938) introduced the concept "diffuse endokrine epithheliale Organe The term was used to characterize the various systems of scattered endocrine cells observed in a number of exocrine (and certain endocrine) organs. The reason for introducing this new concept was to distinguish between diffuse endocrine epithelial glands (such as *e.g* the argyrophil cells of the digestive tract, the parafollicular cells of the thyroid and the numerous endocrine cells in the pancreatic ducts) and the parenchymal endocrine organs (such as the thyroid, the parathyroid and the adrenal cortex) In another communication published several years later Feyrter (1954) introduced the

term "paracrine glands" for this type of diffuse endocrine organs. Thus, Feyrter distinguished between paracrine glands ("diffuse endokrine Organe") and endocrine glands ("kompakte endokrine Organe")

In a series of communications Pearse and co-workers (Pearse, 1966a, b and c, 1968a and b 1969a and b Carvalheira, Welsch and Pearse, 1968) have pointed out certain characteristics shared by some apparently unrelated polypeptide-secreting endocrine cell systems, diffusely distributed in endocrine and exocrine glands A high content of fluorogenic amines, or alternatively the capacity to take up and decarboxylate DOPA argyrophilic reaction high activities of choline-esterase and a high content of acid proteins (carboxyl groups) revealed by "masked metachromasia" reactions (Pearse, 1969b) These characteristics are believed to reflect the production and storage of hormone precursor protein. The list includes the pituitary corticotrophs and melanotrophs, the pancreatic islet cells, the thyroid cells (parafollicular cells) the adrenal medullary cells and the gastrointestinal argyrophil and argentaffin cells. Because of their amine content or of their capacity to take up and decarboxylate DOPA these cells have been referred to as APUD cells (abbreviation for Amine Precursor Uptake and Decarboxylation) Many of the APUD cell systems of Pearse appear to be identical with the paracrine cells of Feyrter. These various systems of scattered endocrine cells are responsible for the secretion of pituitary hormones, insulin, glucagon, calcitonin and probably also for the secretion of gastrointestinal polypeptide hormones (for a discussion see Pearse, 1966c 1969b Aures and Håkanson 1968 Aures Håkanson and Owman 1970) In surprisingly many cases, however the hormones of these putative endocrine cells are still unidentified.

The endocrine like cells of the gastric mucosa are provided with many of the features characteristic of protein-secreting cells a well developed endoplasmatic reticulum, a prominent Golgi apparatus and numerous small round, oval or polymorphous secretory granules (Solcia *et al.* 1965 1967) In addition, their general morphology and the arrangement of their cytoplasmic organelles suggest some functional polarity of these cells. The micro-villi apparatus extending from some of these cells into the glandular lumen may indicate that some constituent of the luminal content, perhaps acting as a chemical stimulus for hormone secretion, may be absorbed by the cell Or alternatively this arrangement may indicate that these endocrine cells in addition perform some exocrine function (for instance, the secretion of IF) On the other hand the preferential arrangement of the secretory granules at the base of the cell suggests the delivery of the secretory product into the interstitial space, surrounding the cell. The capillary vessels, which run just beneath the basement membrane may be engaged in forwarding the secreted material.

From purely morphological observations, Feyrter (1938 1954) suggested that paracrine cells may have multiple functions. Thus, the paracrine cells were believed to have an exocrine as well as an endocrine function (Fig 21) In addition, the possibility was considered that the paracrine cells could have

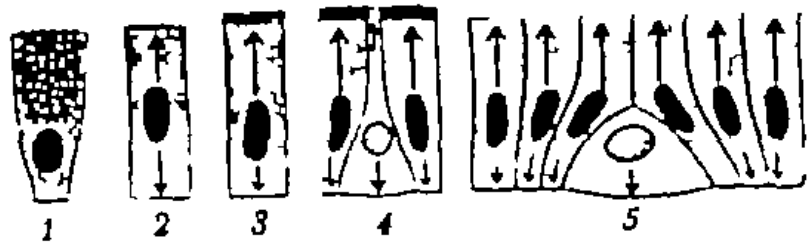


Fig. 21 This illustration is reprinted from the monograph of Feyrter (1938) with the permission of the publisher (J. A. Barth, Leipzig)

a direct local effect on neighbouring exocrine cells (Feyrter, 1938 1954) In the light of current knowledge it may perhaps be suggested that the endocrine function of these cells in the gastric mucosa is represented by the formation and secretion of polypeptide hormones the exocrine function is related to the secretion of proteins (such as IF) which are important for the intestinal absorption of certain dietary agents the local influence, finally is exerted by amines, such as 5-HT dopamine or histamine which *per diffusionem* may stimulate—or inhibit—the secretion of neighbouring exocrine gland cells. There is evidence—from experimental studies (Fyrö 1967 1968 Aures Håkanson and Schauer, 1968 Håkanson and Liedberg 1970a) as well as from *direct morphological observations* (Sikard 1934 Ratzenhofer, 1967 Håkanson Lilja and Ovrlan 1969)—that some of these endocrine cells are to be regarded as neuro-endocrine being under vagal and possibly also sympathetic control

Some of the gastrointestinal polypeptide hormones may affect not only specific exocrine target cells, but possibly also other endocrine cells, such as the *insulin-secreting cells of the pancreatic islets. The existence of an entero-insular axis* has been assumed on the basis of the capacity of gastrointestinal mucosal extracts to release insulin (Moore, Edie and Abram, 1906 Dixon and Wadia, 1926 Heller, 1929 1935 Laughton and Macallum, 1932 Dupré, 1964 Dupré and Beck, 1966) One such insulin-releasing agent is gastrin several other compounds with a B-cytotrophic effect may exist in the digestive tract (Unger, Ketterer, Dupré and Eisentraut 1967) * The possible existence of complex and sequential regulation mechanisms for the control of the secretion of hormones from polypeptide-secreting endocrine cell systems should be kept in mind.

Oral glucose is more effective in mobilizing insulin from the pancreas than is intravenous insulin. One candidate for this insulin-releasing action is the glucagon-like immunoreactivity shown by Unger *et al. (1968) to be released from the upper small* intestine of the dog in response to the intraduodenal administration of glucose.

The function of the various scattered endocrine cell systems (paracrine according to Feyrter, 1954) is not possible to establish in the traditional manner —*e.g.* by extirpation of the gland and observation of subsequent deficiency symptoms. Some information may perhaps be obtained by analyzing the symptoms occurring in disorders caused by malfunctioning of such endocrine cells, for instance carcinoid tumours. These tumours are neoplasms derived from argentaffin or argyrophil cells in the gut, bronchi and biliary duct system. It is evident from numerous observations that these tumours do not constitute a homogenous group of neoplasms. Clinical (Wilkerd and Schaefer, 1952 Thorson ,Biorck, Björkman and Waldenström 1954 Sjoerdsma, Weissbach and Udenfriend 1956 Waldenström, 1958 Oates and Sjoerdsma, 1962 Sjoerdsma and Melmon 1964 Melmon Sjoerdsma and Mason 1965 Brindley and Bonnet 1967 Shanes Dhurandhar and Blackard, 1968) histochemical (Lillie and Glenner, 1960 Stout 1962 Weichert Reid and Creech, 1967) and biochemical (Lembeck, 1953 Ratzenhofer and Lembeck, 1954 Waldenström, Pernow and Silver, 1956 Sandler and Snow 1958 Oates and Sjoerdsma, 1962 Williams and Sandler, 1963 Oates, Melmon Sjoerdsma, Gillespie and Mason 1964) distinctions have been documented. Usually carcinoids of the small intestines, appendix and cecum are argentaffin when properly preserved by prompt fixation. Tumours giving only the argyrophil reaction have also been described. Ultrastructural differences between gastric carcinoids and various intestinal carcinoids have been reported (Luse and Lucy 1960 Bensch, Gordon and Miller, 1965 Toker, 1966 Black and Haffner, 1968) This is perhaps to be expected since the progenitor cells (*i.e.* the argentaffin and the argyrophil cell) from which these tumours arise, comprise several populations of distinctly different cells. From observations on a large number of carcinoid tumours, Black and Haffner (1968) concluded that the enterochromaffin system is made up of a number of distinct cell types which are differently distributed throughout the gut, and also that carcinoids usually correspond cytologically to the type of enterochromaffin cell which is preponderant in a given region *i.e.* ultrastructurally the cells of a carcinoid have a closer resemblance to its progenitor cell than to the cells of other carcinoids.

The clinical symptoms associated with the carcinoid syndrome have been well defined and are principally manifested by flushes, cyanosis, chronic diarrhea, respiratory distress and valvular disease of the heart. 5-HT is often produced at a high rate in carcinoid tumours (see Williams and Sandler, 1963 Schiffman and Rappaport 1965) and this is reflected in markedly increased levels of urinary 5-hydroxyindoleacetic acid, which is the major degradation product of this indolamine. Atypical carcinoids have been described in which the tumours appear to secrete large amounts of 5-HTP (Sandler and Snow 1958 Oates and Sjoerdsma, 1962) histamine (Waldenström Pernow and Silver, 1956) or tryptamine (Eccleston Crawford and Ashcroft 1963) Other agents such as vasoactive polypeptides (kinins) and proteolytic enzymes

(kallicreins) have also been detected in carcinoid tumours and in the blood of patients with such tumours (Oates *et al.* 1964) 5-HT is believed to be a key substance in the development of the clinical symptoms (Thorson *et al.*, 1954) although the symptoms of the carcinoid syndrome cannot be explained by the secretion of 5-HT alone (Robertson Peart and Andrews 1962 Levine and Sjoerdsma, 1963) It appears quite possible that some of the symptoms described are caused by elevated levels of vasoactive polypeptides in the blood. Conceivably the capacity to produce kinins or kallicreins is characteristic also of the normal progenitor cells (cf Said and Mutt 1970)

3 10 *Possible function of amines in gastric enterochromaffin and enterochromaffin-like cells*

Amines such as 5-HT dopamine and histamine can be synthesized and stored by polypeptide-secreting endocrine cells in the thyroid (Falck, Larsson Mecklenburg Rosengren and Svinaeus 1964 Larsson Owman and Sundler, 1966 Owman and Sundler, 1967) anterior pituitary (Carlsson Falck and Hillarp 1962 Dahlström and Fuxe, 1966 Björklund Falck and Rosengren 1967) pancreatic islets (Falck and Hellman 1963 1964 Creutzfeldt 1968) and in the gastrointestinal mucosa (Håkanson and Owman 1966b 1967 Håkanson *et al.*, 1970d) In addition, apart from histamine, other as yet unidentified amines may well occur in those polypeptide-secreting endocrine cells, which lack 5 HT and dopamine (see Björklund and Falck. 1969a and b Håkanson *et al.*, 1970d) The presence of amines appears to be a feature common to all such endocrine cells. The amines may somehow be associated with the general physiological function of these cells, or they may be have a function of their own by exerting a "local hormone" action. It is not possible at present to decide which hypothesis is best justified and the following will be only brief summary of available evidence

According to the first assumption the amines have a secondary role and are involved in some specific cytophysiological function of these endocrine cells. It is reasonable to assume that the amines are stored in the secretory granules (Jaim Etcheverry and Ziehler, 1968a and b Ericson 1970) although the evidence of this is still surprisingly scanty The granules, which are the subcellular element common to all polypeptide or protein-secreting endocrine cells, are believed to contain the secretory products. It may be that the presence of an amine in these granules is necessary for a normal storage and/or release of the true secretory products, i.e. the protein or polypeptide hormone. A few observations from studies with reserpine provide some experimental support for the assumption that the amines and the true secretory products of these cells are functionally interrelated Reserpine causes a simultaneous depletion of histamine and IF in enterochromaffin-like cells of the rat stomach (Håkanson Lindstrand Nordgren and Owman 1970b) Moreover reserpine, which

depletes also other cellular amine stores, is known to cause a release of gastrin from the pyloric gland area (Eaas and Fyrö 1965)

In the second case, the amines are believed to function as local hormones. After being released from the endocrine cell as a result of some stimulus, the amine diffuses into the surrounding tissue, conceivably affecting some adjacent target cell. The smooth muscle of the intestines has been proposed as one important physiological target cell for intestinal 5-HT. It is well known that 5-HT affects intestinal peristalsis (for references, see Haverback and Wirtschafter, 1962 Bülbring and Gershon 1967) but the physiological importance of 5-HT in the control of intestinal motility is poorly understood (see Bennet Bocchnell and Dean 1966 Gershon 1968) even after marked reduction of bowel 5-HT induced by a diet deficient in tryptophan, motility remained normal (Boullin 1964) Judging from the strict localization of the enterochromaffin cells to the epithelium it may perhaps be assumed that these cells —as well as the enterochromaffin-like cells—serve some control function in secretory and/or absorptive mechanisms, and that the putative target cells of the gastrointestinal amines, whether it be histamine, dopamine, 5-HT or some as yet unidentified compound, are to be found among the exocrine gland cells.

The effects of 5-HT dopamine and histamine, as well as those of their immediate natural precursors, on gastric secretion have been studied in chronic gastric fistula rats (Håkanson Lilja, Owman and Thunell, 1967 see also Haverback and Wirtschafter, 1962) Both histamine and histidine stimulated gastric acid secretion, whereas dopamine and 5-HT had an inhibitory effect. While the physiological significance of these pharmacological effects of 5-HT and dopamine is unknown, histamine is claimed to be the common final chemostimulator of gastric acid secretion. This view is historically based on the effectiveness of histamine in stimulating the parietal cell, on the presence of histamine in the gastric juice, and on the high concentration of histamine in the gastric mucosa of several species. This hypothesis was originally proferred by Macintosh (1938) in a review of the subject Code (1965) concluded that stimulation of gastric secretion was a physiological function of histamine, and that no other mediator comes between histamine and the parietal cell. Grossman (1967) on the other hand, believes that in species other than the rat there is no firm evidence linking gastric secretion with endogenous histamine. During the last few years much evidence has accumulated to suggest that in the rat histamine does act as mediator in the control of hydrochloric acid secretion 1) Histamine is present in high concentration in a system of endocrine cells at the base of the mucosa in the oxyntic gland area (Håkanson and Owman 1967 Håkanson Owman and Sjöberg, 1967 Thunberg, 1967 Håkanson *et al.*, 1970d) 2) gastric histamine is produced locally within these cells at a rate which is dependent upon the physiological state of the animal (Thunberg 1967 Aures Håkanson and Schayer, 1968 see also Kahlson *et al.*, 1964) and finally 3) gastric histamine is released from its cellular store as a result of certain physiological stimuli, presumably through

the action of gastrin (KAHLSON *et al.* 1964) It is conceivable that released histamine serves to stimulate the parietal cells by way of diffusion. Moreover KAHLSON has postulated that the gastric histamine content and the activity of gastric histidine decarboxylase are inter related so that depletion of the histamine stores, caused for example by feeding or gastrin injection, induces activation of histidine decarboxylase which serves to replenish the resting levels of gastric histamine Conversely high levels of gastric histamine repress the enzyme activity (KAHLSON *et al.* 1964) However the observation that in the porta-cava shunted rat both the gastric histamine content and the histidine decarboxylase activity are markedly elevated (FISCHER and SNYDER, 1965a and b) seems to disagree with this hypothesis. In the search for an explanation of the mechanism of activation of gastric histidine decarboxylase, it was observed that agents, which cause activation of gastric histidine decarboxylase, are all either parasympathomimetics (carbachol) vagal stimulants (insulin, 2-deoxy glucose, 2-deoxygalactose) or amine-releasing agents, such as reserpine. Histamine itself and histamine liberators such as Compound 48/80 or polymyxin B either did not affect or suppressed the enzyme activity A close parallelism seemed to exist between factors which induce activation of gastric histidine decarboxylase and factors which cause the release of gastrin (see AURES HÅKANSON and SCHAUER 1968) The suggestion was made that in the rat all agents which increase histamine formation act by releasing endogenous gastrin which in turn stimulate gastric histidine decarboxylase activity in the same manner as exogenously administered hormone (AURES HÅKANSON and SCHAUER, 1968) A series of investigations has recently been carried out which seems to support this concept Antrectomy was found to reduce or abolish the enzyme activation induced by feeding insulin and carbachol, but not that induced by histamine (or pentagastrin) (JOHNSON JONES AURES and HÅKANSON 1969 see also JOHNSON WAY and CLARK, 1969 HÅKANSON and LIEDBERG 1970a) These findings suggest that endogenous gastrin, which is stored in the antrum (the pyloric gland area) is the agent responsible for the enzyme activation induced by feeding or by the injection of insulin and carbachol.

There are three major known stimulants of gastric acid secretion that may be natural mediators of the secretory response namely histamine, gastrin and acetylcholine (vagal stimulation) Whether there is a final common pathway for the action of these three agents is unknown. It is known, however that vagal stimulation releases gastrin (UVNÄS 1942 PE THIEN and SCHOFIELD, 1959 OLBE, 1966 OLBE and ELWIN 1966 FYRÖ, 1967 1968) and it is conceivable that in the rat all of these naturally occurring agents are functionally inter related. Acetylcholine (vagal stimulation) releases gastrin which has a two-fold action in that it releases gastric histamine and at the same time activates gastric histidine decarboxylase (KAHLSON *et al* 1964) to replenish the histamine store It is highly probable that all agents that activate the enzyme do so with gastrin as mediator (AURES HÅKANSON and SCHAUER, 1968 JOHNSON *et al* 1969 AURES *et al.*, 1969 HÅKANSON and LIEDBERG 1970a)

It is evident however that if such a simple relationship exists, it exists only in a limited number of species (Aures Davidson and Håkanson 1969) Firstly the cellular storage site of gastric histamine is different in different species only in the rat and mouse is gastric histamine found in endocrine cells, in all other species studied gastric histamine is found in mast cells only (Håkanson *et al.*, 1970d) Secondly specific histidine decarboxylase has been demonstrated in the gastric mucosa of the mouse, rat and hamster only in all other species studied this enzyme is non-measureable (see Aures, Davidson and Håkanson 1969) These circumstances emphasize the need to consider the concept of histamine as the final common chemostimulator of gastric acid secretion separately for each species.

4 SHORT SUMMARY

1 It is suggested that the argyrophil cells of the digestive tract (whether classified as argentaffin, non-argentaffin, enterochromaffin or enterochromaffin-like) constitute an important endocrine system—or perhaps more correctly a number of different, more or less specialized endocrine systems.

2. From a physiological point of view the conventional classification of the endocrine cells of the digestive tract (for instance in argentaffin *versus* non-argentaffin cells) appears to have little justification. The cytochemical and ultrastructural features of these cells are basically very similar suggesting closely related physiological roles. From electron-microscopic studies it may be concluded that five or six different endocrine cell types occur in the gastrointestinal mucosa.

3 The hormones produced and secreted from these cells have not yet been identified. The most plausible candidates are polypeptide hormones such as gastrin, secretin, pancreozymin-cholecystokinin and enteroglucagon. Possibly also agents such as the antipernicious anaemia intrinsic factor originates from these endocrine cells.

4 A common feature of many polypeptide- or protein-secreting endocrine cells is their capacity to produce and store amines, such as histamine, dopamine and 5-hydroxytryptamine. Apparently the various systems of gastrointestinal endocrine cells are endowed with highly specialized mechanisms for the biosynthesis of amines the amine produced and stored may thus differ from one system of endocrine cells to another It is possible that the amines are stored together with the hormones in the secretory granules, which are numerous in these endocrine cells. The amines may regulate the storage and/or release of the true secretory products. Or the amines may be released from the cell as the result of some stimulus, to affect some adjacent target cell.

5 It is suggested that the endocrine cells of the digestive tract may have a variety of functions, including the secretion of polypeptide or protein hormones (*endocrine function*) the secretion of proteins—such as the intrinsic factor—which facilitate the intestinal absorption of dietary compounds (*exocrine function*) and finally the release of amines, which by way of diffusion control the secretion of adjacent exocrine gland cells (*local regulatory function*)

PART II

5 PROPERTIES OF MAMMALIAN HISTAMINE-FORMING ENZYMES

5 1 *General characteristics of mammalian histamine forming enzymes*

Werle (1936) and Holtz and Heise (1937) were first to report on the presence of histidine decarboxylase in mammalian tissues. Following their initial observations the histamine-forming enzymes of mammalian tissues attracted much attention. Considerable disagreement as to the properties of histidine decarboxylase was soon registered. These discrepancies have since been explained by the existence of several histamine-forming enzymes with different characteristics.

The enzymatic formation of dopamine by decarboxylation of L DOPA was recognized by Holtz, Heise and Lüdtke (1938) and the participation of DOPA decarboxylase in the biosynthesis of catecholamines was postulated by Blaschko in 1939 (see also Holtz, 1959) After the discovery of this enzyme a close similarity was noted in the distribution of histidine decarboxylase and DOPA decarboxylase activities in mammalian tissues (see review by Shepherd and Mackay 1967) In these experiments histidine decarboxylase was found to have its pH optimum at 8 0—9.5 and the corresponding value for DOPA decarboxylase was between 6.5 and 7 0. The possibility was soon considered that these two activities might reside in a single enzyme. Later the activity of an enzyme catalyzing the decarboxylation of L 5-HTP was detected in mammalian tissues (Udenfriend Clark and Titus 1953 Clark, Weissbach and Udenfriend 1954) It was found that the ratio of DOPA decarboxylase activity to 5-HTP decarboxylase activity from one tissue to another was roughly constant (Westermann Balzer and Knell, 1958) and it was suggested that DOPA and 5-HTP were decarboxylated by the same enzyme (Langeman 1958 Bertler and Rosengren 1959 Fellman 1959 Rosengren 1960 Lovenberg Weissbach and Udenfriend 1962 Hagen 1962) Thus it seemed possible that the histidine decarboxylating enzyme, having its pH optimum in the range 8.0—9.5 was identical with DOPA/5-HTP decarboxylase. When purified enzyme extracts of ox adrenal and guinea pig and rabbit kidney became available, the preparations were found to decarboxylate not only these three amino acids (Fellman 1959 Rosengren 1960) but also many other natural and purely synthetic aromatic amino acids (Yuwiler, Geller and Eidusion 1959 Lovenberg Weissbach and Udenfriend 1962 Lovenberg Barchas Weissbach and Udenfriend 1963) From these results it was suggested that the non-specific decarboxylase should be designated aromatic L-amino acid decarboxylase(Lovenberg Weissbach and Udenfriend 1962)

Apart from the similar distribution of the enzyme activities in a variety of tissues, the view that aromatic L-amino acids are all decarboxylated by a single

(Kahlson Rosengren and Westling 1958 Kahlson Rosengren Westling and White, 1958 Kahlson Rosengren and White, 1960) This observation was followed by similar findings in some other tissues of the rat (Kahlson Nilsson Rosengren and Zederfeldt 1960 Mackay Marshall and Riley 1960 Håkanson 1961 Mackay Riley and Shepherd 1961 Kahlson and Rosengren 1963) mouse (Rosengren 1963 Maudsley and West 1964) and hamster (Rosengren 1965) The properties of the enzyme of the foetal rat liver were first studied by Ganrot Rosengren and Rosengren (1961) who concluded that foetal rat histidine decarboxylase was different from the histamine-forming enzyme of rabbit kidney The latter enzyme appeared to be identical with DOPA decarboxylase. Foetal rat histidine decarboxylase was not inhibited by DOPA decarboxylase inhibitors and was considered to be specific. These observations initiated much work aimed at characterizing the histamine-forming enzymes of the mammalian organism.

There is general agreement by now that at least two different histamine-forming enzymes, or groups of enzymes, occur in mammalian tissues (Ganrot Rosengren and Rosengren 1961 Mackay Riley and Shepherd, 1964 Weissbach, Lovenberg and Udenfriend 1961 Håkanson 1963 1964 1966 1967a and b Håkanson and Owman 1966b Auers Håkanson and Schauer, 1968 Auers, Davidson and Håkanson 1969) Histidine decarboxylase from the kidney of the rabbit and guinea pig differs in properties from the enzyme obtained from foetal rat liver and rat stomach. One important difference between the two enzymes is the ability of the kidney enzyme to decarboxylate a large number of aromatic L-amino acids (see above) which is reflected in the fact that the non-sepcific enzyme is strongly inhibited by α-methylDOPA, whereas the specific histidine decarboxylase is unaffected. The latter enzyme, on the other hand, is inhibited by *e.g.* α-methylhistidine, which is a weak inhibitor of aromatic amino acid decarboxylase. Another difference is reflected in the different response of these two enzymes to exposure to organic solvents, such as benzene and chloroform (Waton 1956b) Since highly purified preparations of DOPA decarboxylase retain a histidine decarboxylase activity which is inhibited by well-known DOPA decarboxylase inhibitors, it has been suggested that the capacity to decarboxylate histidine is a property of DOPA decarboxylase. However the affinity for L histidine is so low that the ability of DOPA decarboxylase to produce histamine *in vivo* has been questioned (Ganrot Rosengren and Rosengren 1961 Levine Sato and Sjoerdsma, 1965)

5.2. *Properties of aromatic amino acid decarboxylase*

Rosengren (1960) found that the chromatographic distribution of the DOPA and 5-HTP decarboxylase activities of an extract of rabbit kidney cortex were very similar The ratio of the two activities in the enzyme-containing fractions was almost constant and equal to that observed in crude extracts.

The DOPA/5-HTP decarboxylase-containing fractions also contained all the histidine decarboxylase activity of the extract. Furthermore, the decarboxylation of DOPA by the rabbit kidney extracts was found to be competitively inhibited by 5-HTP and the decarboxylation of 5-HTP by the same extract was competitively inhibited by DOPA. Rosengren (1960) also observed that when *o*-tyrosine, *m* tyrosine or caffeic acid were tested as inhibitors of the rabbit kidney preparation, the inhibition constants were the same irrespective of whether DOPA or 5-HTP were used as substrate for the decarboxylase. On the basis of similar inhibition studies with highly purified extracts of ox adrenals Fellman (1959) and Hagen (1962) concluded that both substrates are decarboxylated by one and the same enzyme. There are also inhibition studies to support the view that histidine is decarboxylated by the DOPA/5-HTP decarboxylase. Thus, with purified preparations of DOPA decarboxylase, α-methyl-DOPA and other α-methylated aromatic amino acids were found to be potent inhibitors of all three enzyme activities (Sourkes, 1954 Mackay and Shepherd 1960 Murphy and Sourkes 1961 see also Schayer and Sestokas, 1965) Lovenberg Weissbach and Udenfriend (1962) purified an extract of guinea-pig kidney by DEAE cellulose chromatography and found that the fractions that contained 5-HTP decarboxylase also catalyzed the decarboxylation of other aromatic amino acids (including α methylated derivatives) as well as histidine (see also Weissbach, Lovenberg and Udenfriend 1960) they concluded that the decarboxylation of all of these substrates was catalyzed by a single enzyme aromatic L amino acid decarboxylase.

The pH optimum of DOPA decarboxylation is at about pH 6.5—7.0 (Schales and Schales 1949 Clark, Weissbach and Udenfriend 1954 Hakanson 1967d) The corresponding value for 5-HTP decarboxylation is approximately 9.0 (Clark, Weissbach and Udenfriend 1954) and for histidine decarboxylation 8.5—9.0 (Lovenberg Weissbach and Udenfriend 1962) The K_m value for DOPA decarboxylation was found to be 5.4×10^{-4} at pH 6.8 (Schott and Clark, 1952 Fellman 1959) and 7.4×10^{-5} at pH 7.5 (Rosengren 1960) the K_m for 5-HTP decarboxylation was estimated to 4×10^{-5} at pH 8 1.7×10^{-4} at pH 6.8 (Yuwiler, Geller and Eidusson 1960 see also Clark, Weissbach and Udenfriend 1954) and 1.3×10^{-4} at pH 7.5 (Rosengren 1960) The K_m for histidine decarboxylation catalyzed by the rabbit enzyme at pH 7.8 was given as 10^{-1} by Ganrot Rosengren and Rosengren (1961) Inhibition studies performed with DOPA and 5-HTP suggested that one single enzymic site is involved in the decarboxylation of both substrates (Yuwiler, Geller and Eidusson 1959 1960 Rosengren 1960)

Waton (1956b) observed that addition of small amounts of certain organic solvents increased the rate of histidine decarboxylation in extracts from rabbit kidney Benzene, which was found to be most active, produced five- to ten-fold activation of the enzyme. The potentiating effect of benzene has since been amply confirmed in several laboratories (Schayer, 1957 Mackay Riley and Shepherd 1961 Telford and West 1961b) Lovenberg, Weissbach and

Udenfriend (1962) reported that while the ability of the kidney enzyme to decarboxylate histidine and *p*-tyrosine was increased by benzene, the ability to decarboxylate DOPA and 5-HTP was decreased. No explanation for these effects of benzene can be offered. Conceivably organic solvents, such as benzene and chloroform, alter the enzyme protein configuration thus enhancing the opportunities for attachment of the histidine and *p*-tyrosine molecules to the active site.

5.3 *Properties of specific histidine decarboxylase*

Another histidine decarboxylating enzyme appears to be specific and is probably of greater physiological significance as a catalyst of histamine formation (Ganrot Rosengren and Rosengren 1961, Weissbach Lovenberg and Udenfriend 1961, Levine, Sato and Sjoerdsma, 1965). This enzyme is generally referred to as mammalian histidine decarboxylase or "specific" histidine decarboxylase (EC 4.1.1.22). The "specific" histamine forming enzyme has been found in a limited number of tissues. Foetal rat liver (Ganrot Rosengren and Rosengren 1961, Håkanson 1963), hamster placenta (Håkanson 1967b) and murine mastocytoma (Noll, 1965, Ahres and Noll, 1966) are conspicuously rich sources of this enzyme. Considerable activities have been demonstrated also in gastric mucosa of the rat (Weissbach Lovenberg and Udenfriend 1961, Håkanson and Owman 1966b) and mouse (Håkanson 1967a), in the red bone marrow of the rat (Håkanson 1964) and in various transplantable tumours of the rat (Mackay Riley and Shepherd 1961, Håkanson 1966) (Table 2).

The kinetic properties of foetal histidine decarboxylase could be studied in some detail only after a method for its purification had been developed (Håkanson 1963). Pregnant rats were decapitated 15—20 days after mating and the litters were collected and pooled. In frozen condition (—30 C) the foetal rat tissue maintained its enzyme activity for several weeks. The tissue was homogenized in 0.1 M sodium acetate-acetic acid buffer pH 4.5 and centrifuged at 20 000×g in a refrigerated centrifuge for 20 min. The supernatant was heated at 55 C for 5 min. and centrifuged at 20 000×g for 10 min. the precipitate was discarded. The resulting supernatant was found to contain more than 90 per cent of the initial enzyme activity. The bulk of histidine decarboxylase was precipitated with ammonium sulfate at between 25 and 40 per cent saturation. This precipitate was spun down, re-suspended in 10—20 ml 0.1 M phosphate buffer pH 7.0 and dialyzed against re-distilled water at 4 C for a minimum of 18 hours with frequent changes of water. After centrifugation, the supernatant of the dialyzed extract was again treated with ammonium sulfate. The material which now precipitated at between 28 and 42 per cent saturation contained all the histidine decarboxylase activity. This precipitate was spun down, re-suspended in a small volume of the phosphate buffer and dialyzed against water for at least 6 hours with 3 changes of water.

TABLE 2

Characteristics of histidine decarboxylases from various tissue sources*

Source	pH Optimum	Substrate saturation point	Inhibition by DOPA or α-methyl DOPA	Effect of benzene	Substrat specificity	Classification	References
Mouse							
Mastocytoma	6.0—7.3	low	weak or none	none	histidine	specific	1 6 23 36
Stomach	6.0—7.0	low	—	—	—	specific	10
Maternal kidney	6.0—7 0	low	–	—	—	specific	10
Fetal tissues	6.4—7.0	low	—	—	—	specific	10 22
Rat							
Stomach	6.2—7 2	low	weak	inhibition	histidine	specific	3 12, 14 16 32, 36
Fetal tissues	6.0—7 2	low	weak or none	none	histidine	specific	4, 5 7 11 15
Bone marrow	6.5—6.8	low	none	—	histidine	specific	8, 13
Tumours							
Rous, sarcoma, hepatoma, Walker carcinoma	6.0—6.8	low	weak	none	histidine	specific	9 15 20 25 31
Mast cells	6.5—7 6	low	weak	inhibition	histidine	specific	30 33
Liver	8.0	high	strong	potentiation	aromatic amino acids	non-specific	15 25 35
Kidney	7.5	—	—	potentiation	—	non-specific?	35
Guinea-pig							
Kidney	8.0—9.5	high	strong	potentiation	aromatic amino acids	non-specific	18, 19 20 21 27 32 36
Stomach	8.0	high	—	—	—	non-specific	2, 36
Fetal tissues	8.0	—	—	potentiation	—	non-specific?	16

Rabbit							
Kidney	8.0	high	strong	potentiation	aromatic amino acids	non-specific	3, 28
Fetal tissues	8.0—8.5	—	—	potentiation	—	non-specific	16
Platelets	7.2—7.4	—	—	—	—	specific?	34
Stomach	9.0	high	—	—	aromatic amino acids	non-specific	2, 1
Hamster							
Placenta	6.0—7.2	low	—	—	histidine	specific	11, 29
Stomach	6.6—7.2	low	—	—	histidine?	specific?	2
Fetal tissues	8.0			potentiation	aromatic amino acids?	non-specific	16

It should noted that in some tissues both the specific and the non-specific histamine forming enzyme has been demonstrated (see e.g. Ref. 12). In those cases the properties of the specific enzyme are given.

1 Aures and Clark (1964)
2. Aures, Davidson and Håkanson (1969)
3 Aures, Håkanson and Schauer (1968)
4 Burkhalter (1962)
5. Garrot, Rosengren and Rosengren (1961)
6. Hagen, Werner Ono and Lee (1960)
Håkanson (1963)
Håkanson (1964)
nson (1966)
son (1967)
on (1967b)
n and Owman (1968b)
13 Kahlson and Rosengren (1965)
14 Kahlson, Rosengren, Svahn and Thunberg (1964)
15. Kameswaran and West (1961)
16 Kameswara and West (1962)
17 Kim and Glick (1967)
18 Lovenberg, Barchas, Weissbach and Udenfriend (1963)
19 Lovenberg, Weissbach and Udenfriend (1962)
20 Mackay Reid and Shepherd (1961)
21 Mackay and Shepherd (1960)
22. Nicholaidis and Maslinski (1968)
23 Noll (1965)
24 Pearlman and Waton (1968)
25 Reid, Riley and Shepherd (1963)
26. Reid and Shepherd (1963)
7 Robinson and Shepherd (1962)
28. Rosengren (1960)
29 Rosengren (1963)
30 Rothchild and Schayer (1959)
31 Shepherd and Woodcock, (1968b)
32. Schayer (1957)
33. Schayer (1963)
34 Schayer and Kobayashi (1956)
35. Telfod and West (1961b and c)
36. Weissbach, Lovenberg and Udenfriend (1961)

The procedure described was found to result in a 200-fold purification of the enzyme. Although a single ammonium sulfate fractionation results in only a partial separation of histidine decarboxylase and DOPA decarboxylase (see Table 3) the full procedure markedly reduces the activity of the non-specific enzyme. More than 50 per cent of the histidine decarboxylase activity of the initial homogenate was preserved in the final extract. Even at —30 C the purified enzyme was stable for only a few days. The purification procedure caused an almost complete resolution of the holoenzyme, and pyridoxal 5 phosphate had to be added to the incubation mixture to restore the enzyme activity

TABLE 3

Activities of histidine decarboxylase and DOPA decarboxylase in fractions obtained by precipitating extracts from rat stomachs and foetal mice with increasing amounts of ammonium sulfate.

$(NH_4)_2SO_4$ (% saturation)	Rat stomach		Foetal mice	
	DOPA decarboxylase	Histidine decarboxylase	DOPA decarboxylase	Histidine decarboxylase
20	2±1 (3)	6±2 (3)	1±0 (3)	2±0 (3)
30	7±2 (3)	39±4 (4)	8±4 (3)	39±8 (3)
40	33±3 (3)	43±3 (4)	39±9 (3)	40±1 (3)
50	52±2 (3)	9±3 (4)	36 ± 3 (3)	15±5 (3)
60	7±3 (3)	3±1 (4)	16±2 (3)	4±1 (3)

The purification procedure described above has been found useful for the preparation of histidine decarboxylase not only from foetal rat tissues (Levine, Sato and Sjoerdsma 1965 Levine and Watts 1966 Skidmore and White 1966a 1966b Lelkweber, 1968) but also from other tissue sources, such as hamster placenta (Håkanson 1967b) kidney of pregnant mice (Håkanson 1967a) red bone marrow of the rat (Håkanson 1964) transplantable tumours of the rat (Håkanson 1966) and murine gastric mucosa (Håkanson and Owman 1966b Håkanson 1967a, Autes Håkanson and Schauer, 1968) Minor modifications have been introduced. In some cases it has been found advantageous to elevate the pH of the acetate buffer used in the initial homogenization from 4.5 to 5.5 (Levine and Watts, 1966) sometimes the heating step has been omitted, so has quite often the second ammonium sulfate precipitation. The methods described for the purification of mammalian histidine decarboxylase have recently been reviewed by Schayer (1968) and by Autes and Håkanson (1970)

The process of purification of mammalian histidine decarboxylase (EC 4 1 1.22) may be directed towards 1) high purity in absolute terms or 2) high purity in relative terms i.e. successful separation from other contaminating histamine forming enzymes. These objectives are not always identical. The purpose of the study will be decisive for selection of enzyme source. Hamster

placenta, which is low in DOPA decarboxylase (HÅKANSON 1967b) is the superior source of enzyme for extensive purification and detailed characterization of "specific" histidine decarboxylase. Murine mastocytoma, which is rich in both histidine decarboxylase and DOPA decarboxylase (AURES and CLARK, 1964; AURES and NOLL, 1965; NOLL, 1966) is an excellent source of enzyme for studies on the separation of the two enzymes.

As of yet, column chromatographic procedures have had only limited use in the purification of mammalian histidine decarboxylase (WERLE and LORENZ, 1966) and methods presented up till now have had a preliminary character. Highly purified preparations of histidine decarboxylase have been prepared (HÅKANSON and JOSEFSSON 1970) but the major problem in the purification of mammalian histidine decarboxylase by column chromatography is the extreme lability of the highly purified preparations.

Foetal rat histidine decarboxylase was found to have very characteristic kinetic properties.* The optimal pH varied with the substrate concentration (Fig. 22 and Fig. 23) from pH 6.0 at high histidine concentration

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Mathematical treatment of kinetic data. The kinetic data were analyzed by the Lineweaver Burk transformation of the general rate equation, which was used to give the apparent maximal velocity (V'_{max}) and the apparent Michaelis-Menten constant (K_m). It has rightly been argued that whereas V'_{max} can be fairly reliably determined by the conventional Lineweaver Burk method this transformation of the general rate equation has to be used with caution for the determination of K_m since with this plot the most unreliable values of each experiment has a decisive influence on the result. Thus, in some experiments (see Håkanson, 1967c; Aures, Håkanson and Schauer 1968) the so-called Eadie transformation was used. In conjunction some authors hold this method of analysis to be superior.

The simple reaction scheme according to Michaelis and Menten

$$E + S \underset{2}{\overset{1}{\rightleftharpoons}} ES \underset{4}{\overset{3}{\rightleftharpoons}} E + P + CO_2 \qquad (1a)$$

E denotes the enzyme-coenzyme complex, S represents histidine and P and CO_2 the reaction products. The backward reaction (Reaction 4) may be neglected since wherever possible initial velocities were measured.

From this scheme, the usual rate equation may be derived from the assumption of a steady state:

$$\frac{d(ES)}{dt} = k_1(E)(S) - k_2(ES) - k_3(ES) = 0 \qquad (2a)$$

$$\frac{(E)(S)}{(ES)} = \frac{k_2 + k_3}{k_1}$$

$$v = \frac{d(P)}{dt} = k_3(ES) \qquad (3a)$$

Applying these relations to the conservation equation

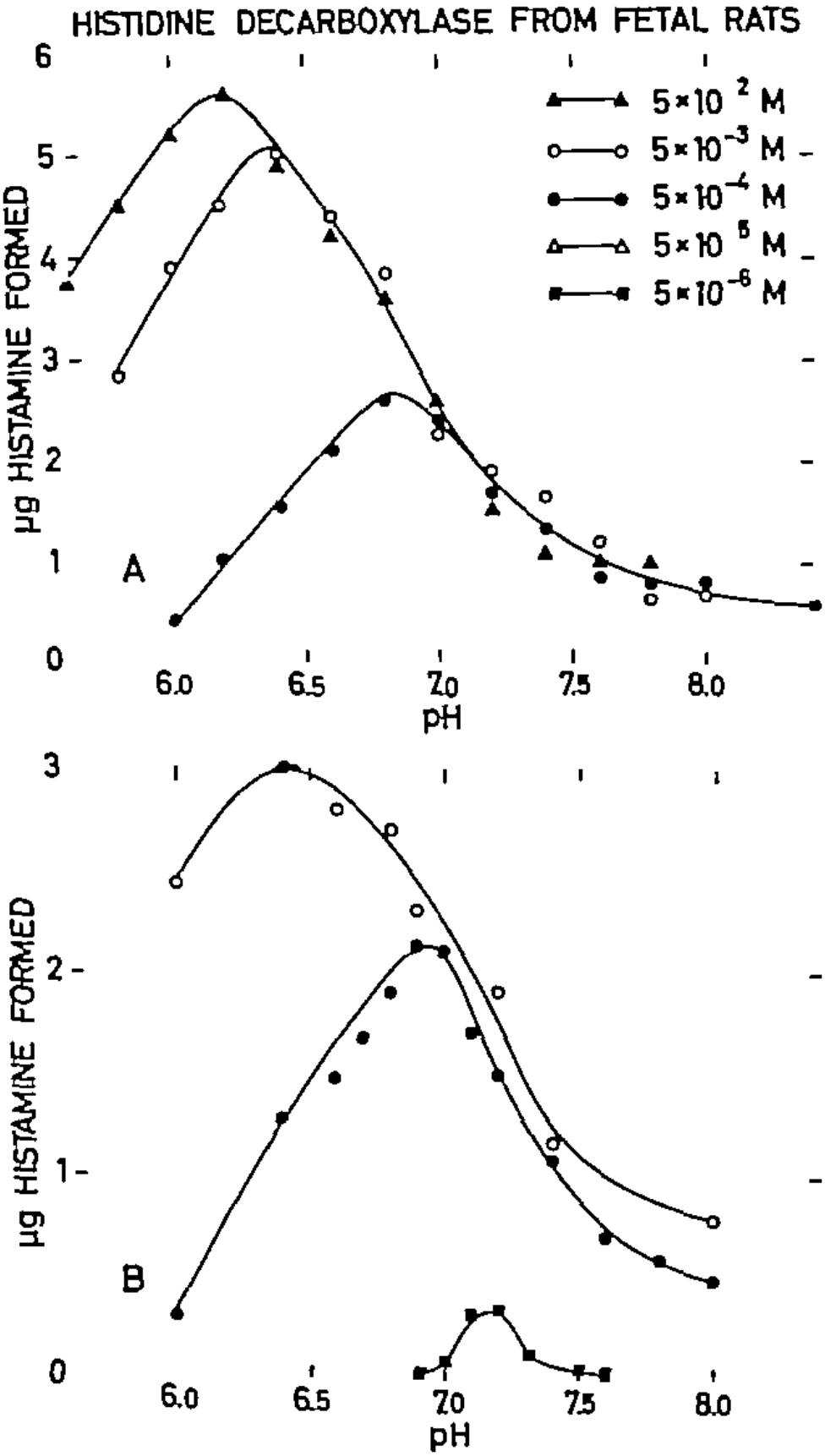
$$(E_t) = (E) + (ES) \qquad (4)$$

and

$$V'_{max} = \left(\frac{K_m}{(S)} + 1\right) \qquad (5a)$$

where

$$K_m = \frac{k_2 + k_3}{k_1} \text{ and } V'_{max} = k_3(E_t)$$


HISTIDINE DECARBOXYLASE FROM FETAL RATS
5×10 ² M
5×10⁻³ M
5×10⁻⁴ M
5×10 ⁵ M
5×10⁻⁶ M
µg HISTAMINE FORMED
A
pH
6.0
6.5
7.0
7.5
8.0
B

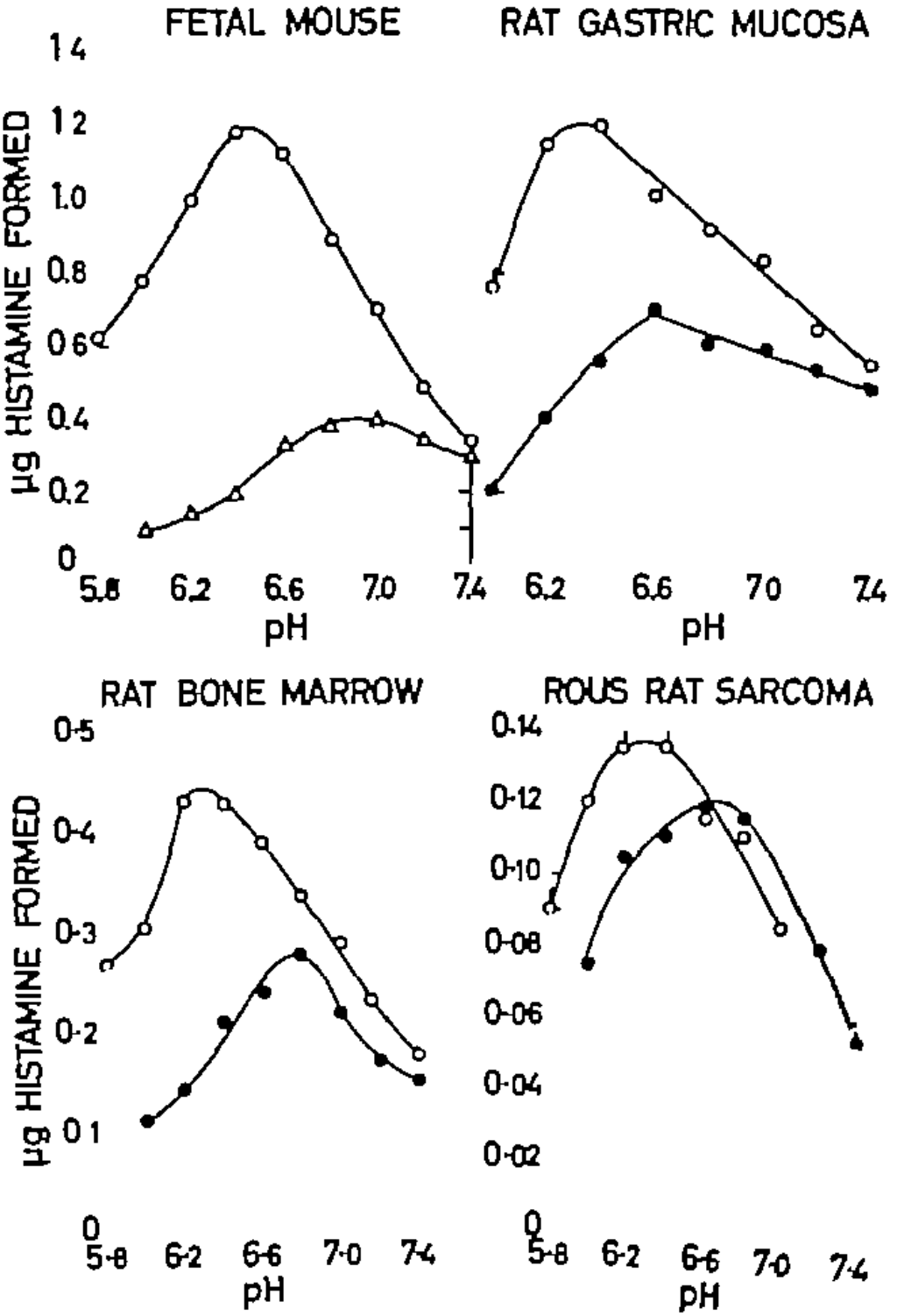


Fig. 22 Histidine decarboxylase from various tissue sources. Enzyme activity is expressed as µg histamine produced. From Håkanson (1963, 1964, 1966) and Håkanson and Owman (1966b)

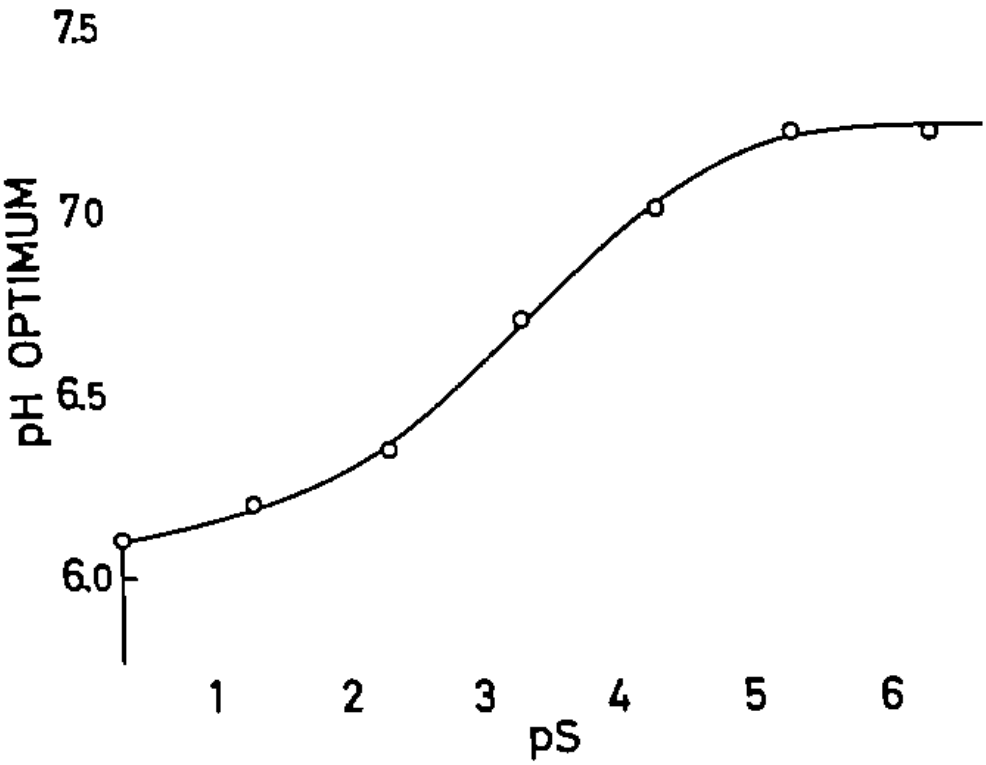


Fig. 23 The influence of substrate concentration (expressed as pS) on the pH optimum of enzymatic histamine formation. From Håkanson (1967b)

to 7.9 at low concentrations. Similar observations were subsequently made with semi-purified preparations of histidine decarboxylase from foetal mice, hamster placenta, renal cortex of pregnant mice, red bone marrow and transplantable tumours of the rat and murine gastric mucosa (Fig. 22). The substrate saturation point of the enzyme turned out to be highly dependent on pH. At pH 7.6–8.0 a low K_m was observed; at more acid conditions K_m was markedly increased (Fig. 24). It is evident from these observations which are summarized in Table 4 that several histamine forming enzymes, or groups of enzymes, with different properties occur in mammalian tissues. Until precise chromatographic procedures have become available for the separation and identification of the various enzyme species, their kinetic characteristics will be the most important criterion in distinguishing between them. It is not enough to demonstrate that a particular histamine-forming enzyme is different from DOPA decarboxylase by means of inhibitors or by its being unaffected by the addition of benzene.* At present the only reliable method for positive identification of mammalian histidine decarboxylase is through kinetic analysis of the partially purified

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*Neither is the responsiveness of histidine decarboxylase activity to corticoid stimuli a reliable criterion as suggested by Telford and West (1961) and Telford (1965) since conceivably the response is related to complex effects on the cell or on the tissue storing the enzyme rather than on the enzyme protein directly. The same enzyme may respond differently depending on the [illegible] which the enzyme is contained.

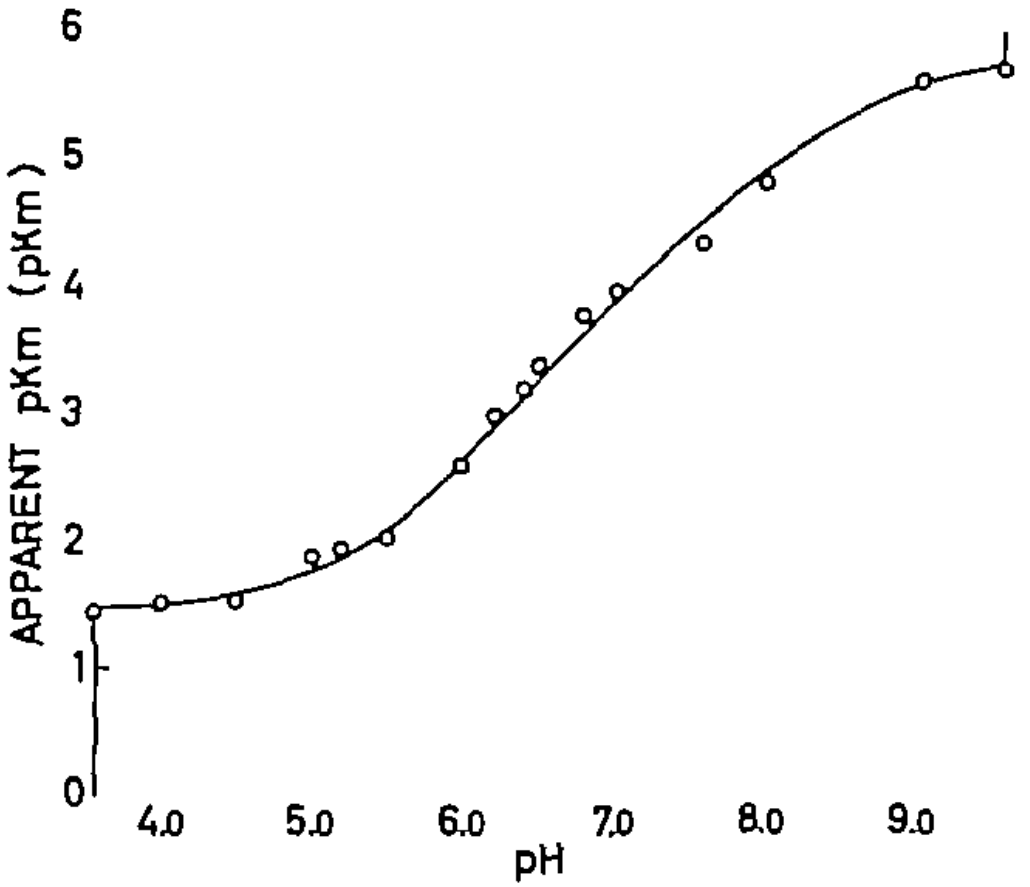


Fig. 24 pK_m *versus* pH (see Table 7)

enzyme. Available data indicate that mammalian histidine decarboxylase from a variety of tissues, such as foetal rat tissues, hamster placenta, murine mastocytoma, murine gastric mucosa, mouse kidney and rat tumours, has the following characteristics (Table 5 6 and7) 1) With high substrate concentration maximal reaction velocity is observed at pH 6 0 the maximal apparent V_{max} is at a pH value close to 5 6 (Fig 25) 2) The pH optimum of the reaction is inversely related to the substrate concentration with very low histidine concentration optimal pH is close to 7 2 (Fig 23) 3) the K_m value decreases markedly with increasing pH In contrast, the non-specific histamine forming enzyme (possibly identical with DOPA decarboxylase) has its pH optimum at about 9 and requires very high histidine concentrations for saturation at all pH values.**

** These differences have some important practical consequences. It is possible, and advisable, to select incubation conditions such as to minimise the interference of the histamine-forming capacity of DOPA decarboxylase, for instance by choosing pH 6.0—6.9 and by restricting the histidine concentration t that which is saturating or near -saturating t that particular pH valu

TABLE 4

Differences in properties between specific" and non-specific histidine decarboxylase.

	Specific enzyme	Non-specific enzyme
pH optimum	5.8—7.2a)	8.0—9.5
K_m	3.7×10^{-4} — 1.9×10^{-3}b)	$\sim10^{-1}$
Substrates	L-histidine	L histidine, aromatic L-amino acids
Effect of PALP	potentiation	potentiation
Effect of benzene	none	potentiation
Inhibitor c)	α-methylhistidine	α-methylDOPA

a) The pH optimum is inversely related to the substrate conc. (Fig.23)
b) The K_m value decreases with increasing pH (Fig 24)
c) Carbonyl-trapping agents inhibit both enzymes indiscriminately

TABLE 5

Apparent K_m values reported by other laboratories.*

pH	Apparent Km (K_m)
5.8	6.8×10^{-3} (d)
6.0	7.1×10^{-4} (f)
6.4	4.7×10^{-4} ( ) 2.7×10^{-4} (f)
6.6	2.0×10^{-3} (e)
6.7	6.8×10^{-4} (d) 5.0×10^{-4} (h)
6.8	1.2×10^{-4} f) 3.7×10^{-4} (g)
7.0	4.6×10^{-4} ( )
7.2	4.4×10^{-3} (f)
7.4	1.85×10^{-3} ( )
7.6	1.9×10^{-3} (f)
7.8	$\sim0\times10^{-3}$ (b) 1.4×10^{-4} (d)

Crude enzyme preparations.

(a) Burkhalter (196)
(b) Gamrot, Rosengren and Rosengren (1961)
(c) Kim and Glick (1967)
(d) Mackay Riley and Shepherd (1961)
( ) Niedzielski and Maslinski (1968)
(f) Noll (1965)
(g) Skidmore and Whitehouse (1966b)
(h) Weinbach, Lovenberg and Udenfriend (1961

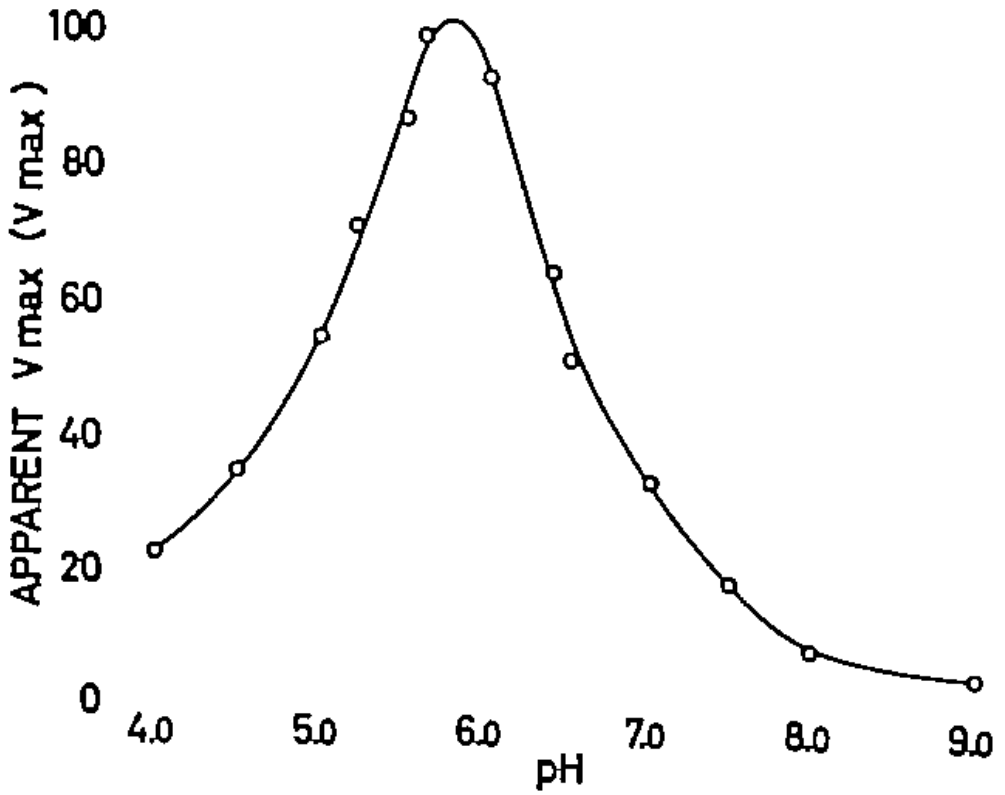


Fig. 25. pH-dependent variation of apparent V_{app} (expressed as per cent of the maximum V_{max} value within the experimental pH region and calculated from double-reciprocal plots of initial velocities *versus* substrat concentration) (see Table 6)

TABLE 6

pH	Apparent V_{max} (V_{max}) Foetal rat	Hamster placenta	Foetal mouse	Rat gastric mucosa	Mean values
4.0		23			23
4.5		35			35
5.0		70	69	27	55
5.2	72				72
5.5		100		74	87
5.6	100				100
6.0		82	100	100	94
6.4			89	42	65
6.5	45			58	52
7.0	28	30	56	21	34
7.5			27	11	19
8.0		7	14	6	9
9.0		3	6		5

The values are from the following publications: Håkanson (1967 and b); Areskog, Håkanson and Schauer (1968).

V'_{max} is given in per cent of maximal V'_{max}.

TABLE 7

pH	K_m values of semi-purified preparations of histidine decarboxylase								
	Rat foetus	Mouse foetus	Hamster placenta	Mouse kidney	Rat gastric mucosa	Rous rat sarcoma	Rat bone marrow	Mouse gastric mucosa	Mean values**
3.5	3.7×10^{-3}								3.7×10^{-3}
4.0	2.7×10^{-3}		3.0×10^{-4}						2.9×10^{-3}
4.5			2.7×10^{-3}						2.7×10^{-3}
5.0	1.1×10^{-3}	1.0×10^{-3}	2.6×10^{-3}	1.0×10^{-3}	1.0×10^{-3}	4.0×10^{-3}			1.3×10^{-3}
5.2	1.0×10^{-3}								1.0×10^{-3}
5.5	6.0×10^{-3}		1.3×10^{-2}						9.5×10^{-3}
6.0	2.0×10^{-3}	3.2×10^{-3}	3.2×10^{-3}	2.5×10^{-3}	2.0×10^{-3}	8.0×10^{-3}		2.5×10^{-3}	2.5×10^{-3}
6.2	9.3×10^{-4}						3.1×10^{-3}		9.3×10^{-4}
6.4	4.7×10^{-4}				8.2×10^{-4}	1.6×10^{-3}			6.4×10^{-4}
6.5					4.0×10^{-4}				4.0×10^{-4}
6.8	1.5×10^{-4}								1.5×10^{-4}
6.9	1.2×10^{-4}								1.2×10^{-4}
7.0		8.0×10^{-5}	7.8×10^{-5}	1.0×10^{-4}	1.5×10^{-4}	1.6×10^{-4}	1.0×10^{-4}	2.5×10^{-4}	1.1×10^{-4}
7.6	2.0×10^{-5}				1.0×10^{-4}				6.0×10^{-5}
8.0	1.0×10^{-5}	6.4×10^{-6}	1.0×10^{-5}	1.3×10^{-6}	4.0×10^{-5}	1.0×10^{-5}			1.4×10^{-5}
9.0	3.2×10^{-6}	1.7×10^{-6}	3.8×10^{-6}	8.0×10^{-7}					2.4×10^{-6}
9.5			1.9×10^{-6}						1.9×10^{-6}

From Håkanson (1963, 1964, 1966, 1967a and b); Håkanson and Owman (1966b); Aures, Håkanson and Schauer (1968).

** Mean values were calculated from the data of the 5 left columns only since these results are much more accurate than those in the remaining 3 columns.

5.4 *Cofactor requirements of mammalian histamine forming enzymes*

With very few exceptions (Rosenthaler, Guirard Chiang and Snell, 1965) pyridoxal-5 -phosphate (PALP) is the coenzyme of amino acid decarboxylases of bacterial as well as of mammalian origin (Ono and Hagen 1959 Rothschild and Schayer, 1959 Awapara, Sandman and Hanley 1962)

DOPA/5-HTP decarboxylase however has proved difficult to resolve completely in inactive apoenzyme and free coenzyme. In extracts from certain tissues, the coenzyme seems to be rather tightly bound to the apoenzyme (Clark, Weissbach and Udenfriend, 1954) in such a case it can be very difficult to prove that a coenzyme participates in the reaction. It has been shown, however that addition of PALP to highly purified or semipurified preparations of DOPA decarboxylase from *e.g.* ox adrenal and rabbit pineal gland causes a marked increase in enzyme activity (Fellman 1959 Hårdanson and Owman 1966a) Furthermore, carbonyl trapping agents, which react with PALP are inhibitors of DOPA/5-HTP decarboxylase (Clark, 1963 Pletscher, Gey and Burkard 1966) Together these observations indicate that PALP is the coenzyme also of DOPA decarboxylase.

Fellman (1959) showed that substances which combine with SH-groups (N-ethyl maleimide and mercuric ions) inhibit ox adrenal DOPA decarboxylase. This may indicate that the non-specific aromatic amino acid decarboxylase contains SH-groups, possibly at its active center No metal ion tested activated DOPA decarboxylase, and EDTA did not inhibit.

Foetal rat *histidine decarboxylase* is almost completely resolved into free apoenzyme by the purification procedure used (Hårdanson 1963) Prolonged dialysis against carbonyl trapping agents such as semicarbazide enhances the resolution of the holoenzyme (Hårdanson 1967c) Of all the compounds initially tested (pyridoxal, pyridoxal-5 phosphate, pyridoxine, pyridoxamine, pyridoxamine-5 phosphate and the cyanohydrin derivative of pyridoxal 5 -phosphate) only PALP was capable of reactivating the enzyme (Hårdanson 1967c) Only small amounts of the coenzyme were required for maximal activation. While the addition of PALP readily activated the enzyme, the cyanohydrin derivative failed to do so. If both PALP and the cyanohydrin derivative were added to the incubation mixture, the enzyme-activating effect of PALP was inhibited, possibly through competitive inhibition. This observation is at variance with the results of Bonavita and Scardi (1958a) on glutamic-oxaloacetic transaminase but nevertheless support their conclusion that PALP is attached to the apoenzyme by the carbonyl group as well as by the phosphate ester group. In agreement with this assumption it was observed that pre incubating the enzyme with pyridoxal caused a moderate inhibition of the coenzyme activity of PALP possibly through pyridoxal occupying the carbonyl attachment site. The equilibrium constant of the coenzyme histidine apodecarboxylase complex was found to change with pH in a manner which suggests

that the reactants involved are particular ionic species of the molecules (HÄKANSON 1967c)

EDTA or EGTA in concentrations of 10^{-3} M did not interfere with the formation of histamine (see also WEISSBACH, LOVENBERG and UDENFRIEND, 1961 AURES and CLARK, 1964) In concentrations below 10^{-4} M metal ions (Mg^{++} Cu^{++} and Fe^{+++}) were inert. There was thus no evidence that metal ions are essential for the molecular mechanism of histamine biosynthesis. Iodoacetamide (final concentrations 5×10^{-4} M) added either before or after PALP failed to inhibit mammalian histidine decarboxylase indicating that SH-groups are not involved in the reaction.

In subsequent studies it was observed that phospho-5 pyridoxaminotriazole (PPIT) a condensation product of PALP and 4-amino-1 2 4-triazole, was capable of activating mammalian L histidine apodecarboxylase (AURES, HÄKANSON and WIBERMAN 1969) Previously PPIT had been found potent in activating L DOPA decarboxylase (GONNARD and NGUYEN CHI 1959) PPIT is known to be a stable compound and GONNARD concluded that PPIT *per se* acted as coenzyme (GONNARD DUHAULT CARLIER, NGUYEN PHILIPPON and BOIONE, 1967) This was in disagreement with the concept of BRAUNSTEIN and SNELL BRAUNSTEIN and SHEMYAKIN 1953 METZLER, IKAWA and SNELL 1954) but predicts that only compounds with a free or potentially free carbonyl group should act as coenzyme. However it was observed that PPIT reacts with certain amino acids (but not with all) forming condensation products with ultraviolet absorption and fluorescence spectra identical with those of their aldimines with PALP (AURES HÄKANSON and WIBERMAN 1967) It was concluded that the activating effect of PPIT on L histidine apodecarboxylase is the result of an initial transaldimination of PPIT at the enzyme site leaving the conventional holoenzyme (apoenzyme-PALP aldimine) and free aminotriazole. The affinity of PPIT for the apoenzyme may differ from that of PALP itself but after the holoenzyme complex is established, the enzyme-substrate reaction proceeds in a normal fashion.

5.5 *Histamine forming enzymes of gastric mucosa*

SCHAYER (1956b 1957) was the first to show that the glandular stomach of the rat had a high histidine decarboxylating activity and that this enzyme was different from the histidine decarboxylase of rabbit kidney This observation was later confirmed by a number of authors (KAHLSON *et al.* 1964 HÄKANSSON and OWMAN 1966b LEVINE and WATTS 1966) and the experiments of HIGHT, SHEPHERD and WOODCOCK (1965) and SHEPHERD and WOODCOCK (1968a) established beyond doubt that in the rat the glandular stomach is a major source of histamine formation *in vivo*

The kinetic properties of a crude preparation of rat stomach histidine decarboxylase was first studied by WEISSBACH, LOVENBERG and UDENFRIEND (1961) who demonstrated a much lower K_m value for this enzyme than

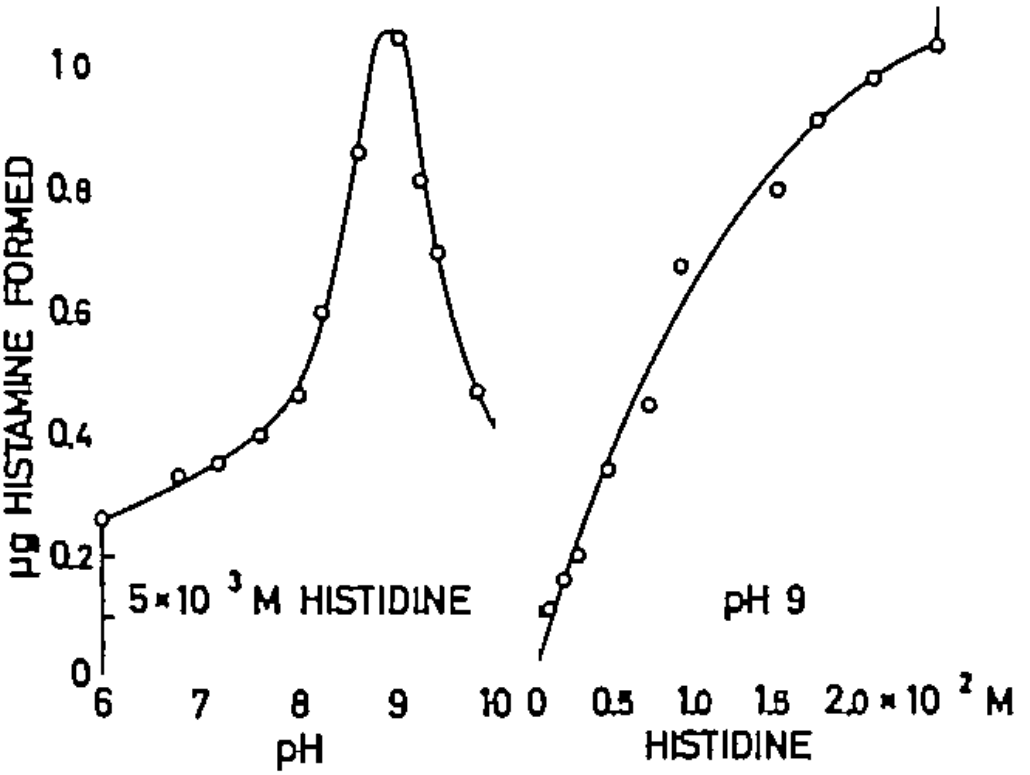


Fig. 26. Non-specific histamine-forming enzyme of rabbit stomach. (Left) Reaction velocity *versus* pH. (Right) Reaction velocity *versus* substrate concentration.

for the formation of histamine catalyzed by DOPA decarboxylase. This initial observation was confirmed by HÅKANSON and OWMAN (1966b) and by AURELL, HÅKANSON and SCHAUER (1968) using a semi-purified preparation of rat stomach histidine decarboxylase. The purification procedure was similar to that used for foetal rat histidine decarboxylase. It could be shown that the pH optimum of the enzyme varied with the substrate concentration (Fig 22) and that the K_m value increased with increasing hydrogen ion concentration (Table 7) Gastric histidine decarboxylase was thus found to have kinetic properties very similar to those of the histamine-forming enzyme of other tissue sources such as the foetal rat or the hamster placenta. It was apparent, however already from the studies of WEISSBACH *et al.* (1961) that the histamine forming enzyme of the guinea-pig and rabbit stomach (see also DAWSON MAUPAILEY and WEST 1965 HÅKANSON and OWMAN 1966b AURELL DAVIDSON and HÅKANSON 1969) was different from of the rat stomach. The histamine forming enzyme of the guinea pig and rabbit stomach was found to have kinetic properties similar to those reported for histamine formation catalyzed by DOPA decarboxylase. Thus, with these preparations the optimum pH for the histamine formation was close to 9 and the K_m value at this pH was very high (Fig. 26) Moreover it could be established that also the rat stomach had high activities of DOPA decarboxylase, in addition to the "specific" histamine-forming enzyme (HÅKANSON 1965 HÅKANSON and OWMAN 1966b AURELL, HÅKANSSON and SCHAUER, 1968)

The histidine decarboxylase of the glandular rat stomach is activated by a number of physiological and pathophysiological (Code and Hallenbeck, 1961 Kahlson *et al* 1964 Castellucci, 1965 Lilja and Svensson 1967 Fischer and Snyder, 1965a and b Johnson 1967 Håkanson and Liedberg, 1970a) and pharmacological stimuli (Kahlson Rosenberg and Thunberg 1967 Aures Håkanson and Schauer, 1968) It has been suggested that the adaptivity of the enzyme reflects a physiological control function of histamine in gastric secretion and that depletion of the gastric histamine stores caused for instance by gastrin (Kahlson *et al.* 1964) triggers the events leading to increased histidine decarboxylase activity and thus to the rapid replenishment of the histamine content of the gastric mucosa (Kahlson *et al.*, 1964)

Fasting and feeding have marked effects on the histidine decarboxylase activity of the rat stomach After 48 hours of starvation, the enzyme activity is reduced to very low levels Intake of food at this stage results in a 5-10-fold activation of gastric histidine decarboxylase within 1—2 hr The activity of gastric DOPA decarboxylase, on the other hand was not significantly affected by feeding or fasting (Aures Håkanson and Schauer, 1968) A number of drugs were tested for their effect on gastric amino acid decarboxylases. Gastrin was most effective in activating the histidine decarboxylase the activation occurred very rapidly after 1 hr the enzyme was maximally activated and the activity stayed high for another hour Insulin, 2-deoxyglucose, 2-deoxygalactose, reserpine and carbachol were also quite potent in increasing the level of gastric histidine decarboxylase (Aures Håkanson and Schauer, 1968) None of these compounds had any effect on gastric DOPA decarboxylase. Histamine and histidine failed to activate histidine decarboxylase in fact, both these compounds caused a reduction of the enzyme activity in fasted rats. Polymyxin B and Compound 48/80 did not activate gastric histidine decarboxylase, neither did they affect gastric DOPA decarboxylase (Aures Håkanson and Schauer, 1968) One possible explanation for the enzyme-activating effect of certain drugs and certain treatments is that they release endogenous gastrin, which probably is the mediator of the enzyme activation. For a discussion of this concept see section 3 10

Very little is known about the mechanism by which gastrin activates the histidine decarboxylase of the enterochromaffin-like cell system in the rat stomach. Observations by Snyder and Epps (1968) suggest that the increased histidine decarboxylase activity is a result of accelerated synthesis of enzyme protein. In this context the observation of a local protein synthesis-stimulating effect of gastrin on certain parts of the digestive tracts gains added significance (Johnson Aures and Håkanson 1969 Johnson Aures and Yuen 1969)

Observations on the regional and topographical distribution of gastric histidine decarboxylase and DOPA decarboxylase in the rat indicate that most of the histidine decarboxylase is contained in the histamine-storing enterochromaffin like cells of the oxyntic gland area, whereas the major portion of DOPA decarboxylase is found in the 5-HT-containing enterochromaffin cells of the

pyloric gland area (Aures Håkanson and Schauer, 1968) It appears well established, however that the enterochromaffin-like cells in addition also contain DOPA decarboxylase (Håkanson and Owman 1966b Håkanson Lilja and Owman 1967 Aures Håkanson and Schauer, 1968 section 3.6) although in considerably lower quantities than the enterochromaffin cells. No histidine decarboxylase activity has been demonstrated in the latter cell system and there was no evidence that the mast cells of the stomach wall add significantly to the activities of either histidine decarboxylase or DOPA decarboxylase (Aures Håkanson and Schauer, 1968)

High activities of gastric DOPA decarboxylase were found in all mammals studied the regional distribution of the enzyme generally agreed very well with the distribution of the enterochromaffin cells (Håkanson *et al.* 1970d) Gastric histidine decarboxylase, on the other hand, could be demonstrated only in the mouse, rat and hamster in the other species the activity was either non-measurable or too low to permit identification of the enzyme. This was the case also under conditions which are known to result in maximal stimulation of gastric histidine decarboxylase activity in the rat, *i.e.* after the intake of food or after the injection of gastrin (Aures Davidson and Håkanson 1969) This finding, which is in agreement with previous reports from some laboratories (Weisbach, Lovenberg and Udenfriend 1961 Håkanson and Owman 1966b Aures, Håkanson Owman and Sporrong, 1968 Kim and Glick, 1969 Håkanson Lilja and Owman 1969) disagrees with those of some other groups (Kahlson Rosengren Svahn and Thunberg 1964 Kahlson Rosengren and Thunberg 1967 Werle and Lorenz, 1964 Lorenz, Pfleger and Werle, 1967 Lorenz, Hahlbach, Gerant and Werle, 1969) These conflicting observations may perhaps to some extent reflect differences in the strains of animals used and in the treatment of the animals but it appears more likely that they reflect differences in experimental technique. Lorenz and co-workers have been using a fluorometric method for the determination of histidine decarboxylase activity and Kahlson and co-workers a radiometric method, the so-called HFC assay For the discussion and evaluation of these methods see Aures Davidson and Håkanson (1969) and section 2.1 It must be emphasized, also in this context, that it is not enough to register formation of histamine the catalyzing agent has to be further characterized to permit an identification with the "specific" histidine decarboxylase.

Radwan and West (1967) described a third histamine-forming enzyme in the ruminal portion of the rat stomach, with a pH optimum of 5.5 The presence of this enzyme could not be confirmed by Aures, Håkanson and Schauer (1968) Kobayashi and Maudsley (1969) however recently reported that the enzyme occurred predominantly in male rats and convincingly documented its occurrence in the rumen and its kinetic properties. It appears that this enzyme is insoluble and unaffected by fasting and feeding. Very little is known of the factors regulating the activity of this enzyme, as well as of its properties and possible physiological significance. Kinetically this enzyme

resembles bacterial histidine decarboxylase, and the possibility that the enzyme activity reflects bacterial contamination has still not been definitely excluded.

SCHAYER (1960a and b 1963a 1964) and SCHAYER and GANLEY (1959) have reported that histidine decarboxylase in many tissues of the rat and mouse, such as skin, muscle and kidney can be markedly activated by the injection of adrenaline, histamine liberators and endotoxins. It is apparent that although gastric histidine decarboxylase and the inducible" histidine decarboxylase of SCHAYER may be similar kinetically the physiological and pharmacological factors which control their activities are quite different.

5 6. *Molecular mechanism of enzyme catalyzed histamine formation*

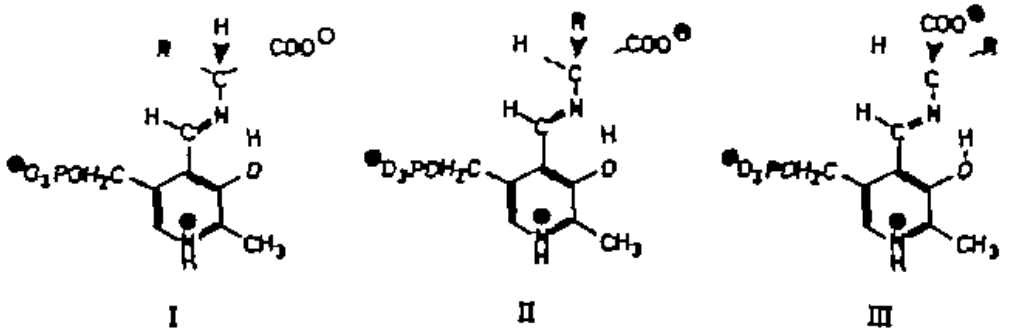
General mechanism of amino acid decarboxylation PALP acts as coenzyme in a number of enzyme reactions involving α-amino acids in particular It is generally accepted that for pyridoxal-enzymes the coenzyme constitutes the catalytic nucleus of the active site and that the surrounding protein structure provides the steric arrangement which accounts for the specificity of the reaction. A general scheme for PALP-catalyzed reactions was proposed by BRAUNSTEIN and SHEMYAKIN (1953) and by METZLER, IKAWA and SNELL (1954) based on their studies of the non-enzymic interaction of pyridoxal with amino acids. When solutions of pyridoxal and amino acids are mixed an immediate yellowing occurs as the result of an imine (Schiff base) formation between the two reactants. The Schiff base is a highly reactive product the subsequent transformation of the amino acid depends upon the reaction conditions and may involve deamination racemization, decarboxylation, αβ-eliminations or cleavage Addition of appropriate metal ions increases the rate of deamination and αβ-elimination reaction but inhibits decarboxylation. This may be due partially to the increased rate of other pyridoxal-catalyzed reactions that are promoted by metal ions alternatively it may result from formation of chelates in which the binding of the carboxyl group to metal ion slows its release as carbon dioxide.

General mechanisms were proposed to explain the above results. All avilable evidence suggested that the non-enzymatic reactions proceed by mechanisms closely resembling those of the corresponding enzymatic reactions. It is apparent therefore, that PALP constitutes the catalytic nucleus of the active center of each of the many pyridoxal-dependent enzymes. SNELL (1958) suggested that the apoenzyme can be visualized as replacing the metal ion in non-enzymatic reactions and in so doing plays a similar but even more effective role in the enzymatic reactions.

The original model suggested that the amino acid substrate reacted with the free carbonyl-group of enzyme-bound PALP forming a Schiff base which was subsequently degraded or modified in a specific manner depending on the stereochemical configuration of the active site. The observations of BONAVITA and SCARDI (1958a and b) introduced a slightly different concept, according

to which PALP was attached to the apoenzyme via an aldimine bond and reaction with substrate involved an initial transaldimination in which the amino acid substrate formed a Schiff base with the enzyme-bound PALP displacing the apoenzyme amino nitrogen. There are reasons to believe that in some cases the coenzyme forms an aldimine with the -amino group of a lysine residue of the apoenzyme (Fischer, Krnt Snyder and Krebs 1958 Dempsey 1960 Hughes Jenkins and Fischer 1962) Snell (1958) pointed out that the binding of the carbonyl group does not exclude the intermediary -zomethine formation between enzyme-bound PALP and substrate. On the contrary such binding may be partially responsible for substrate specificity since only certain amino compounds would compete successfully with groupings from the protein for the carbonyl group of the coenzyme Although the formyl and phosphate groups have been emphasized as the primary site of binding other functional groups of the pyridine ring may be considered. There is no evidence as yet that the phenolic hydroxyl group and the ring nitrogen are directly involved in coenzyme binding

It should be noted that Gonnard and Nouyen Chi (1959) and Murakami and Makino (1966) have refuted the catalytic mechanism proposed by Braunstein and Snell on the basis of observations showing that certain carbonyl-substituted derivatives of PALP could act as coenzyme. This has been demonstrated for a variety of amino acid decarboxylases, including mammalian histidine decarboxylase and DOPA decarboxylase. Gonnard Duhault Camier, Nouyen Phillipon and Boione (1964) interpreted the results as indicating that these derivatives function coenzymatically *per se* this is contradictory to the theory of Braunstein and Snell, which requires a free—or potentially free—carbonyl group of PALP The interpretation of Gonnard has been criticized by Torchinsky (1963) and Aurea Hakanson and Wiseman (1969) who have pointed out that the results may equally well be explained by an initial transaldimination of the PALP-aldimine at the active sites of the apoenzyme, leaving the conventional holoenzyme and stoichiometric amounts of liberated carbonyl trapping compound.



I II III

Fig. 27 Various steric conformations of the pyridoxal-5 -phosphate amino acid imine. The arrow indicates the position (perpendicular to the α-carbon) which is activated by th cofactor pi system. Conformation III is appropriate for decarboxylation.

DUNATHAN (1966) has emphasized the specific geometrical requirements for each type of chemical reaction catalyzed by PALP enzymes. The main function of the cofactor pi system is to weaken the sigma bonds around the α-carbon of the amino acid. For this purpose the stereochemical relationship of the sigma bond to the adjacent pi orbitals is important. Experimental results have indicated a clear preference for the geometry in which the bond to be broken lies in a plane perpendicular to that of the pyridoxal imine system. Conformation III as illustrated in Fig. 27 is appropriate for α-decarboxylation. The position of the enzymatic site binding the carboxyl anion of the amino acid is probably the most important factor in controlling the conformation which determines the reaction specificity. If a change is made in the configuration at the α-carbon of the amino acid and if the carboxyl binding site continues to control the conformation about the α-carbon to nitrogen bond, a different group on the α-carbon may be subject to the activating influence of the PALP ring. From theoretical considerations, however, it can be predicted that an α-decarboxylase should never show reactions involving labilizations of groups other than the carboxyl group since only the carboxyl anion can occupy the "activated" position. On the other hand, the stereochemical relationship between PALP and the carboxyl binding site is such that α-decarboxylases should be able to accept as substrates also D-amino acids. In Fig. 28 R and H may exchange positions at the active site while the carboxyl anion remains in the activated position. However, in the case of mammalian histidine decarboxylase and DOPA decarboxylase absolute stereospecificity exist, permitting enzymatic transformation of the substrate L forms only. This would indicate that the holoenzyme offers specific binding sites for other groupings on the amino acid substrate apart from the amino group and the carboxyl group; apparently this third binding site strictly determines the three-dimensional conformation

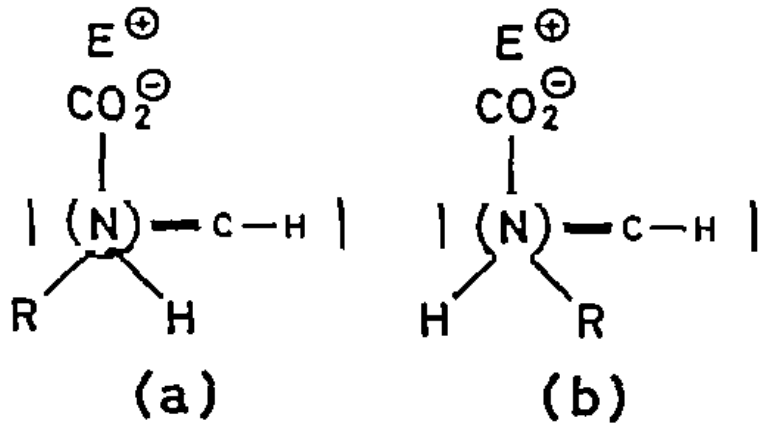


Fig. 28. Various conformations of the pyridoxal-5'-phosphate-amino acid imine as viewed along the α-carbon to nitrogen bond. The aldehydic carbon of PALP is shown (=C—) and the atoms of the pyridine ring are indicated by the rectangular box. The anionic carboxyl group is attached to a cationic group at the enzyme active site and occupies the activated position (perpendicular to the α-carbon). Theoretically both L- and D-forms, (a) and (b) can be decarboxylated, since for the reaction the positions of R and H are irrelevant.

of the enzyme-substrate complex and prevents the holoenzyme from accepting also the D-form of the substrate. With histidine this third binding site probably involves the imidazole ring with DOPA as substrate the corresponding structure may be the catechol. It should be noted that since amino acid decarboxylases appear to accept also α-methylated derivatives the α-hydrogen is not necessary for the decarboxylase activity (see MANDELES, KOPELMAN and HANES, 1954) and presumably not involved in binding to the apoenzyme.

In the interaction between pyridoxal-enzymes and amino acid substrates the first step is considered to be the transaldimination reaction which involves the enzyme-bound Schiff base of the coenzyme and the amino acid substrate (Fig. 29 I and II). It has been suggested that the reactants may be in particular ionic species of the amino acid and of the enzyme-bound PALP (HIRANSON 1963, 1966, 1967a, b, c and d, see also below). The first step in the reaction scheme is considered to be the weakening of the bonds in the α-position. The system of conjugated double bonds present in the aldimine will withdraw electrons to the electrophilic nitrogen atom of the pyridine ring to withdraw electrons to the nitrogen atom which was formerly the α-nitrogen of the amino acid. The bonds in the sigma bonds around the α carbon are thus weakened clearing the way for reactions which then results respectively in the release of a proton, or of CO_2 or of the carbanion. Subsequent rearrangements would lead to racemization, transamination, cleavage or decarboxylation of the amino acid. In the case of decarboxylation, the Schiff base looses CO_2 to form an intermediate structure (III). Addition of a proton leads to structure (IV) which is the Schiff base of the amine produced by decarboxylation of the amino acid. Transaldimination of this Schiff base gives the free amine with regeneration of the holoenzyme (the amine of PALP and apoenzyme).

Molecular mechanism of histamine formation. It has been suggested that the 4-formyl group of PALP is attached to histidine apodecarboxylase by an aldimine bond and that the reaction with the substrate involves transaldimination: the amino acid substrate forms a Schiff base with the enzyme-bound PALP displacing the apoenzyme amino nitrogen (HIRANSON 1967d). However as pointed out by JENCKS (1963) the reactants are probably particular ionic species of the amino acid and of the aldimine. From consideration of non-enzymatic reactions it may be assumed that the free amine form of the amino acid reacts with the protonated form of the enzyme-bound Schiff base. This assumption has important consequences as regards the kinetics of PALP-catalyzed enzyme reactions. The requirement that only the free base form of the substrate should react causes the K_m to become pH dependent. Moreover it may be expected that if the active ionic species of the enzyme occurs in high concentrations at a pH value much below the pK of the substrate-NH_2 group, the pH optimum of the reaction will depend on the substrate concentration. At saturating substrate concentrations the optimal pH will be determined exclusively by the pK values for the dissociation of the enzyme-substrate complex; with very low substrate concentrations the fact that

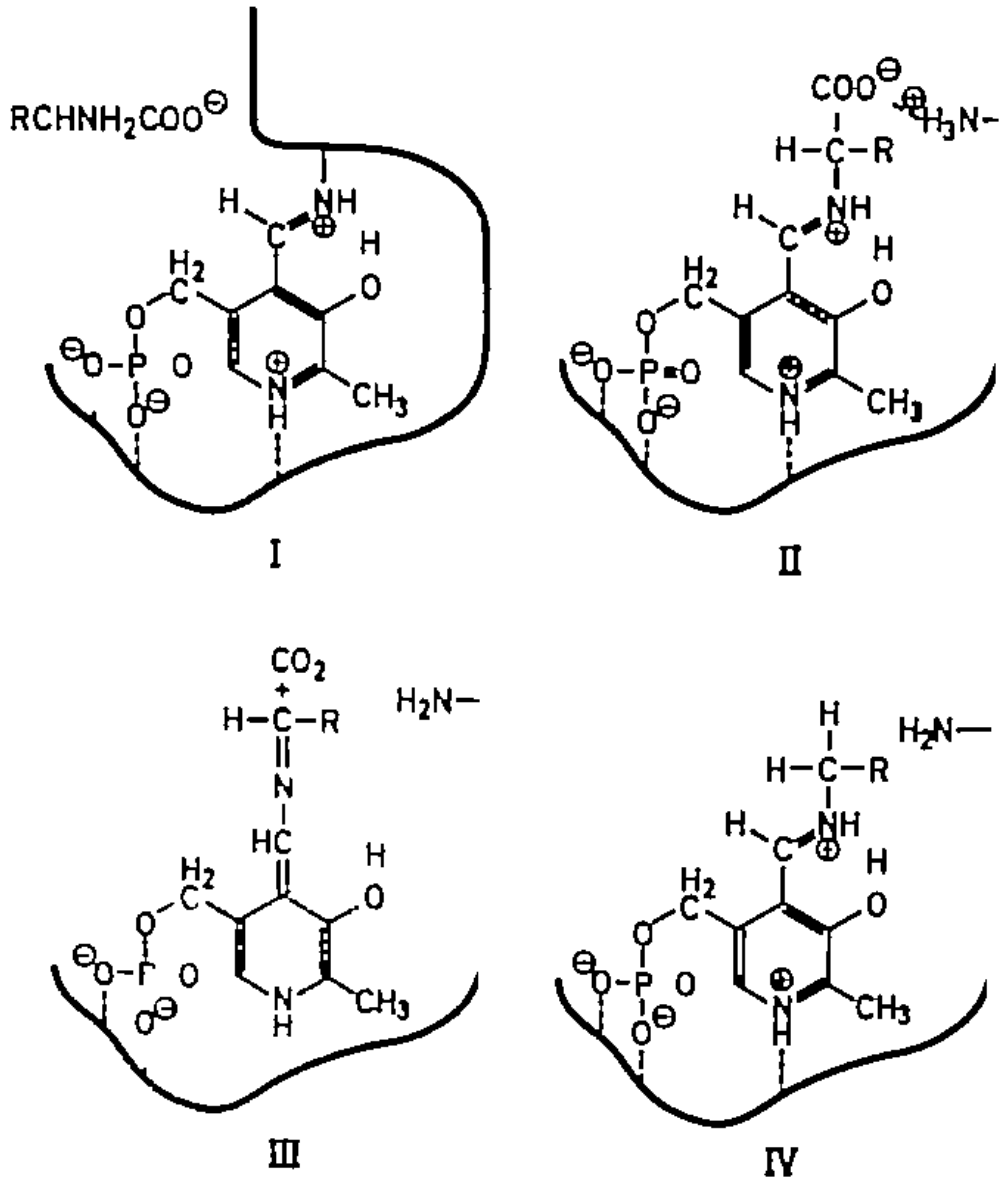


Fig. 29 Histidine decarboxylase Schematic presentation of the sequence of events leading to the formation of histamine. Non-protonated histidine and enzyme-bound protonated pyridoxal-5 -phosphat imine are about to react (I) by a process of transaldimination (II) Electrons are w thdrawn and decarboxylation takes place (III) the protonated Schiff base of histamine and pyridoxal-5 phosphate exchs with the non-protonated amino group of the enzyme activ ate (IV) which results in the liberation of histamine and the reconstitution of the holoenzyme (the protonated imine of pyridoxal 5 phosphate and enzyme amino nitrogen)

the reactive species of the substrate prevails at pH values above the pK of the substrate NH will shift the pH optimum towards more alkaline values. These effects may of course be masked by the influence of other ionizing groups of the substrate or of the enzyme Nevertheless, the kinetics of certain PALP enzymes seem to provide support for the assumption that only the non-protonated form of the substrate is involved in the reaction Davis and Metzler (1962) observed that the K_m of threonine dehydratase (deaminase) (EC 4 2.1 16)

decreased with increasing pH and concluded that the substrate anion containing a non-protonated amino group reacts with the enzyme. Some amine oxidases are PALP-dependent (Werle and Pechmann 1949 Sinclair, 1952 Davison 1956 Kapeller-Adler and MacFarlane, 1963 Yasunobu and Yamada, 1963 Blaschko and Buffoni, 1965 Buffoni, 1966 Suzuki 1966) Human plasma benzylamine oxidase (McEwen 1965a) appears to be fairly similar to pig plasma benzylamine oxidase (Buffoni, 1966) which is a cupric-PALP enzyme (Yasunobu and Yamada, 1963) The pH optimum of human plasma benzylamine oxidase is inversely related to the substrate concentration and the K_m decreases with increasing pH (McEwen 1965b) From these and similar data, Buffoni (1966) recently claimed that all plasma amine oxidases act on the non-protonated form of the substrate.

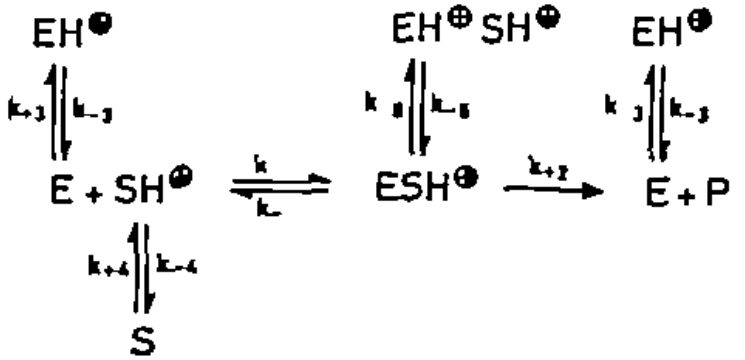
The kinetic properties (Fig 22—25) of mammalian histidine decarboxylase (EC 4 1 1.22) suggest that only the anionic form of histidine reacts with the enzyme. The observations summarized in section 5.3 are all understandable on the basis of an interaction between the protonated form of the enzyme-bound Schiff base and the free amine form of the substrate. It may be argued that the reaction between non-protonated Schiff base and protonated substrate (Eq 1b) is kinetically indistinguishable from the reaction involving non protonated substrate and protonated aldimine (Eq. 2b) (see also Jencks, 1963)

$$v = k\ (RNH_3^+)\ (>C=N-) \tag{1b}$$

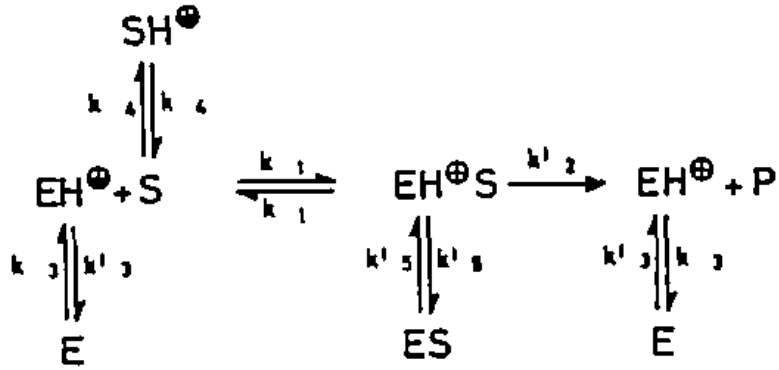
$$v = k\ (RNH_2)\,(>C=NH^+-) \tag{2b}$$

However some differences in the kinetics of PALP-enzyme-catalyzed reactions may be predicted from assumptions as to the ionic nature of the reactants involved The alternative enzyme reactions may be represented by the following simplified reaction schemes.

A. Non-protonated enzyme and protonated substrate.



B. Protonated enzyme and non protonated substrate



From these reaction schemes alternative kinetic equations may be derived from the usual assumption of a steady state.

A.

$$V = v\left[\frac{K_m}{(SH^+)}\left(1+\frac{(H^+)}{K_e}\right)+1+\frac{(H^+)}{K_{es}}\right] \qquad (3b)$$

where

$$K_m = \frac{(E)\,(SH^+)}{(ESH^+)} = \frac{k_{-1}+k_{+2}+k_{+3}}{k_{+1}}$$

$$K_e = \frac{(E)\,(H^+)}{(EH^+)} = \frac{k_{-3}}{k_{+3}} \quad K_{es} = \frac{(ES)\,(H^+)}{(ESH^+)} = \frac{k_{-2}}{k_{+2}} \quad v = k_{+2}(ESH^+)$$

$V = k_{+2}(E_t)$ and (E_t) is the total enzyme concentration. If the total substrate concentration (S_t) is very large

$$V = v\left(1+\frac{(H^+)}{K_{es}}\right) \qquad (4b)$$

With a diminishing H^+ concentration v approaches V
If (S_t) is very small

$$V = v\left[\frac{K_m}{(S_t)}\left(1+\frac{K_e}{(H^+)}\right)\left(1+\frac{(H^+)}{K_e}\right)\right] \qquad (5b)$$

$$K_e = \frac{(S)\,(H^+)}{(SH^+)} = \frac{k_{-1}}{k_{+1}}$$

The value of (H^+) for maximum v at low (S_t) is $(H^+) = \sqrt{K_e K_e}$. Thus, with low (S_t) the reaction has a pH optimum which is close to the negative logarithm of $\sqrt{K_e K_e}$ but with increasing substrate concentration the pH optimum shifts towards more alkaline values. The apparent (i.e. experimentally established) Michaelis-Menten constant $(K_{m'})$ is defined with respect to the

total amount of substrate present and is related to the "true" Michaelis-Menten constant (K_m) through the following equation

$$K_m = K_m \frac{(1+\frac{(H^+)}{K_e})(1+\frac{K_e}{(H^+)})}{1+\frac{(H^+)}{K_{es}}} \qquad (6b)$$

With a diminishing H^+ concentration K_m increases. The changes in pH optimum and K_m predicted from scheme A are contrary to experimental observations and this reaction scheme is consequently not an acceptable model.

B.

$$V = v\left[\frac{K_m}{(S)}(1+\frac{K_e}{(H^+)}) + 1 + \frac{K_{es}}{(H^+)}\right] \qquad (7b)$$

If (S_t) is very large (and hence (S))

$$V = v(1+\frac{K_{es}}{(H^+)}) \qquad (8b)$$

With an increasing H^+ concentration v approaches V
If (S_t) is very small

$$V = v\left[\frac{K_m}{(S_t)}(1+\frac{(H^+)}{K_e})(1+\frac{K_e}{(H^+)})\right] \qquad (9b)$$

The value of (H^+) for maximum v at low (S_t) is $\sqrt{K_e K_{es}}$. With this model the pH optimum of the reaction is inversely related to the substrate concentration in agreement with experimental observations.

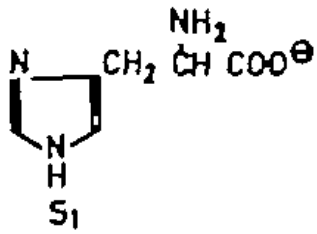
$$K_m = K_m \frac{(1+\frac{K_e}{(H^+)})(1+\frac{(H^+)}{K_e})}{1+\frac{K_m}{(H^+)}} \qquad (10b)$$

With an increasing H^+ concentration K_m increases as has also been observed experimentally with several PALP enzymes. The kinetic analysis of the two alternative models may thus provide some support for the hypothesis that the non protonated species of the amino acid is the "true" substrate. This may also be the case with mammalian DOPA decarboxylase (Hägerman 1967d). It may in fact, be a general characteristic of all PALP-catalyzed transformations of amino compounds.

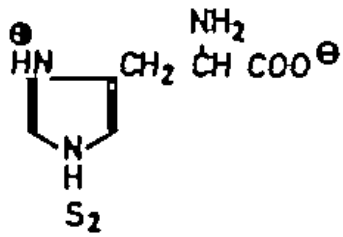
The assumption that the enzyme reacts only with the non-protonated form of histidine is also supported by the following analysis of the pH-dependent variation of the K_m value

It has been suggested that a constant true K_m value can be arrived at from the assumption that the enzyme attacks a specific ionic variety of histidine (HARRISON 1963 1966b) The various ionic species of histidine existing within the experimental pH region are given below The corresponding conservation equation for histidine is

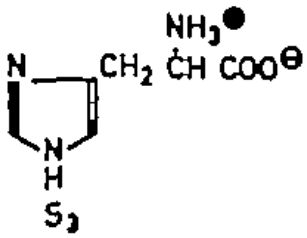
$$(S_t) = (S_1) + (S_2) + (S_3) + (S_4) \tag{1c}$$



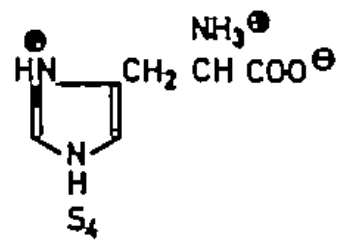
S_1



S_2



S_3



S_4

The concentration of each ionic variety of histidine is governed by the law of mass action and given by the following equations, which are derived from the conservation equation. (The K values are approximated to 10^{-6} and 10^{-9} the carboxyl group is assumed to be charged within the entire experimental pH region.)

$$(S_t) = (S_1)\ (1 + \frac{(H^+)}{10^{-6}} + \frac{(H^+)}{10^{-9}} + \frac{(H^+)^2}{10^{-15}}) \tag{2c}$$

$$(S_t) = (S_2)\ (1 + \frac{10^{-6}}{(H^+)} + \frac{10^{-6}}{10^{-9}} + \frac{(H^+)}{10^{-9}}) \tag{3c}$$

$$(S_t) = (S_3)\ (1 + \frac{(H^+)}{10^{-6}} + \frac{10^{-9}}{10^{-6}} + \frac{10^{-9}}{(H^+)}) \tag{4c}$$

$$(S_t) = (S_4)\ (1 + \frac{10^{-6}}{(H^+)} + \frac{10^{-9}}{(H^+)} + \frac{10^{-15}}{(H^+)^2}) \tag{5c}$$

A k_m value, which is fairly constant between 5.5 and 9.5 may be calculated from the apparent $K_m(K_{m'})$ by the relation.

$$K_{m'} = k_m f_{S_1} \quad K_{m'} = k_m \left(1 + \frac{(H^+)}{10^{-6}} + \frac{(H^+)}{10^{-9}} + \frac{(H^+)^2}{10^{-15}}\right) \text{ (Table 8)} \quad (6c)$$

The other ionic species of histidine (S_3, S_5 and S_7) give K_m values which vary markedly within the experimental pH region. However the enzyme may attack several ionic varieties of histidine indiscriminately

$$(S_1) = (S_0)\left(1 + \frac{(H^+)}{10^{-6}}\right) \quad (S_3) = (S_1) + (S_2) \quad (7c)$$

$$(S_4) = (S_6)\left(1 + \frac{(H^+)}{10^{-6}}\right) \quad (S_6) = (S\ ) + (S\ ) \quad (8c)$$

$$(S_7) = (S_7)\left(1 + \frac{10^{-6}}{(H^+)}\right) \quad (S_7) = (S_5) + (S\ ) \quad (9c)$$

$$(S_4) = (S_8)\left(1 + \frac{10^{-6}}{(H^+)}\right) \quad (S_8) = (S_5) + (S\ ) \quad (10c)$$

A k_m value, which is constant between pH 4.5 and 9.5 may be calculated from the apparent $K_m(K_{m'})$ by the relation

$$K_{m'} = K_m f_{S_0} \quad K_{m'} = K_m \left(1 + \frac{(H^+)}{10^{-5}}\right) \quad (11c)$$

The other ionic species of histidine (S_2, S_7 and S_8) give K_m values which vary markedly within the experimental pH region (Table 8)

Both relations (6c and 11c) are based on the assumption that the enzyme reacts with specific ionized varieties of histidine (S or S) and the role of functioning groups on the enzyme is not considered. Evidently the K_m values obtained from these formulae must be regarded as approximations. Work is in progress to identify the substrate attachment sites of the holoenzyme.

The process of enzymatic histamine formation involves three separate molecules apoenzyme, PALP and histidine. Normally reaction takes place between histidine and the complex of apoenzyme and PALP However under artificial conditions it is possible to study also the reaction between the apoenzyme and the histidine PALP aldimine (HÅKANSON 1967c) The reaction between histidine and PALP can be followed spectrophotometrically and appears to go to completion in the presence of high amounts of histidine and at pH values above 5 A high rate of histamine formation was observed after the addition of apoenzyme to a solution containing the Schiff base of histidine and PALP The kinetics of the decarboxylation of the Schiff base were different from that of the conventional holoenzyme-catalyzed histidine decarboxylation. The pH optimum of the aldimine decarboxylation was close to 6.2 at all substrate concentrations tested. The K_m value was found to decrease from 5×10^{-4}

TABLE 8

Km calculated from Km

The hypothetical substrate species is given in parenthesis

pH	Km (S_1)	Km (S_2)	Km (S_3)	Km (S_4)	Km (S_5)	Km (S_6)	Km (S_7)	Km (S_8)
3.5	3.7×10^{-10}	1.2×10^{-7}	1.2×10^{-4}	3.7×10^{-3}	1.2×10^{-7}	1.2×10^{-4}	3.7×10^{-3}	3.7×10^{-3}
4.0	2.9×10^{-9}	2.9×10^{-7}	2.9×10^{-4}	2.9×10^{-3}	2.9×10^{-7}	2.9×10^{-4}	2.9×10^{-3}	2.9×10^{-3}
4.5	2.7×10^{-8}	8.4×10^{-7}	0.8×10^{-3}	2.7×10^{-3}	0.8×10^{-6}	8.2×10^{-4}	2.7×10^{-3}	2.7×10^{-3}
5.0	1.2×10^{-7}	1.2×10^{-6}	1.2×10^{-3}	1.2×10^{-3}	1.3×10^{-6}	1.2×10^{-3}	1.3×10^{-3}	1.2×10^{-3}
5.2	2.1×10^{-7}	1.4×10^{-6}	1.4×10^{-3}	0.9×10^{-3}	1.6×10^{-6}	1.4×10^{-3}	10^{-3}	0.9×10^{-3}
5.5	1.3×10^{-6}	2.3×10^{-6}	2.3×10^{-3}	7.3×10^{-3}	3.0×10^{-6}	2.3×10^{-3}	9.5×10^{-3}	7.2×10^{-3}
6.0	0.7×10^{-6}	1.3×10^{-6}	1.3×10^{-3}	1.3×10^{-3}	2.5×10^{-6}	1.3×10^{-3}	2.5×10^{-3}	1.3×10^{-3}
6.	0.9×10^{-6}	5.7×10^{-7}	5.7×10^{-4}	3.6×10^{-4}	1.5×10^{-6}	5.7×10^{-4}	9.3×10^{-4}	3.6×10^{-4}
6.4	1.1×10^{-6}	4.6×10^{-7}	4.6×10^{-4}	1.8×10^{-4}	1.6×10^{-6}	4.6×10^{-4}	6.4×10^{-4}	1.8×10^{-4}
6.5	10^{-6}	3.0×10^{-7}	3.0×10^{-4}	0.9×10^{-4}	1.3×10^{-6}	3.0×10^{-4}	4.0×10^{-4}	10^{-4}
6.8	0.8×10^{-6}	1.3×10^{-7}	1.3×10^{-4}	2.1×10^{-5}	0.9×10^{-6}	1.3×10^{-4}	1.5×10^{-4}	2.1×10^{-5}
6.9	0.8×10^{-6}	1.1×10^{-7}	1.1×10^{-4}	1.4×10^{-5}	0.9×10^{-6}	10^{-4}	1.2×10^{-4}	1.4×10^{-5}
7.0	10^{-6}	10^{-7}	10^{-4}	10^{-5}	1.1×10^{-5}	10^{-4}	1.1×10^{-4}	10^{-5}
7.6	2.3×10^{-6}	5.6×10^{-6}	5.6×10^{-5}	1.4×10^{-5}	2.4×10^{-6}	5.9×10^{-5}	5.8×10^{-5}	1.5×10^{-5}
8.0	1.3×10^{-5}	1.3×10^{-6}	1.3×10^{-5}	1.3×10^{-7}	1.3×10^{-6}	1.4×10^{-5}	1.3×10^{-5}	1.4×10^{-7}
9.0	1.2×10^{-4}	1.2×10^{-6}	1.2×10^{-5}	1.2×10^{-6}	1.2×10^{-6}	2.4×10^{-6}	1.2×10^{-6}	2.4×10^{-6}
9.5	1.4×10^{-4}	4.6×10^{-10}	4.6×10^{-7}	1.4×10^{-10}	1.4×10^{-6}	1.9×10^{-6}	4.6×10^{-7}	0.6×10^{-10}

at pH 5 to 3×10^{-7} at pH 9 (HÅKANSON 1967c) There is thus a marked difference between the pH-dependent change of the K_m of aldimine decarboxylation and that of the conventional enzymatic decarboxylation of histidine. If the pH-dependent change in the K_m value of histidine decarboxylation, particularly notable between pH 6 and 8 is caused by a lower availability of "true" substrate at more acid pH, it is to be expected that this pH-dependent change is abolished when the reaction takes place between apoenzyme and the Schiff base.

In conclusion, it may be noted that also the formation of holoenzyme (the reaction between PALP and apoenzyme amino nitrogen) should proceed according to the rule that only the non-protonated amino group reacts. This may provide a starting point for a mathematical analysis of this interaction as well, ultimately permitting the identification of the various ionic species of the reactants engaged in the formation of the holoenzyme complex.

6. SHORT SUMMARY

1 Several histamine-forming enzymes exist in mammalian tissues. One is non-specific and probably identical with DOPA decarboxylase. Another one appears to be specific and hence is referred to as specific histidine decarboxylase. DOPA decarboxylase has a wide distribution in the mammalian organism. The specific histidine decarboxylase has been demonstrated only in a limited number of tissues. The kinetics of the formation of histamine as catalyzed by DOPA decarboxylase seems to justify the conclusion that the histamine forming capacity of DOPA decarboxylase is negligible *in vivo*. The pH optimum of the reaction is at 8.0—9.5 and very high concentrations of histidine are required for saturation of the enzyme. The kinetic properties of histidine decarboxylase are quite different. The pH optimum is dependent upon the substrate concentration: at high concentrations the pH optimum is at about 6.0; at low concentrations the corresponding value is 7.2. The Michaelis-Menten constant increases markedly with diminishing pH. This behaviour can be explained by the assumption that only the non-protonated species of histidine is decarboxylated by the enzyme. This assumption is supported by mathematical analysis of the kinetics of enzymatic histamine formation.

2. Gastric mucosa of all species studied is rich in DOPA decarboxylase. In addition, gastric mucosa of the mouse, rat and hamster contains histidine decarboxylase, kinetically identical with histidine decarboxylase from foetal rat tissues and hamster placenta. Histidine decarboxylase in the glandular portion of the rat stomach is located in the histamine-containing enterochromaffin like cells of the oxyntic gland area; these cells also contain DOPA decarboxylase. The major portion of gastric DOPA decarboxylase, however, appears to be located in the 5-HT-containing enterochromaffin cells, which are devoid of histidine decarboxylase. Gastric histidine decarboxylase is adaptive. The mechanism of activation of gastric histidine decarboxylase has been studied in some detail only in the rat. Starvation markedly reduces the enzyme activity; this reduction is reversed by the injection of gastrin, by the intake of food, and by other treatments known to cause the release of endogenous gastrin. The activity of DOPA decarboxylase in the rat stomach is resistant to all the treatments which affect the histidine decarboxylase activity.

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ACTA PHYSIOLOGICA SCANDINAVICA
SUPPLEMENTUM 341

Noradrenaline Uptake Mechanisms in the Mouse Atrium

A Biochemical and Histochemical Study

BY

CHARLOTTE SACHS

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From the Department of Histology Karolinska Institutet, Stockholm, Sweden

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KUNGL. BOKTRYCKERIET P. A. NORSTEDT & SÖNER

Summary

Isolated mouse atria were incubated *in vitro* in Krebs-Ringer bicarbonate buffer containing ^{3}H noradrenaline and the radioactivity taken up and accumulated was determined. In representative types of experiments, ^{3}H-noradrenaline and ^{3}H metabolites were determined by specific chemical procedures and the amount of metabolites recovered was in most cases very small. The sorbitol space ($\approx$ extracellular space) was found to be about 23 % and could be efficiently cleared in 5 min of incubation.

The extraneuronal uptake was studied by using atria from mice pretreated with 6-hydroxydopamine, a drug known to cause a selective destruction of the adrenergic nerves. Incubation in a low concentration of ^{3}H noradrenaline (10^{-7} M 30 min) resulted in a relatively low extraneuronal uptake and retention of ^{3}H noradrenaline, corresponding to 12 % of the uptake in innervated atria. This value increased with an increasing concentration of ^{3}H noradrenaline in the medium, being about 32 % after 10^{-6} M and 75 % after 10^{-4} M, 30 min. There was no true accumulation of amine in the tissue, compared with the medium. The noradrenaline taken up extraneuronally was easily washed out and was not observed to be localized in any specific tissue structures, but was evenly distributed in the muscle cells of the atrium.

The uptake and accumulation of ^{3}H noradrenaline was investigated in atria from untreated mice, and mice pretreated with nialamide (a monoamine oxidase inhibitor) and reserpine + nialamide or H44/68 (a tyrosine hydroxylase inhibitor). The initial uptake was the same in atria from the four different groups, whereas, after longer incubation times, markedly less ^{3}H noradrenaline was recovered in atria from reserpine + nialamide pretreated animals. This latter finding certainly reflects an impaired retention of the amine taken up, and is not an effect of reserpine on the axon membrane pump. The neuronal uptake accumulation capacity is very rapid and efficient, and the concentration of amine in the varicosity of the adrenergic nerve was calculated to be about 30,000 times higher than in the medium, after incubation in 10^{-7} M ^{3}H noradrenaline for 30 min. It was concluded that the axon membrane pump is most important for the initial uptake, while the granular uptake mechanism is essential for the total uptake, storage and retention of noradrenaline accumulated in the neuron.

The neuronal noradrenaline uptake and accumulation was considerably

reduced at 0° C and showed temperature and pH optima of 37—40° C and pH 7.4 respectively. The uptake disclosed stereospecificity with a small preference for the *l*-isomer probably localized to the amine storage granules.

Studies on the effect of various doses of nialamide on the uptake in atria from reserpinized mice showed the uptake and accumulation of ^{3}H noradrenaline to increase, and the formation of deaminated metabolites to decrease with an increasing dose of nialamide. It was concluded that, in order completely to prevent the deamination of noradrenaline taken up in the nerves, monoamine oxidase has to be efficiently inhibited, since a relatively small enzyme activity can cause a decreased accumulation of noradrenaline.

The dose of H44/68 (*the methylester of α-methyl-p-tyrosine*) used caused a 60 % reduction of the heart noradrenaline content. The uptake and accumulation of ^{3}H noradrenaline was the same as that in untreated mice, while the net uptake was markedly increased. It was found, however that the original noradrenaline content could not be completely restored by exogenously administered noradrenaline, which could be due to the possibility that exogenous noradrenaline does not easily mix with the endogenous noradrenaline pool(s). Another possibility is that H44/68 impairs the storage capacity for noradrenaline.

Introduction

During the last decade considerable interest has been focused on tissue catecholamine uptake mechanisms, especially those in adrenergic nerves, due to their significant role in the inactivation of circulating catecholamines and transmitter released at the nerve endings. The fact that these uptake mechanisms can be affected by certain drugs, such as reserpine, guanethidine or tricyclic antidepressants, some of which are clinically important, is also of great interest.

There is at present strong evidence for the view that adrenergic nerves possess two main uptake-accumulation mechanisms operating at different levels of the nerve. One the so called "membrane pump" is energy requiring and probably located at the axonal membrane, while the other is considered to be restricted to the intraneuronal amine storage granules. An extensive literature on this subject has appeared in recent years (see among others, Carlsson *et al.* 1963 Euler and Lishajko 1963a, Stjärne 1964 Carlsson 1966a, b Malmfors 1965 1967 Hamberger 1967 Iversen 1967)

The present study is concerned with the uptake and accumulation of NA[1] in the isolated mouse atrium *in vitro* with special reference to the specific neuronal uptake. Biochemical isotope and histochemical techniques have been used. The extraneuronal uptake has been possible to elucidate by pretreatment of the animals with large doses of 6-OH DA, a drug known to cause within a short time, a severe damage of the NA uptake-storage mechanisms of the adrenergic nerves and later a destruction of the nerves (Tranzer and Thoenen 1967 1968 Malmfors and Sachs 1968b Jonsson and Sachs 1970a) The stereospecificity of the NA uptake and the effect of temperature and pH of the incubation medium on the NA uptake has been investigated. Experiments using reserpine, MAO or tyrosine hydroxylase inhibition were designed with a view to obtaining information on the relative importance of the different uptake processes, and on the significance of different factors regulating the uptake, storage and retention of exogenously administered NA.

[1] *Abbreviations used*: NA = noradrenaline; NM = normetanephrine 6-OH DA = 6-hydroxydopamine MAO = monoamine oxidase; COMT = catechol-O-methyltransferase PCA = perchloric acid.

Material and methods

Albino mice (NMRI 25—30 g) and in a few experiments albino rats, (Sprague Dawley about 200 g) were used for the studies.

Immunosympathectomized mice obtained from Dr L. Olson, were prepared by giving the antiserum to nerve growth factor NGF (Wellcome Research Laboratories England through Dr D C. Edwards) Each mouse received 0.05 ml of serum/g body weight, *s.c* immediately after birth and on the four following days. The mice were sacrificed when 9 months of age (weight, 25—30 g)

The animals were sacrificed by dislocation of the neck under ether anaesthesia and from the atria right and left auricles were carefully cut out (and in some experiments irides from rats were dissected out under a microscope see Malmfors 1965) The tissue was immediately immersed in cool Krebs-Ringer bicarbonate buffer pH 7 4

In vitro incubation

The incubation medium used was a Krebs-Ringer bicarbonate buffer[2] containing 10^{-8}—10^{-4} M *dl* ^{3}H NA and 0 2 mg/ml ascorbic acid (Merck *p.a.*) saturated with 93.5 % O_2 and 6.5 % CO_2 (Umbreit *et al.* 1951) Unlabelled *dl* NA was added to the medium in order to obtain concentrations higher than 10 M In some experiments *l* ^{14}C-NA (10^{-7} or 5×10^{-7} M) and *dl* ^{3}H metaraminol (10^{-7}—10^{-3} M) were used. Other substances added to the incubation medium were *d* NA, *l* NA reserpine malamide and guanethidine. At the start of the incubation each flask containing Krebs-Ringer bicarbonate buffer and atria (or irides) was gassed for 10—15 sec and then sealed with a tight fitting rubber stopper The tissue was incubated for various periods of time at 37 C (except for studies on the effect of temperature on ^{3}H NA uptake) using a metabolic shaker The incubation volume was one ml per two atria (or irides) When the effect of changes in pH of the incubation medium on H NA uptake was studied Krebs-Ringer phosphate buffer[3] containing

Krebs-Ringer bicarbonate buffer (g/liter) NaCl 6.92 KCl 0.35, $CaCl_2$ 6 H_2O 0.28, KH_2PO_4 0.16, $MgSO_4$ H_2O 0 29 $NaHCO_3$ 2.10, glucose 1.8.

Krebs-Ringer phosphate buffer (g liter) NaCl 7 45 KCl 0.38, $CaCl_2$ 6 H_2O 0.30, KH_2PO_4 0.17 $MgSO_4$ 7 H_2O 0 31 Na_2HPO_4 2 H_2O 0.42, N HCl 1 98 ml, glucose 1.8.

ascorbic acid, saturated with 100 % O_2 was used. After the incubation the atria were immersed in cool buffer for a few sec and then in most cases re-incubated for 10 min in isotope-free buffer at 37 C, in a volume of two ml buffer per two atria. When incubations were performed for longer periods of time, the incubation flasks were regassed every 30 min. There was no significant decrease of radioactivity of the medium after termination of the incubation.

After the incubation procedure, two atria were carefully blotted on a filter paper and weighed together on a Cahn electrobalance. Mean wet weight of two atria was 5 2 mg (range 3.5—6.4 mg) Since the uptake values per weight unit were found to be the same for right and left atria, no care was taken to get atria from the same animal for each analysis.

Determination of total radioactivity

The atria were dried overnight at 37 C on filter paper in a closed vessel and then combusted in counting vials in an oxygen atmosphere to water and CO_2 according to Gupta (1966) and Maurer (1968) The ^{3}H-water formed in this way was condensed by chilling the glass vial for about 10 sec in liquid nitrogen. No loss of radioactivity occurs during this procedure. Ten ml of a toluene scintillation solution[4] was added and the radioactivity measured in a Packard Model 3002 Tri-Carb Liquid Scintillation Spectrometer In some experiments the uptake of *l*- ^{4}C-NA in mouse atria and rat irides was studied and in these cases the tissue was dissolved in 0.5 ml Soluene^{TM} 100 (Packard Instr Co., Inc.) and 10 ml toluene scintillation solution was added before determination of radioactivity The samples were counted for 10 min. Efficiency was determined by adding a standard amount of ^{3}H toluene or ^{14}C-toluene to representative samples and recounting the vials. All values are expressed per weight unit.

Specific determination of H NA, H NM and H-deaminated metabolites

In representative experiments ^{3}H NA, H NM and ^{3}H-deaminated metabolites were determined by specific chemical analytical procedures. Two atria were after incubation in ^{3}H NA, homogenized and extracted with 4 ml cool 0.4 N PCA. The extracts were chromatographed on ion-exchange columns (Dowex

Scintillation solution for combustion 900 ml of toluene scintillation solution containing 5.0 g PPO (2.5-diphenyloxazole) and 0.5 g DM POPOP (1 4-bis 2(4-methyl-5-phenyloxazolyl)-benzene) per liter of toluene, 50 ml ethanol, 50 ml dioxane and 80 g naphtalene.

50W X4 diameter 4 0 mm and height at pH 1 120 mm) The amines were adsorbed to the resin, whereas the acid and neutral catabolites passed through in the effluent (Carlsson and Waldeck 1963) The amines were eluted with N HCl. The first 7 ml were discarded, NA was thereafter eluted with 8 ml and NM with 13 ml N HCl (see Fig. 1) There was a 7 % overlap of NA into the NM fraction, which was corrected for in the calculations. The acid eluates containing ^{3}H NA and ^{3}H NM respectively were freeze-dried in counting vials, and the radioactivity was taken up in 1 ml ethanol and 10 ml toluene scintillation solution. The effluent was after acidification (pH 2) shaken with 300 ml ether (Rutledge and Jonason 1967) the volume reduced to dryness under a hood, and the radioactivity taken up in 1 ml ethanol/0 01 N HCl and 5 ml toluene scintillation solution, from which a 5 ml aliquot was counted. Recovery for NA was 82 ± 2.9 % (n = 7) and for NM, 84 ± 7 4 % (n = 4) Recovery for ^{3}H-deaminated metabolites has not been determined. No corrections for recovery have been made.

NA assay

NA content in whole heart was determined according to Bertler *et al* (1958) Each determination was performed on six pooled mouse hearts which had been homogenized with an Ultra Turrax apparatus (Janke and Kunkel Freiburg) and extracted with 10 ml ice-cold 0 4 N PCA. To the extracts 0 1 ml ascorbic acid (?0 mg/ml) and 0.2 ml Na_2EDTA (100 mg/ml) were added and thereafter chromatographed on ion-exchange columns (Dowex 50W X4 diameter 4 0 mm and height at pH 1 50 mm) NA was eluted with 8 ml N HCl and the isolated NA was after oxidation, measured fluorimetrically Recovery of NA was 79 ± ? 4 % (n = 5)

In vivo infusion of H NA

Infusions of ^{3}H NA *in vivo in mice were performed by cannulation of a tail* vein. The animals were anesthetized with nembutal (50 mg/kg *i.p.*) Each mouse received 0 1 mg/kg ^{3}H NA (113 mCi/mM) during 14 min from an automatic infusion apparatus. The rate of infusion was 16 µl/min. The animals were immediately killed six hearts pooled for each determination and extracted with 10 ml 0.4 N PCA. NA was isolated by ion-exchange chromatography (see *NA assay* above) and an aliquot taken for determination of ^{3}H NA by liquid scintillation counting The rest was used for fluorimetric determination of NA (Bertler *et al.* 1958)

Determination of sorbitol space

Sorbitol space of mouse atria was determined by incubation of the atria in Krebs-Ringer bicarbonate buffer containing 1.25 μCi/ml ^{14}C-sorbitol (7.0 mCi/mM) according to Morgan *et al.* (1961). After incubation, two atria were blotted on filter paper, weighed and homogenized in 1.5 ml ethanol and centrifuged (12 000 × g, 10 min). A 1 ml aliquot of the supernatant was added to 10 ml toluene scintillation solution and the radioactivity determined. After correction for efficiency the sorbitol space was calculated by comparing the radioactivity equilibrated in the tissue with the radioactivity in the medium.

Determination of MAO-activity

MAO-activity of atria was determined according to the method of Wurtman and Axelrod (1963). Atria from one mouse were after weighing, homogenized in 0.5 ml 1.15 % KCl using a glass homogenizer with a tightly fitting glass pestle. Of the homogenate 25 μl was added to 250 μl 0.5 M phosphate buffer pH 7.4 and 25 μl ^{14}C-tryptamine (2.5 μCi/ml) in a centrifuge tube. The tubes were incubated at 37° C for 20 min and the reaction was terminated by adding 0.2 ml 2 N HCl. Toluene (6 ml) was added and the tubes shaken for 5 min. After centrifugation (3,000 × g for 10 min) 4 ml of the supernatant was added to 10 ml of a toluene scintillation solution in a counting vial and the radioactivity determined. Boiled tissue homogenate or buffer instead of the sample, was used for obtaining blank values. Each determination was performed in duplicate and MAO activity was expressed as cpm/mg tissue.

Fluorescence histochemistry

Mouse atria were studied with the histochemical fluorescence method of Falck and Hillarp (Falck *et al.* 1962, Falck 1962, Carlsson *et al.* 1962, Falck and Owman 1965, Corrodi and Jonsson 1967). Some hearts were perfused with Indian ink in order to demonstrate the blood vessels and the adrenergic nerves in the same preparation. The tissue was prepared as whole mounts on microscope slides with the endocardium directed upwards, dried about one hr in a desiccator containing phosphorous pentoxide (see Malmfors 1965) and treated with formaldehyde gas of optimal humidity generated from paraformaldehyde (see Hamberger 1967) at 80° C for one hr. Some atria were freeze dried, treated with formaldehyde gas and sectioned (8μ). The slides were thereafter examined in a fluorescence microscope. For details see Dahlström and Fuxe (1964) and Falck and Owman (1965).

Combined fluorescence histochemistry and ^{3}H NA measurements

Atria from the immunosympathectomized mice were after incubation *in vitro* in ^{3}H NA, prepared as whole mounts, exposed to formaldehyde gas and examined in the fluorescence microscope to check the completeness of the immunosympathectomy. After examination, the atria were scraped off of the slides, homogenized and the radioactivity determined (Olson *et al.* 1968).

Drugs and substances

The following drugs and substances were used: *dl* noradrenaline 7 ^{3}H HCl 5—10 Ci/mM, *dl* metaraminol 7 ^{3}H HCl 6.5 Ci/mM, tryptamine 2 ^{14}C bisuccinate 10.9 mCi/mM (New England Nuclear Corp. Boston, Mass.) Sorbitol ^{14}C 7 mCi/mM, *l* ^{14}C-noradrenaline bitartrate 57 mCi/mM (The Radiochemical Centre Amersham England) *dl* noradrenaline HCl (Calbiochem) *l* noradrenaline bitartrate (Sigma) *d* noradrenaline (Sterling Winthrop Lab.) nialamide (Niamid® Swedish Pfizer) guanethidine sulfate (Ismeline®) reserpine (Serpasil®) reserpine phosphate, dissolved in 5.5 % glucose (Swedish Ciba) the methylester of α-methyl-*p*-tyrosine HCl (H44/68) 6-hydroxydopamine HCl (H88/39) *dl* metaraminol HCl (H99/13 AB Hässle).

Concentrations of drugs and substances were calculated as free base. When used for injection they were dissolved in 0.9 % NaCl, except for nialamide which was dissolved in a small amount of N HCl diluted with 0.9 % NaCl and pH was adjusted to about 5 with N NaOH. The volume for *i.p.* administrations was 0.5 ml and for *i.v.* injections (in tail vein) 0.2 ml.

The purity of H NA, ^{14}C-NA and ^{3}H metaraminol was checked with *ascending paper chromatography* on Whatman nr 1 paper. The systems used were n-butanol/N HCl 4 1 n-butanol/acetic acid/aqua 4 1 5 and 2 N HCl/isopropanol 63 35. The distribution of radioactivity on the chromatograms showed only one peak which R_f-value was identical to that of the standard substance.

Results

Metabolism of ^{3}H-NA during in vitro incubation

In order to obtain information on the metabolism of ^{3}H NA during *in vitro* incubation of mouse atria under the various experimental conditions used in this study ^{3}H NA, ^{3}H NM and ^{3}H-deaminated metabolites were determined by specific analytical procedures in representative types of experiments (Table I and Fig 1) This information was furthermore of importance for the evaluation of results from experiments where total ^{3}H radioactivity was determined. For the detailed schedules of the pharmacological pretreatments see below

When atria from *untreated mice* were incubated for 30 min in a medium containing 10^{-7} M ^{3}H NA, very small amounts of metabolites were detected, they made up about 3 % of the total radioactivity recovered. Relatively more metabolites were found when the medium contained higher concentrations of ^{3}H NA and after longer incubation times. The relative amount of metabolites was generally somewhat lower when the tissue was reincubated in an isotope free medium for 10 min, after the ^{3}H NA incubation.

After *nialamide* pretreatment only small amounts of ^{3}H NM, and no detectable amounts of ^{3}H-deaminated metabolites were found after incubation in 10^{-7} M ^{3}H NA. However incubation in 10^{-6} M ^{3}H NA for 120 min (wash-out omitted) yielded a relatively large amount of ^{3}H NM, 24 % Pretreatment with *reserpine and nialamide* resulted in 73—87 % unchanged ^{3}H NA, and the recovered metabolites consisted mainly of ^{3}H NM (see Fig. 1 B, *cf* Fig. 1 A) *H44/68* pretreatment gave a metabolite pattern which was similar to that derived from untreated animals. Since the amount of metabolites recovered in mouse atria was rather low in most cases, total radioactivity has generally been determined The radioactivity values obtained, will thus mainly represent *unchanged ^{3}H NA and therefore total ^{3}H radioactivity has in most cases* been presented as ^{3}H NA. In one series of experiments using reserpine pretreatment, when a considerable catabolism of NA is known to occur ^{3}H NA was determined specifically

After pretreatment with *6-OH DA* there was an increase of the relative amounts of ^{3}H NM and ^{3}H-deaminated metabolites formed during the incubation, while the absolute amounts were practically the same as those from untreated animals. Reincubation of the tissue in isotope-free medium for 10 min

TABLE I *Metabolism of H-V4.* Atria from differently pretreated mice were incubated *in vitro* in a H NA containing medium. Determinations of H NA, H NM and H-deaminated metabolites were performed either directly after incubation or after 10 min reincubation in fresh buffer. The figures are expressed as per cent of the sum of the total H-radioactivity recovered. Each val represents the mean ± S.E.M. from 4—9 determin tions.

Pretreatment	Time of incubation (min)	H NA in medium (M)	Time of reincubation in fresh buffer (min)	H NA (%)	H NM (%)	H-deaminated metabolites (%)
Untreated	30	10^{-4}	0	96.7±1.6	2.3±1.3	1.0±0.4
	30	10^{-4}	10	97.0±1.0	2.0±0.7	1.0±0.3
	120	10^{-4}	0	92.5±4.5	6.2±3.8	1.3±0.7
	120	10^{-4}	10	97.7±2.2	2.0±0.2	0.3±0.2
	120	10^{-3}	0	79.5±2.1	10.5±2.7	10.0±1.9
Nialamide (100 mg/kg *i.p.* 2 hr)	30	10^{-4}	0	96.2±2.3	3.8±2.4	N.D.
	30	10^{-4}	10	97.1±2.9	2.9±2.7	N.D.
	120	10^{-4}	0	98.5±1.5	1.5±1.5	N.D.
	120	10^{-4}	10	98.2±1.7	1.8±1.7	N.D.
	120	10^{-3}	0	75.9±6.5	24.1±6.9	N.D.
Reserpine (10 mg/kg *s.c.* 16 hr) + *Nialamide* (100 mg/kg *i.p.* hr)	30	10^{-4}	0	87.0±1.0	12.2±0.8	0.8±0.3
	30	10^{-4}	10	81.2±3.5	16.9±2.9	1.9±1.0
	120	10^{-4}	0	79.2±2.7	20.3±0.2	0.5±0.2
	120	10^{-4}	10	81.0±6.6	18.0±5.4	1.0±0.0
	120	10^{-3}	0	73.5±5.2	26.5±5.2	N.D.
H44/68 (500 mg/kg *i.p.* 16 hr)	30	10^{-4}	0	95.6±1.6	1.7±1.2	2.7±0.6
	30	10^{-4}	10	98.2±3.0	1.3±0.5	0.5±0.2
	120	10^{-4}	0	96.6±1.5	1.7±1.7	1.7±0.4
	120	10^{-4}	10	98.5±1.5	1.5±1.2	N.D.
	120	10^{-3}	0	75.9±2.1	10.6±1.9	13.5±2.2
6-H dihydrothebaine (2 × 50 mg/kg *i.v.* 16 and 2 h)	30	10^{-4}	0	62.5±3.9	32.3±4.3	5.2±0.7
	30	10^{-4}	10	71.9±4.2	22.4±3.3	5.7±1.0
	120	10^{-4}	0	59.9±1.9	34.1±1.7	6.0±0.9
	120	10^{-4}	10	70.5±4.9	23.2±4.7	6.3±0.8
	120	10^{-3}	0	80.9±0.9	11.7±2.1	7.4±1.6

N.D. = not detectable

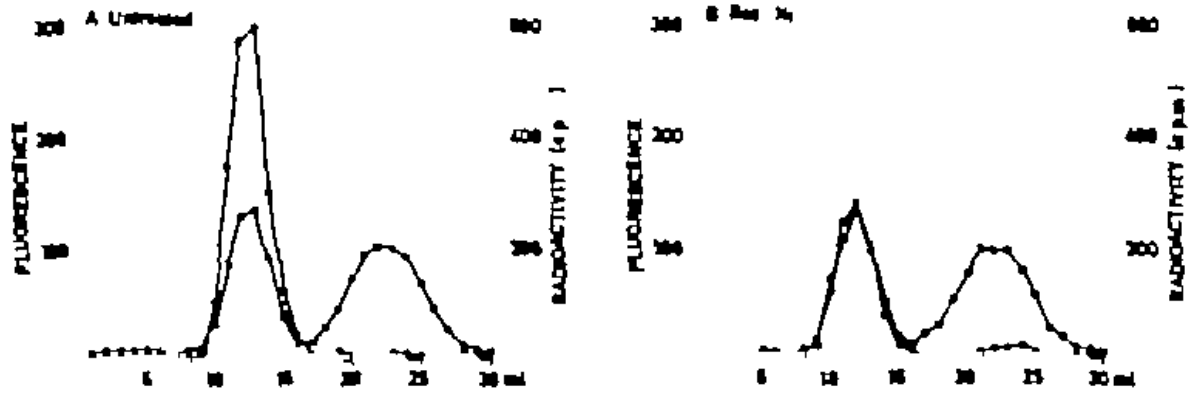


Fig. 1 Ion-exchange chromatography of extracts from mouse atria, which had been incubated in 10^{-7} M H NA for 30 min at 37° C.

A. U treated

B. Pretreated with reserpine (10 mg/kg i.p. 16 hr) + nialamid (100 mg/kg i.p. 2 hr)

● ● ● radioactivity ○——○——○ fluorescence (native 285 mμ activation 515 mμ emission) NA (10 μg) and NM (10 μg) had been added to the extracts as carriers. The left peak corresponds to NA and the right to NM.

resulted in a relative increase in the wash-out of metabolites compared with untreated. The total uptake of radioactivity after 6-OH DA pretreatment, however was found to be considerably less than the total uptake in intact atria (see below)

Sorbitol space

Sorbitol space[3] was determined by incubation of atria in Krebs-Ringer bicarbonate buffer containing ^{1}C-sorbitol at 37 C (Fig 2) There was a rapid equilibration of sorbitol in the tissue—between 10 and 30 min the equilibration curve flattened off After 30 min incubation, the sorbitol space was about 23 % (22.8 % ± 1 1 n = 1[2])

Clearance of the sorbitol space was studied by incubating atria in ^{1}C-sorbitol for 30 min and then transferring the tissue to isotope-free buffer for further incubation for different periods of time (Fig. 3) A rapid clearance of C-sorbitol from the tissue was observed within 5 min and after 10 min only some 4 % was left. The clearance of the sorbitol space in 10 min at 0° C was, however markedly diminished compared to that seen at 37 C—about 36 % of the radioactivity initially present, remained in the tissue. In atria which had

$$\text{sorbitol space} = \frac{\text{dpm } ^{14}\text{C-sorbitol/mg atrial tissue}}{\text{dpm } ^{14}\text{C-sorbitol/}\mu\text{l medium}} \times 100.$$

TABLE I *Metabolism of H-NA.* Atria from differently pretreated mice were incubated *in vitro* in a H-NA containing medium. Determinations of H-NA, H-NMI and H-deaminated metabolites were performed either directly after incubation or after 10 min reincubation in fresh buffer. The figures are expressed as per cent of the sum of the total H-radioactivity recovered. Each value represents the mean ± S.E.M. from 4–9 determinations.

Pretreatment	Time of incubation (min)	H-NA in medium (M)	Time of reincubation in fresh buffer (min)	H-NA (%)	H-NMI (%)	H-deaminated metabolites (%)
Untreated	30	10^{-7}	0	96.7±1.6	2.3±1.3	1.0±0.4
	30	10^{-7}	10	97.0±1.0	2.0±0.7	1.0±0.3
	120	10^{-7}	0	92.5±4.5	6.2±3.8	1.3±0.7
	120	10^{-7}	10	97.7±2.2	2.0±0.2	0.5±0.2
	120	10^{-6}	0	79.5±2.1	10.5±2.7	10.0±1.9
Nialamide	30	10^{-7}	0	96.2±2.3	3.8±2.4	N.D.
(100 mg/kg i.p. 4 hr	30	10^{-7}	10	97.1±2.9	2.9±2.7	N.D.
	120	10^{-7}	0	98.5±1.5	1.5±1.5	N.D.
	120	10^{-7}	10	98.2±1.7	1.8±1.7	N.D.
	120	10^{-6}	0	75.9±6.5	24.1±6.5	N.D.
Reserpine	30	10^{-7}	0	87.0±1.0	12.2±0.8	0.8±0.3
(10 mg/kg i.p. 18 h	30	10^{-7}	10	81.2±3.5	16.9±2.9	1.9±1.0
Nialamide	120	10^{-7}	0	79.2±2.7	20.3±0.2	0.5±0.2
(100 mg/kg i.p. 4 hr	120	10^{-7}	10	81.0±6.6	18.0±5.4	1.0±0.0
	120	10^{-6}	0	73.5±5.2	26.5±5.2	N.D.
H7	30	10^{-7}	0	95.6±1.6	1.7±1.2	2.7±0.6
(300 mg/kg i.p. 16 hr)	30	10^{-7}	10	98.2±5.0	1.3±0.5	0.5±0.2
	120	10^{-7}	0	96.6±1.5	1.7±1.7	1.7±0.4
	120	10^{-7}	10	98.5±1.5	1.5±1.2	N.D.
	120	10^{-6}	0	73.9±2.1	10.6±1.9	15.5±2.2
6-Hydroxydopamine	30	10^{-7}	0	62.5±3.9	32.3±4.3	5.2±0.7
(2×50 mg/kg s.c.	30	10^{-7}	10	71.9±4.2	22.4±3.3	5.7±1.0
16 and 2 hr)	120	10^{-7}	0	59.9±1.9	34.1±1.7	6.0±0.9
	120	10^{-7}	10	70.5±4.9	23.2±4.7	6.3±0.8
	120	10^{-6}	0	80.9±0.9	11.7±2.1	7.4±1.6

N.D. = not detectable

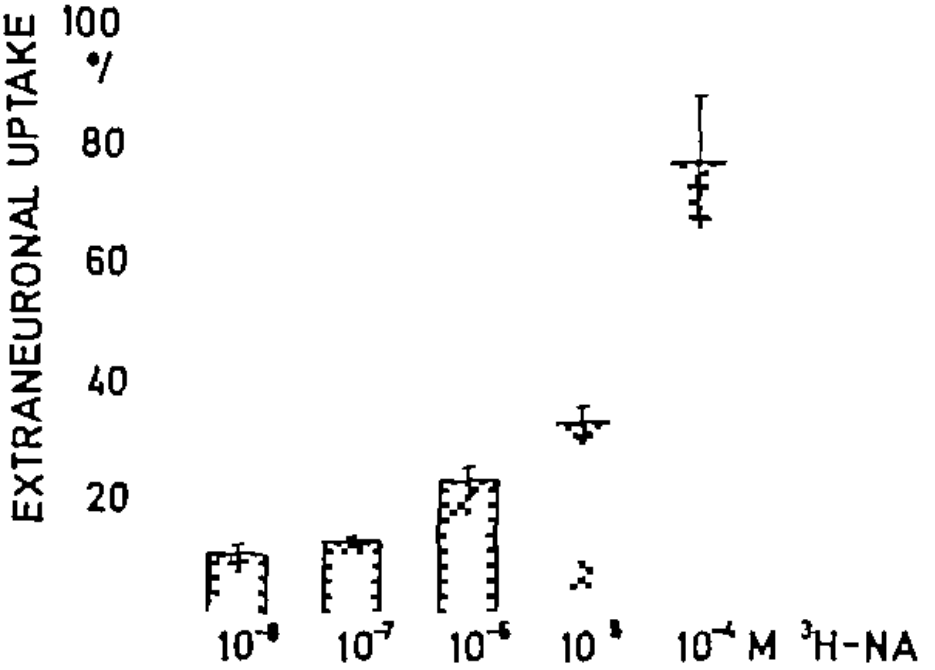


Fig. 4. Uptake of radioactivity in atria from 6-OH DA (2 × 50 mg/kg i.v. 16 and 2 hr) pretreated mice incubated in various H NA concentrations in the medium. The values are expressed as per cent of the uptake in atria from untreated animals. The incubation time was 30 min, followed by 10 min reincubation in fresh buffer. Each column represents the mean ± S.E.M. from 6–12 determinations.

to that of atria from 6-OH DA pretreated mice. The ^{3}H NA taken up after 6-OH DA pretreatment can thus, be taken as a measure of the extraneuronal uptake.

There was a gradual increase in the extraneuronal uptake, with increasing concentration of ^{3}H NA in the medium. The uptake was about 12 % of untreated after incubation in 10^{-7} M ^{3}H NA for 30 min followed by a 10 min wash-out, whereas using a medium concentration of 10^{-4} M ^{3}H NA this value was about 75 % (Fig 4). These figures are calculated on total radioactivity values obtained and would be somewhat lower when corrected for formed metabolites. The uptake ratios[6] were in all cases below one. The extraneuronal uptake of radioactivity increased with time although this uptake calculated as a percentage of the uptake in atria from untreated animals, identically incubated, was approximately constant during the whole incubation period (Fig. 5).

Fluorescence histochemistry of atria from 6-OH DA pretreated mice incubated in a high concentration of NA revealed no specific structures able to

$$\text{Uptake ratio} \quad \frac{\text{g NA/mg atrial tissue}}{\text{ng NA/}\mu\text{l medium}}$$

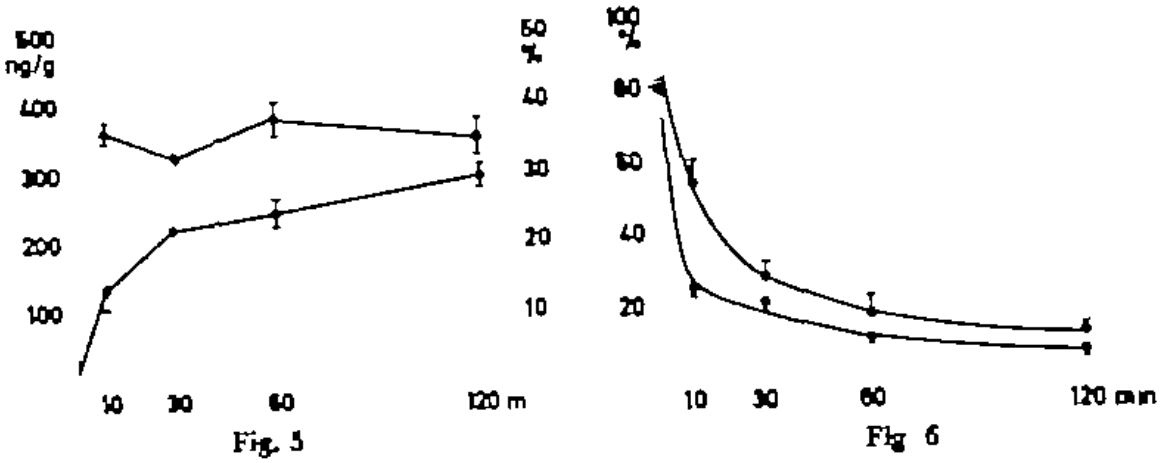


Fig. 5 Fig 6

Fig 5. Time-course of H-radioactivity uptake (H NA, 10^{-5} M) in tria from 6-OH DA pretreated mice (2×50 mg/kg *i.v* 16 and 2 hr) (●——●——●) The tissue was reincubated in fresh buffer for 10 min. Extraneuronal uptake is expressed as per cent of that in intact tria for corresponding incubation times (○——○——○) Each point represents the mean ± S.E.M. from 4—8 determinations.

Fig. 6. Disappearance of H radioactivity previously taken up during a 30 min incubation of tria from 6-OH DA (2×50 mg/kg *i.v* 16 and 2 hr) pretreated mice. Concentrations of H NA in the medium were 10^{-5} M (● ● ●) or 10^{-6} M (○——○——○) The values are expressed as per cent of the amount present at zero time Each point represents the mean ± S.E.M from 6—8 determinations.

take up and accumulate NA The NA fluorescence observed was diffusely located in the whole atrial tissue as observed in stretch preparations (micrograph 3 B) In sections (8μ) from atria it was seen that the NA fluorescence was evenly distributed throughout the muscle fibres (micrograph 2 C) The intensity of the fluorescence was moderate and slightly higher than in control after incubation in 10^{-5} M NA for 30 min and very intense after 10^{-4} M 30 min No differences in fluorescence intensity were observed in control atria and atria incubated in NA concentrations below 10^{-5} M 30 min.

NM in a concentration of 2×10^{-6} M had no effect on the uptake of ^{3}H NA (10^{-5} M 30 min) in atria from untreated or 6-OH DA pretreated mice, while NM in a concentration of 2×10^{-5} M caused an about 20 % inhibition of the uptake both in untreated (21.2 % ± 0.9 n = 4) and 6-OH DA (20.4 % ± 0.8, n = 4) pretreated. Reserpine (10 mg/kg *i.p* 16 hr) and nialamide (100 mg/kg *i.p* 2 hr) did not cause any change of ^{3}H NA uptake (10^{-7} 10^{-6} or 10^{-5} M ^{3}H NA, 30 min and 10 min wash-out) in atria from 6-OH DA pretreated mice compared to the uptake in atria from mice pretreated with 6-OH DA alone.

The H NA uptake (10^{-7} M 30 min) at 0° C in atria from 6-OH DA treated mice was about 30 % (32.8 ± 2.6 % n = 4) lower than that at 37 C,

TABLE II NA content in whole heart of mice after various pretreatments. Six hearts were pooled for each determination and each value represents the mean ± S.E.M. from 4–12 determinations. Recovery: 79 %. Corrections for recovery have been made.

Pretreatment	ng/g	%
Untreated	0.59±0.040	100±6.8
Nialamide (100 mg/kg *i.p.* 2 hr)	0.69±0.061	117±10.3
Reserpine (10 mg/kg *i.p.* 16 hr) + *Nialamide* (100 mg/kg *i.p.* 2 hr)	0.05±0.015	8±2.5
H44/68 (500 mg/kg *i.p.* 16 hr)	0.24±0.034	41±5.8
6-Hydroxydopamine (2×50 mg/kg *i.v.* 16 and 2 hr)	0.04±0.021	7±3.6

while the uptake at 25 C and 45 C was the same as 37 C. Varying the pH of the medium (pHs 6, 7.4 or 8.0) did not affect the ^{3}H NA uptake (10^{-7} M, 30 min, 37 C) in atria from 6-OH DA treated mice.

The retention of radioactivity taken up was investigated in atria from 6-OH DA pretreated animals by subsequent incubation of the tissue in fresh buffer for different periods of time (Fig 6, *cf* 18 A). There was a rapid wash-out of ^{3}H radioactivity previously taken up (10^{-7} M ^{3}H NA, 30 min) about 45 % had disappeared in 10 min. The disappearance was more pronounced when atria had been incubated in a higher ^{3}H NA concentration (10^{-5} M) but the wash out curves showed a similar general appearance for the two concentrations.

Uptake and accumulation of NA — effects of reserpine, MAO and tyrosine hydroxylase inhibition

Whole hearts or atria from the following groups of mice have been studied: A. untreated. B. pretreated with nialamide (100 mg/kg *i.p.* 2 hr). C. pretreated with reserpine (10 mg/kg *i.p.* 16 hr) and nialamide (100 mg/kg *i.p.* 2 hr). D. pretreated with H44/68 the methylester of α-methyl-β-tyrosine (500 mg/kg *i.p.* 16 hr).

NA content. The NA content of whole heart from the different groups of mice is shown in Table II. The NA content in *atria* from untreated mice was indirectly estimated after an injection of 10 μCi ^{3}H NA per mouse, *i.v.* 30 min.

The atria and the rest of the heart were weighed and homogenized separately in 0 4 N PCA and the ^{3}H NA taken up was determined. The uptake of ^{3}H NA in two atria was found to be 10 % of that taken up in whole heart. If the circulatory and diffusional conditions are presumed to be the same and if the uptake and the NA content per nerve unit is constant in atria and in the rest of the heart, then the uptake would reflect nerve density and NA content. Atria would consequently contain 10 % of the NA present in the whole heart, corresponding to a NA concentration in atria of about 1.3 μg/g This value is in good agreement with that obtained by an enzyme assay for NA (Jonsson and Sachs, unpublished observations)

Fluorescence histochemistry In *untreated* mice the adrenergic nerves form a ground-plexus in the endocardium, as observed in the fluorescence microscope (*cf* Ehinger *et al* 1966 Jonsson and Sachs 1969 1970a) (micrograph 1) The nerve fibres run 3—4 together in bundles exhibiting a rather strong fluorescence intensity Single fibres with pronounced varicosities leave the bundles. After Indian ink perfusion the vessels appeared dark in the fluorescence microscope and the adrenergic nerves could be seen both in the direct vicinity of the vessels and located outside, independently of the vessels. In the myocardium single nerve terminals could be seen to run in between the heart muscle cells (micrograph 2 A and B) Incubation of atria in 10^{-6} or 10^{-5} M NA for 30 min, gave a fluorescence morphological picture quite similar to that of atria incubated in amine-free buffer except that 10^{-6} M NA gave a slight increase in the background fluorescence. Using 10^{-4} M NA, a very intense background fluorescence was observed making it difficult to judge any differences of the fluorescence intensity of the nerves. Incubation of atria in Krebs-Ringer buffer up to 130 min, the longest incubation time studied, did not cause any changes in morphology or fluorescence intensity of the adrenergic nerves.

Nialamide treatment (micrograph 5) resulted in a slight increase in the fluorescence intensity of the non-terminal axons and this was more pronounced after incubation in NA (10^{-6} or 10^{-5} M, 30 min)

Reserpine treatment induced a complete disappearance of the NA fluorescence in the adrenergic nerves, and incubation in NA (10^{-6} or 10^{-5} M, 30 min) did not restore the fluorescence of the adrenergic nerves. Incubation of atria (10^{-6} or 10^{-5} M NA, 30 min) from *reserpine + nialamide* pretreated mice resulted in a restitution of the nerve fluorescence, but the non-terminal axons were more prominent and the terminals exhibited a rather smooth appearance (micrograph 6, *cf* Hamberger *et al.* 1964 Jonsson and Sachs 1969) After *H44/68* pretreatment only a few faint adrenergic nerve terminals could be observed. After incubation in NA (10^{-6} or 10^{-5} M, 30 min) there was a resti-

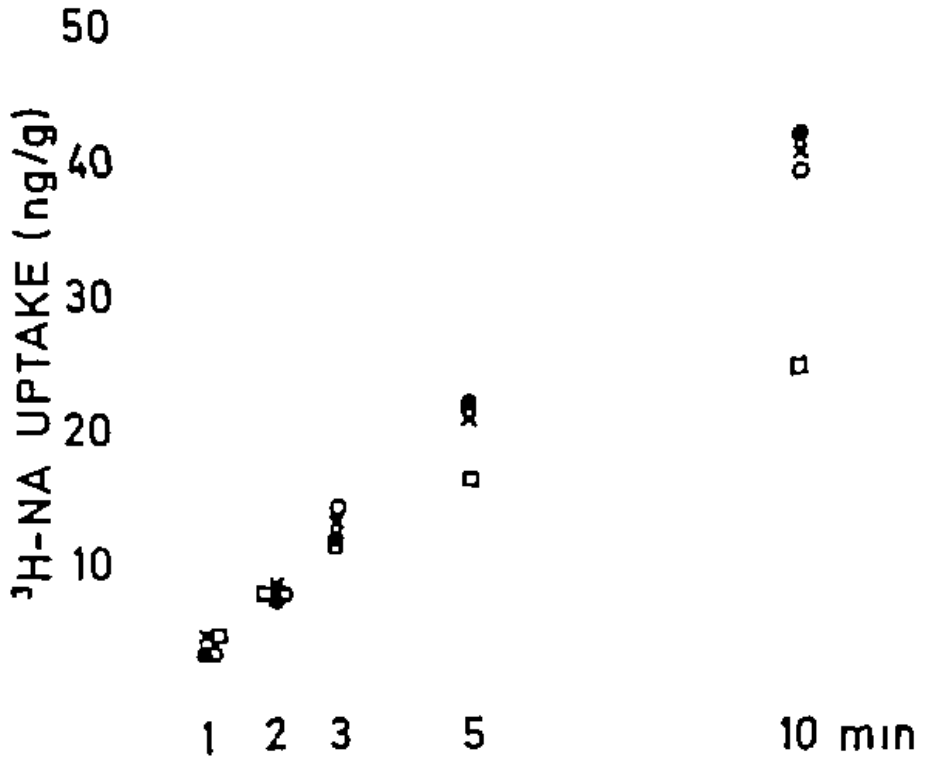


Fig. 7 Time-course of H NA uptake (10^{-7}M) in atria from differently pretreated mice. No reincubation in fresh buffer has been made The values ha been corrected for radioactivity present in the sorbitol space.

● untreated
○ nialamide (100 mg/kg *i.p.* 2 hr)
□ reserpine (10 mg/kg *i.p.* 16 hr) + nialamide (100 mg/kg *i.p.* 2 hr)
× H44/68 (500 mg/kg *i.p.* 16 hr)

Each point represents the mean of 8 determinations. S.E.M. was ± 2–5 %.

tution of fluorescent nerves with a morphology and fluorescence intensity quite similar to normal (micrograph 7 *cf* Malmfors and Sachs 1968a, Jonsson and Sachs 1969)

Uptake and accumulation of ^{3}H NA in vitro The uptake of ^{3}H NA (10^{-7} M) was linear and very similar for the four different groups up to 3 min of incubation, while after 5 min the uptake in atria from reserpine + nialamide pretreated mice started to deviate from linearity and was considerably lower at 10 min (Fig. 7) For the other groups the deviation from linearity started after about 10 min Using a medium concentration of 0.5×10^{-7} M ^{3}H NA the uptake was found to be approximately linear with time up to about 60 min, except for the reserpine + nialamide group (Fig 8) At higher medium concen-

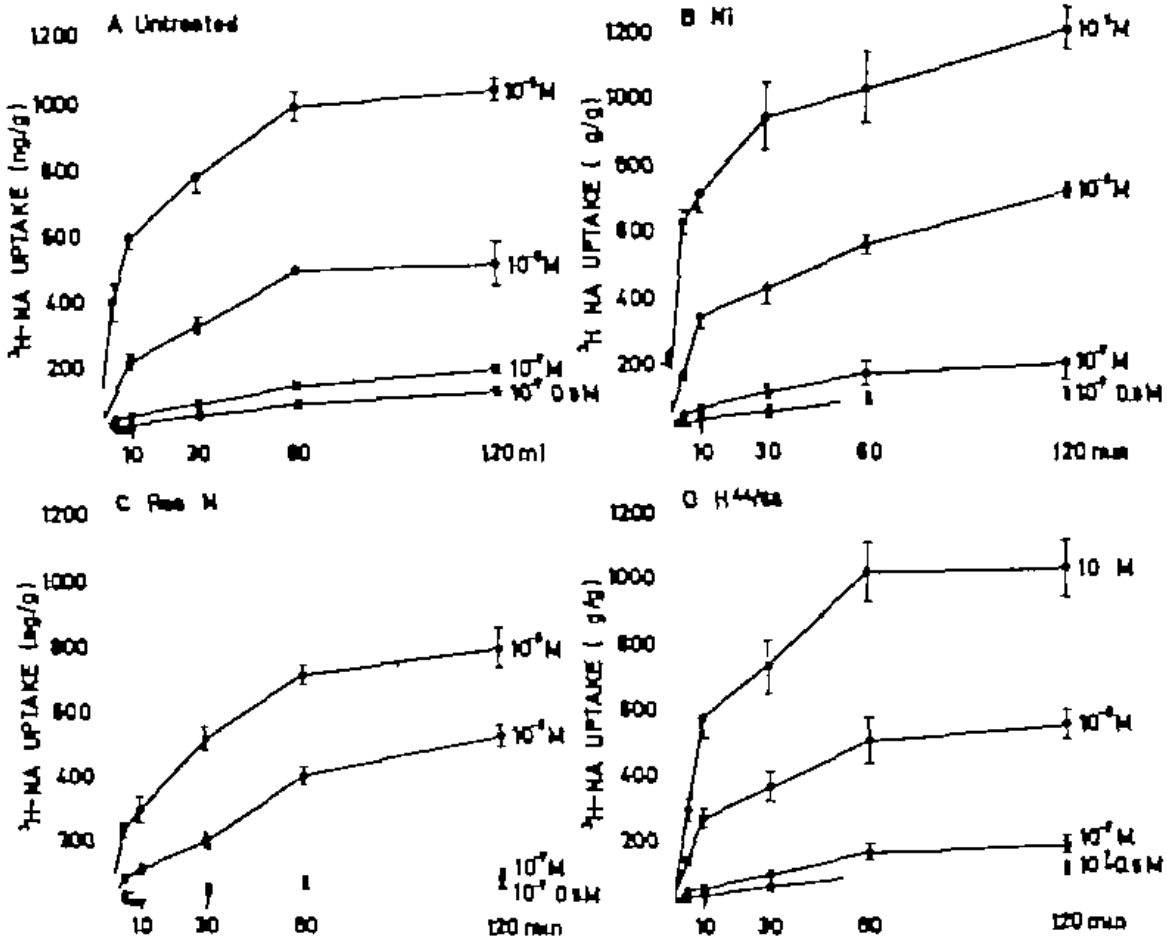


Fig 8. Time-course of H NA uptake ($0.5 \times 10^{-}$ 10^{-} 10^{-6} nd 10^{-5} M) in tria from variously pretreated mice. The tissue was reincubated in isotope-free medi m for 10 min, after the incubation.

A. untreated

B. nialamide (100 mg/kg i.p. 2 hr)

C. reserpine (10 mg/kg i.p. 16 h) + nialamide (100 mg/kg i.p. 2 hr)

D. H44 68 (500 mg/kg i.p. 16 hr)

Each point represents the mean ± S.E.M. from 4—16 determinations.

trations of ^{3}H NA deviation from linearity occurred earlier between 10 and 30 min.

Uptake and accumulation of ^{3}H NA was studied for five different concentrations of ^{3}H NA in the medium, using an incubation time of 30 min and a 10 min reincubation in isotope free buffer (Fig 9). The *uptake ratios* in atria from untreated mice were calculated to be about 6 at 10^{-7} M and 0.5 at 10^{-5} M NA. Atria from nialamide pretreated mice showed an uptake-accumulation of ^{3}H NA which was 30—60 % higher than in atria from untreated mice, with a tendency of increased uptake compared with the control when using lower

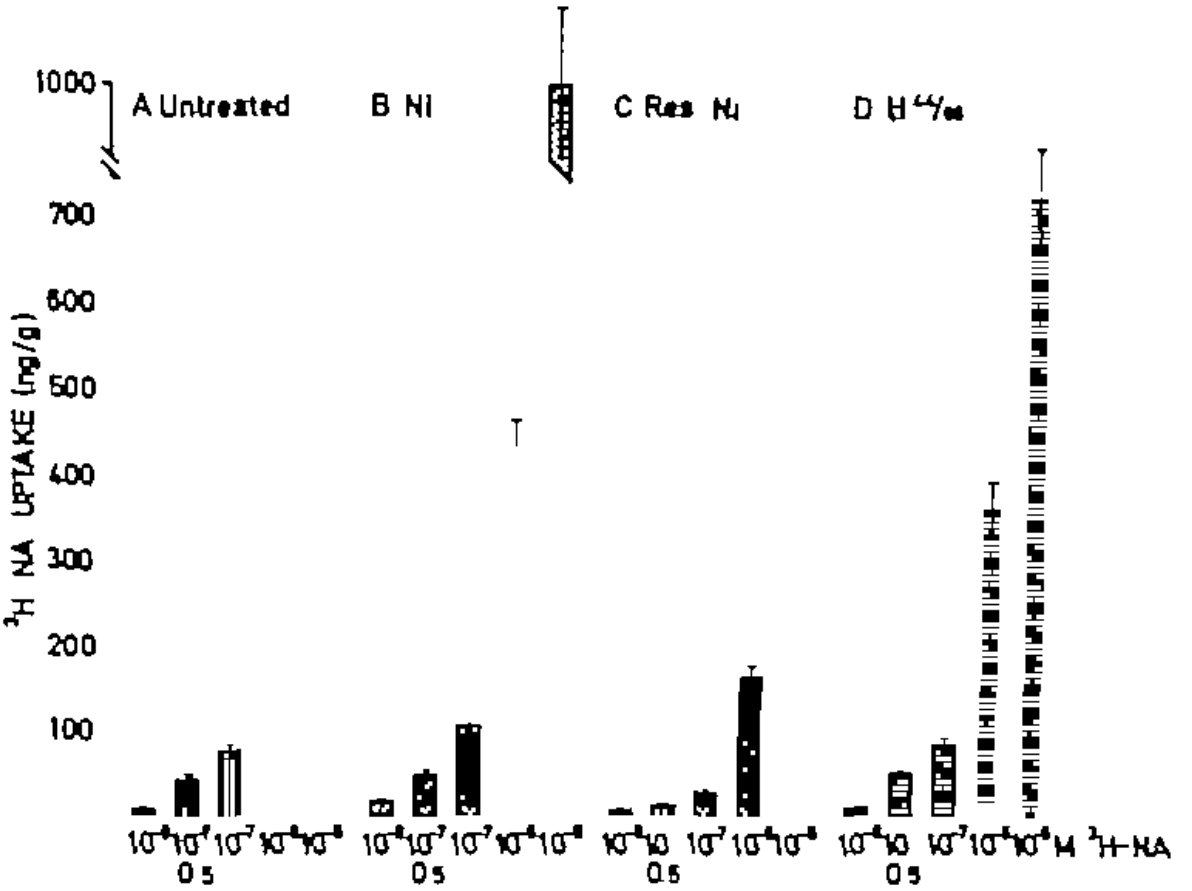


Fig 9 Uptake and accumulation of H NA in tria from mice pretreated with different drugs. The tria were incubated in various concentrations of H NA. The incubation time was 30 min, followed by 10 min reincubation in isotope-free buffer

A. untreated

B. nialamide (100 mg/kg *i.p.* 2 hr)

C. reserpin (10 mg/kg *i.p.* 16 hr) + nialamide (100 mg/kg *i.p.* 2 hr)

D. H44/68 (500 mg/kg *i.p.* 16 hr)

Each column represents the mean ± S.E.M. from 5–9 determinations.

concentrations of ^{3}H NA in the medium. Reserpine + nialamide pretreatment caused a reduction of the uptake-accumulation which was most pronounced with lower concentrations of ^{3}H NA in the medium, but with fairly great variations. The reduction was less pronounced when reincubation in isotope free buffer was omitted. H44/68 pretreatment resulted in a slightly higher uptake and accumulation of ^{3}H NA for each concentration used, compared to the uptake in untreated atria.

Reciprocal analysis of the 5 min uptake values in atria from the different groups of mice, according to Lineweaver and Burk (1934) revealed that the uptake process obeyed Michaelis-Menten kinetics, $Km = 3.26 \times 10^{-6}$ M (Fig 10)

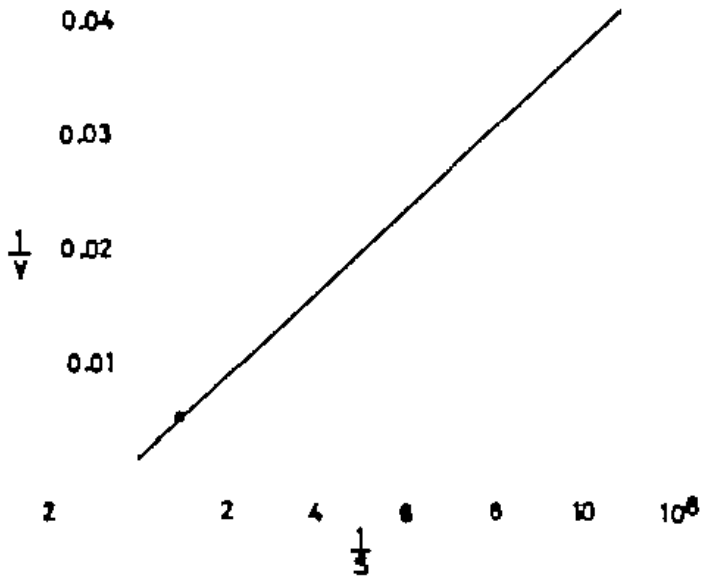


Fig 10 H N \ uptake in atria from untreated mice, plotted according to Lineweaver and Burk. The incubation time was 5 min. No reincubation in isotopo-free buffer was performed. The values are corrected for radioactivity present in the sorbitol space Each point is the mean of 5 determinations. The line was calculated by linear regression analysis, correlation coefficient = = 0.98. Velocity (V) = uptake of H NA ng/g/5 min. Substrate concentration (S) = M H NA. Km = 3.26×10^{-6} M and \ max = 854 7

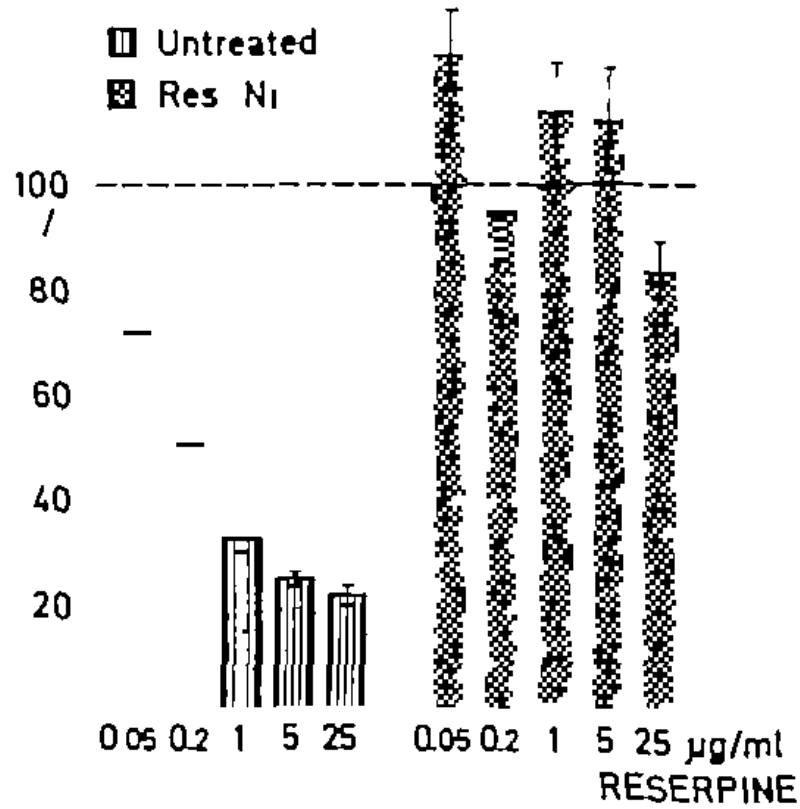


Fig 11 Effect of various concentrations of reserpine *in vivo* on the ptake of H-NA (10^{-7} M) in tria of untreated or reserpine (10 mg/kg *i.p.* 16 hr) + nialamide (100 mg/kg *i.p.* 2 hr) pretreated mice The incubation time was 30 min, followed by 5 ml reincubation in fresh buffer The lues are expressed as per cent of control value (untreated, 67 3 ± 10.6 ng/g; reserpine + talamide 43 4 ± 3.8 ng/g) Each column represents the mean ± S.E.M of 6–8 determinations.

TABLE III *Uptake of H NA in vivo in whole hearts after various pretreatments* H NA (0.1 mg/kg) was infused *i.v.* during 14 min and the animals were sacrificed immediately afterwards. Six hearts were pooled for each determination.

Pretreatment	H NA infusion	NA content (g/g)	Total H-NA uptake (g/g)	H-NA net uptake (ng/g)	Exchange (ng/g)
Untreated	—	544 ± 55.1	—	50	48
	+	594 ± 15.5	98 ± 2.9		
H44/68	—	229 ± 15 4	—	81	27
(500 mg/kg *i.p.* 16 hr)	+	310 ± 17.9	108 ± 5.1		
6-Hydroxydopamine	—	53 ± 5.0	—	18	4
(2 × 50 mg/kg *i.v.* 16 and 2 hr)	+	71 ± 3.9	22 ± 1.8		

The figures represent the mean ± S.E.M from 7 determinations. The values are corrected for recovery

The effect of reserpine (0 05—25 μg/ml) *in vitro* on the uptake of ^{3}H NA, was studied in atria from untreated and from reserpine + nialamide pretreated mice (Fig. 11) In atria of untreated animals there was a gradual decrease of radioactivity recovered from the tissue with higher concentrations of reserpine in the medium. In atria from reserpine + nialamide pretreated animals there was no apparent difference between the various reserpine concentrations, except for 25 μg/ml when the uptake was somewhat reduced.

Uptake and accumulation of NA in vivo In experiments *in vivo* slow *i.v.* infusions of ^{3}H NA (0.1 mg/kg during 14 min) were performed in untreated, H44/68 and 6-OH DA pretreated mice. The animals were sacrificed immediately afterwards and six whole hearts were pooled for each determination and extracted with PCA. After isolation of NA by ion-exchange chromatography the NA content was determined fluorimetrically and the ^{3}H NA taken up, estimated by liquid scintillation counting (Table III) H44/68 pretreatment resulted in a reduction of the endogenous NA content to 42 % of untreated and 6-OH DA a reduction to 10 % of untreated. The total uptake of ^{3}H NA was 11 % higher in hearts from H44/68 pretreated mice than in normal, while the uptake after 6-OH DA pretreatment was reduced to 22 % of normal. The net uptake expressed as the difference between the mean NA content of the

TABLE IV *NA content of whole heart after in vivo administration of* (-)-NA (1.5 mg/kg) was injected *i.p.* in variously pretreated mice. The mice were sacrificed after 1 or 4 hours. Six hearts were pooled for each determination. The figures are corrected for recovery and represent the mean of 3 determinations.

Pretreatment	1 hr		4 hr		Control	
	μg/g	%	μg/g	%	μg/g	%
Untreated	0.68	117	0.56	97	0.58	100
H44/68 (500 mg/kg *i.p.* 16 hr)	0.47	81	0.41	71	0.24	41
6-Hydroxydopamine (2 × 50 mg/kg *i.v.* 16 and 2 hr)	0.06	10	0.09	16	0.04	7

The control value from untreated mice was set to 100 %.

^{3}H NA infused group and the mean NA content of the group infused with fresh buffer was 62 % higher after H44/68 treatment compared to untreated. The exchange of ^{3}H NA with endogenous NA was 9 % in untreated, 19 % in H44/68 pretreated and 8 % in 6-OH DA pretreated mice.

The NA content in mouse heart after injection of a high dose of unlabelled NA (1.5 mg/kg *i.p.*) in untreated, H44/68 and 6-OH DA pretreated mice is shown in Table IV.

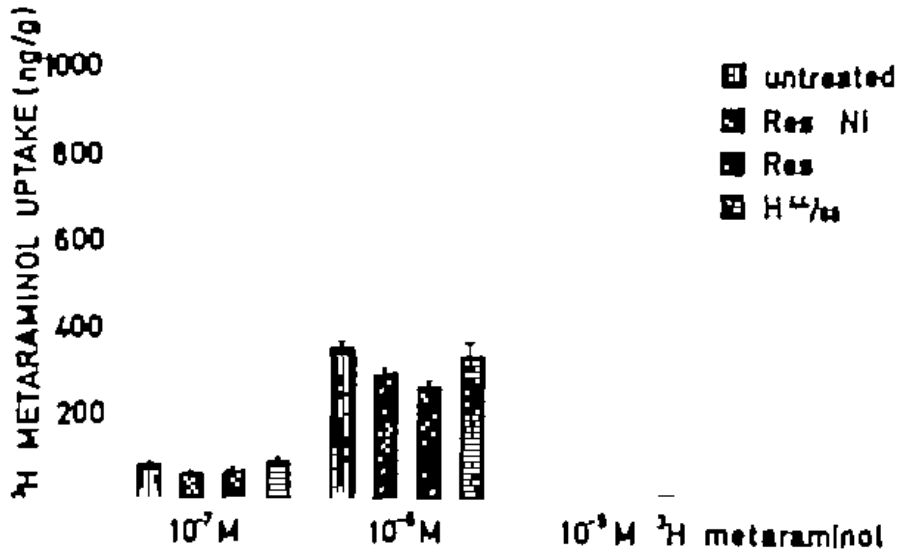


Fig. 12. Uptake of H metaraminol (10^{-7}, 10^{-6} or 10^{-5} M) in vitro from untreated reserpine + nialamide reserpine or H44/68 pretreated mice. The incubation time was 30 min followed by 10 min preincubation in fresh buffer. Each column represents the mean ± S.E.M. from 8 determinations.

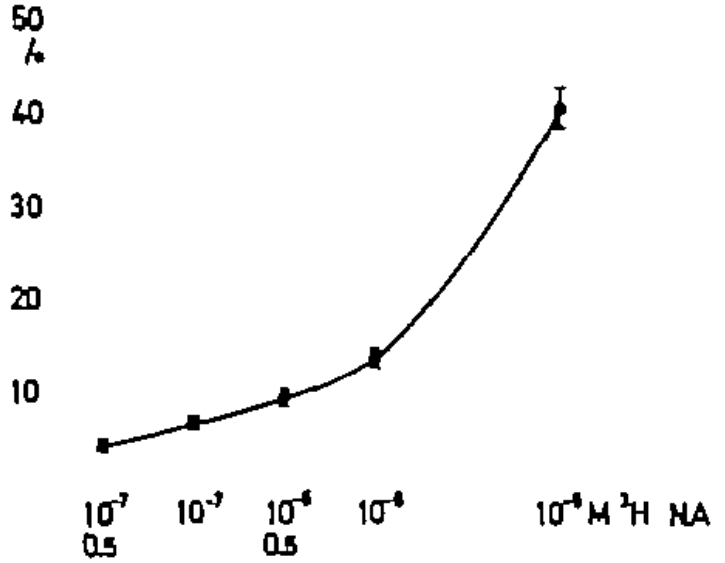


Fig. 13. Uptake of H NA in atria at 0° C, with varying H-NA concentration in the medium. The incubation time was 30 min, followed by 10 min reincubation t 0° C in fresh buffer The values are expressed as per cent of the uptake at 37° C (wash-out t 37° C) Each point represents the mean ± S.E.M. of 6—8 determinations.

Uptake and accumulation of ^{3}H metaraminol in vitro The uptake of H metaraminol *in vitro* was studied in atria from untreated mice and mice pre treated with reserpine + nialamide, reserpine and H44/68 (Fig 1?) in order to compare this uptake in the different groups, with that of ^{3}H NA. The ^{3}H metaraminol uptake was found to be rather similar in all groups for each concentration used.

The ^{3}H metaramnol uptake in atria from 6-OH DA pretreated mice was after incubation in 10^{-7} M (30 min and 10 min wash-out) 10.3 % ± 0.1 of untreated, while after 10^{-6} M and 10^{-5} M the uptake was ?1.8 % ± 1 6 and 41 1 % ± 3 6 respectively (n = 4)

Effect of temperature and pH on the uptake and accumulation of H NA in vitro

The uptake of ^{3}H NA at 0° C was considerably reduced compared to that found at 37 C (Fig. 13) being about 7 % after 10^{-7} M ^{3}H NA for 30 min. The uptake increased, with increasing concentration of ^{3}H NA in the medium and reached a value of about 40 % after 10^{-5} M for 30 min. The ^{3}H NA uptake at different temperatures of the incubation medium is presented in Fig. 14 disclosing an uptake optimum between 37 and 40° C. Fluorescence histochemistry revealed no changes in morphology or fluorescence intensity of the adrenergic nerves, for the various temperatures used.

The effect of pH on the ^{3}H NA uptake was studied by incubation of atria in Krebs-Ringer phosphate buffers, of different pHs (Fig 15) There was an increase of ^{3}H NA uptake with increasing pH of the medium, reaching a

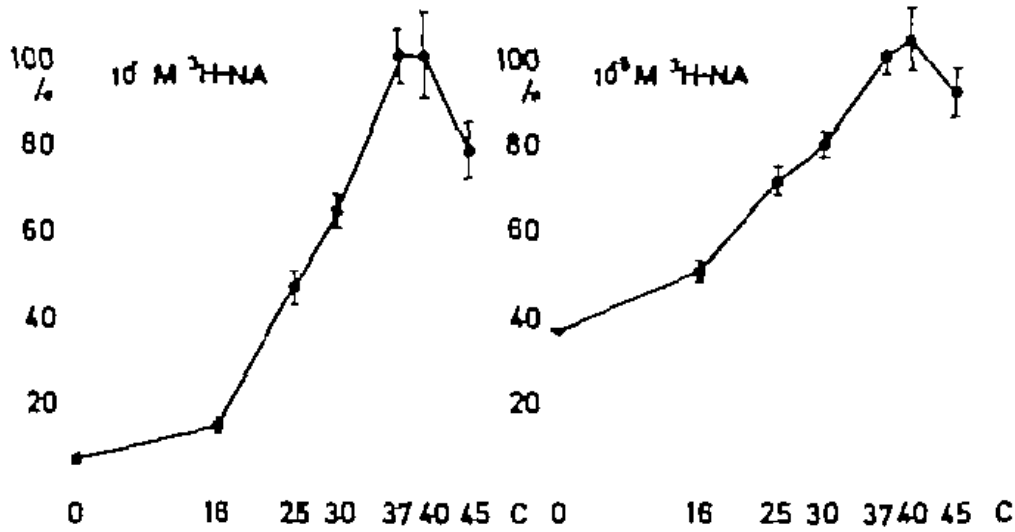


Fig. 14. Effect of temperature on the uptake of H-NA (10^{-7} M or 10^{-8} M) in mouse atria. The incubation time was 30 min. No reincubation in fresh buffer has been made. The values are expressed as per cent of the 37° C uptake value and are corrected for the amount of radioactivity located in the sorbitol space. Each point represents the mean ± S.E.M. of 6—8 determinations.

maximum at pH 7.4. In a control experiment the NA concentration (10^{-8} M) of media of different pHs (5.8, 7.4 and 8.0) which had been used for 30 min incubations, were determined fluorimetrically. No differences were observed in the NA concentration of the various media. Fluorescence histochemical studies were made on atria, incubated for 30 min in Krebs-Ringer phosphate buffer of

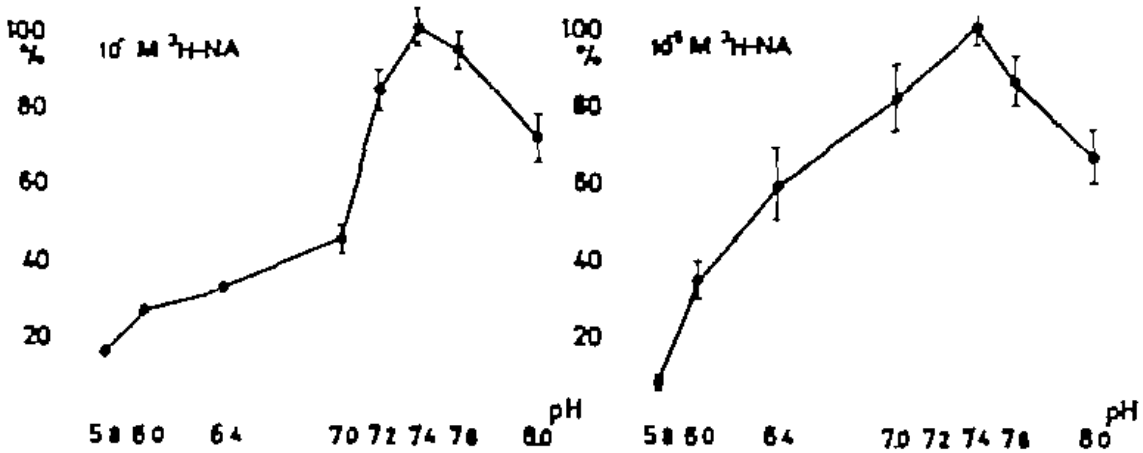


Fig. 15. Effect of pH on the uptake of H-NA (10^{-7} M or 10^{-8} M) in mouse atria. The incubation time was 30 min and no reincubation in fresh buffer has been made. The values are expressed as per cent of the uptake at pH 7.4 and are corrected for the amount of radioactivity located in the sorbitol space. Each point represents the mean ± S.E.M. of 6—8 determinations. The H-NA uptake at pH 7.4 was after incubation in 10^{-7} M H-NA, 68.2 ± 4.30 ng/g and in 10^{-8} M H-NA, 680.6 ± 76.5 ng/g.

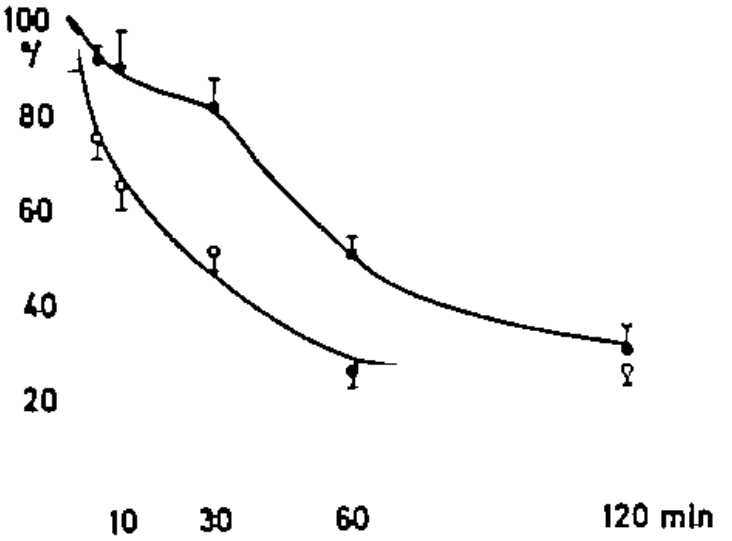


Fig 16. Time-course of disappearance of H-NA previously taken up (10^{-7} M, 30 min) in mouse atria at pH 8.0 (● ● ●) and pH 6.0 (○——○——○) The wash-out was performed in fresh buffer of pH 7 4 The values are expressed as per cent of the amount present in the tissue at zero time. Each point represents the mean ± S.E.M. of 6—10 determinations.

pH 5.8, 7 4 or 8.0 After incubation in pH 5 8 there was a decrease in the fluorescence intensity of the adrenergic nerves and they showed a somewhat fuzzy appearance (micrograph 4) while pH 8.0 and 7 4 had no effect on the fluorescence morphology

The retention of ^{3}H NA taken up at pH 6.0 and pH 8.0 was studied by re-incubation of the atria in isotope-free buffer (pH 7 4) for different periods of time (Fig. 16) There was an increased disappearance of ^{3}H NA previously

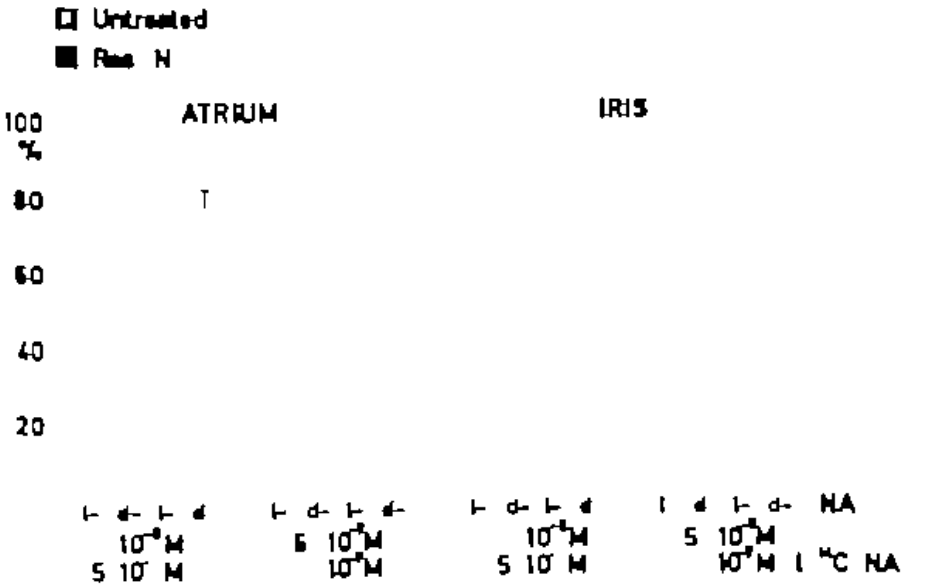


Fig 17 Effect of *d*-NA or *l*-NA (5×10^{-7} or 10^{-6} M) on the uptake of *l*-^{14}C-NA (10^{-7} or 5×10^{-7} M) in mouse atria and rat irides from untreated and reserpine (10 mg/kg i.p. 16 hr) + nialamid (100 mg/kg i.p. 2 hr) pretreated animals. The incubation time was 30 min and no reincubation in fresh buffer was made. The values are expressed as per cent of control tissues. Each column represents the mean ± S.E.M. of 4—6 determinations.

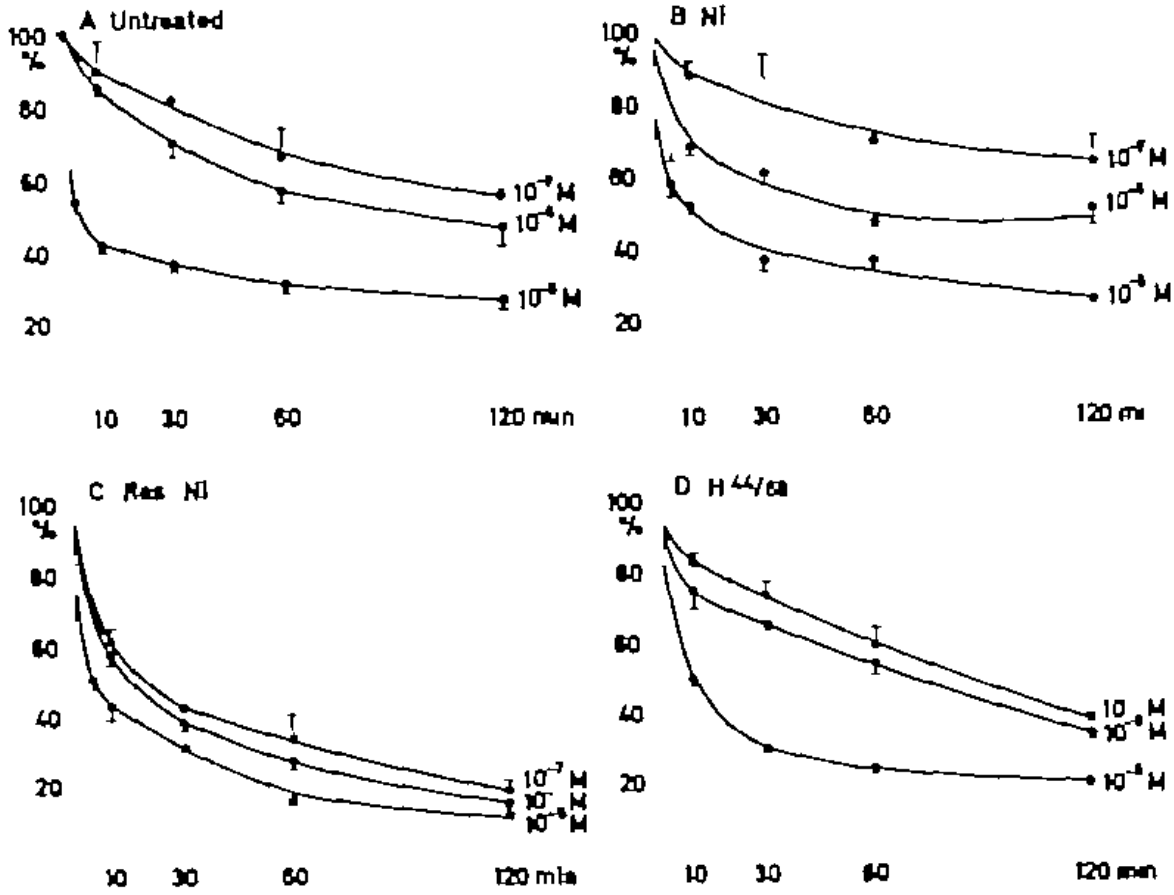


Fig. 18. Time-course of disappearance of H NA previously taken up *in vitro* (10^{-} 10^{-6} or 10^{-6} M, 30 mi) in mouse atria. The tissue was after the incubation transferred to fresh buffer and reincubated for various periods of time. The values are expressed as per cent of the amount present the tissue t zero time. Each point represents the mean ± S.E.M. of 6—14 determinations. The S.E.M. t zero time was ± 5—11 %.

A. untreated
B. nialamide (100 mg/kg *i.p.* 2 hr)
C. reserpine (10 mg/kg *i.p.* 16 hr) + nialamide (100 mg/kg *i.p.* 2 hr)
D. H44/68 (500 mg/kg *i.p.* 16 hr)

taken up at pH 6.0 compared to 7.4 during the whole time period studied. The wash-out curve after pH 8.0 was similar to that after pH 7.4 during the first 30 min, but thereafter showed a more rapid decline (*cf* Fig. 18 A)

Effect of d-or l NA on the uptake and accumulation of l ^{14}C-NA in vitro

The effect of *d* or *l* NA on the uptake of *l* ^{14}C-NA was investigated in mouse atria and rat irides from untreated and reserpine + nialamide pretreated animals (Fig 17) *l* NA caused a slightly more effective inhibition of the *l* ^{14}C-NA

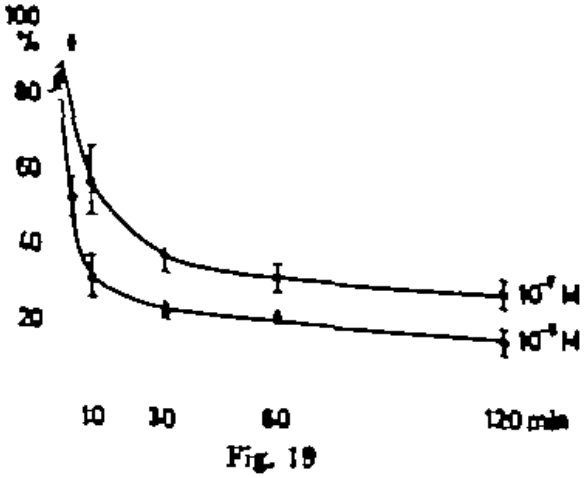


Fig. 19

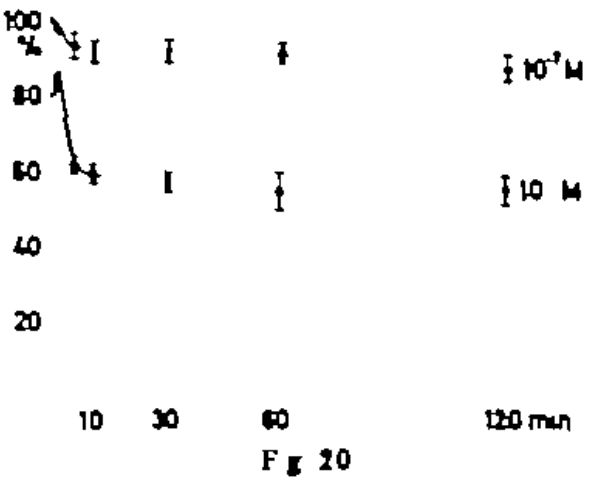


Fig. 20

Fig. 19. Time-course of disappearance of H-NA (10^{-7} or 10^{-6} M) previously taken up in mouse atria during 5 min incubation. The values are expressed as per cent of the amount present in the tissue at zero time. Each point represents the mean ± S.E.M. of 4–6 determinations.

Fig. 20. Time-course of disappearance at 0° C of H-NA (10^{-7} or 10^{-6} M 30 min) previously taken up in atria at 37° C. The tissue was after incubation transferred to fresh buffer of 0° C for reincubation for various periods of time. The values are expressed as per cent of the amount present in the tissue at zero time. Each point represents the mean ± S.E.M. of 8 determinations.

uptake than the *d*-isomer in both atria and atrides from untreated animals. There seemed to be no clear-cut difference, however, between the *d* and *l* isomers after reserpine + nialamide pretreatment.

Disappearance of ^{3}H-NA taken up in vitro

The time-course of disappearance of ^{3}H-NA previously taken up in atria from untreated, nialamide or H44/68 pretreated mice was practically identical (Fig. 18 A, B, D). Reserpine + nialamide pretreatment caused, however, a more pronounced wash-out (Fig. 18 C). The disappearance of ^{3}H-NA taken up was in all cases more rapid with higher concentrations of ^{3}H-NA in the medium.

At the end of an incubation the sorbitol space of atria, in the experimental conditions used in this study, was calculated to contain 4 ng ^{3}H-NA/g atrial tissue when using a medium concentration of 10^{-7} M ^{3}H-NA and 400 ng/g using 10^{-6} M. A 10 min wash-out of atria previously incubated in 10^{-7} M ^{3}H-NA (30 min) caused a disappearance of 8 ng ^{3}H-NA/g atrial tissue, corresponding to a 10 % wash-out of that initially present in the tissue (80 ng/g). Of the amount washed out, 7 ng/g was calculated to originate from extraneuronal sites (including ^{3}H-NA present in the sorbitol space). When using

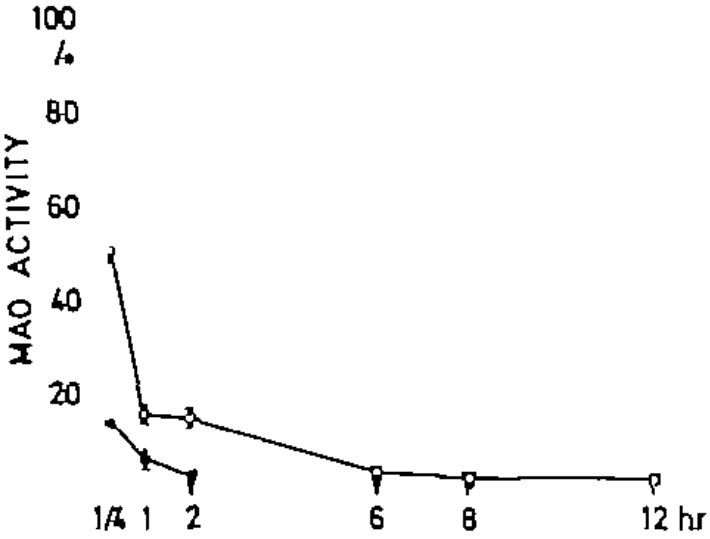


Fig 21 Effect of nialamide (10 mg/kg ○—○—○ or 100 mg/kg ●—●—● i.p.) on MAO activity in atria from mice pretreated with reserpine (10 mg/kg i.p. 16 hr) The values are expressed as per cent of control. Each point represents the mean ± S.E.M. of 6—10 determinations.

10^{-6} M ^{3}H NA (30 min) the disappearance during a 10 min wash-out period was about 910 ng/g (=42.5 % of that present at zero time 1585 ng/g) of which about 650 ng/g came from extraneuronal sites (including ^{3}H NA present in the sorbitol space) These calculations are based on results presented in Figs. 4 6, 9 and 18 A.

The disappearance of ^{3}H NA taken up in atria, previously incubated for 5 min (10^{-7} M or 10^{-6} M) was more pronounced compared to that of atria incubated for 30 min (Fig. 19 *cf* 18 A) Of the amount present at the beginning of the reincubation, 57 % at 10^{-7} M and 31 % at 10^{-6} M remained in the tissue after a 10 min wash-out. On the other hand the disappearance of ^{3}H NA was found to be similar after incubation times of 30 or 60 min.

When the reincubation was performed at 0° C, the disappearance of ^{3}H NA was considerably reduced compared to that at 37 C (Fig. 20)

Effect of MAO activity on the uptake and accumulation of H NA in vitro

The effect of the potent MAO inhibitor nialamide, on the MAO activity in atria from mice pretreated with reserpine was investigated. Injection of 100 mg/kg nialamide *p* caused a reduction of MAO activity to about 14 % of the control value within 15 min After 1—? hr this value was about ? % Ten mg/kg nialamide reduced the MAO activity to about 52 % within 15 min, while after 6 hr the value was 3 % and remained so for 12 hr which was the longest time studied (Fig ?1)

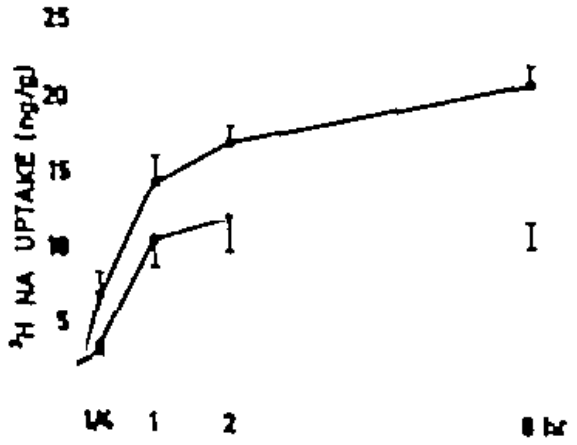


Fig. 22

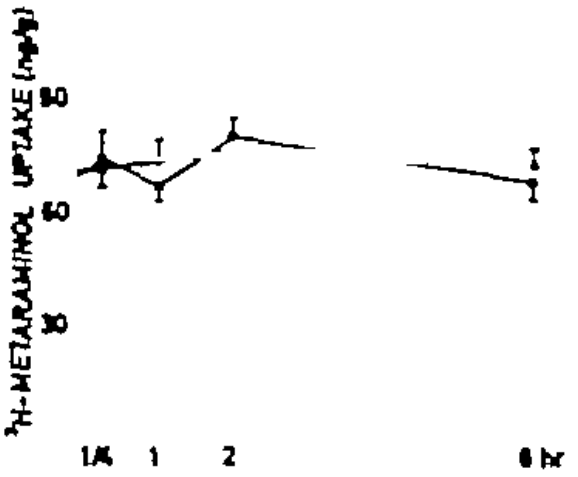


Fig. 23

Fig. 22. Effect of nialamide on the uptake of H NA (10^{-7} M 30 min and 10 min wash-out) in atria from mice pretreated with reserpine (10 mg/kg i.p. 16 hr) The animals were sacrificed t various periods of time after the nialamide injection (10 mg/kg ○——○——○ or 100 mg/kg ● ● ● i.p.) H NA was isolated and determined specifically Each value represents the mean ± S.E.M. from 5–8 determinations.

Fig. 23. Effect of nialamide on the uptake of H metaraminol (10^{-7} M, 30 ml and 10 min wash-out) in atria from mice pretreated with reserpine (10 mg/kg i.p. 16 hr) The animals were sacrificed t various periods of time after the nialamide injection (10 mg/kg ○——○——○ or 100 mg/kg ● ● ● i.p.) Each value represents the mean ± S.E.M. from 8 determinations.

The MAO activity of atria from reserpine pretreated animals was similar to that of normal. The inhibitory effect of nialamide was unchanged after incubation of the atria in buffer alone for 40 min. Atria from untreated mice incubated in buffer for 40 min showed, however a somewhat reduced MAO activity about 20 % compared to that of atria taken directly for determination of the enzyme activity

Uptake and accumulation of ^{3}H NA in atria of reserpine pretreated mice was studied after injection of 10 or 100 mg/kg nialamide i.p. (Fig 22) The ^{3}H NA was determined specifically since the amount of metabolites formed especially the deaminated ones, increases after reserpine treatment (Kopin *et al.* 1962) The ^{3}H NA uptake and accumulation was higher at all points of time studied, using the higher dose of nialamide.

The uptake of ^{3}H metaraminol, an amine which is not metabolized by MAO was studied in an analogously designed experiment. The uptake values were approximately equal after reserpine alone as for 10 and 100 mg/kg nialamide and for all points of time studied (Fig 23)

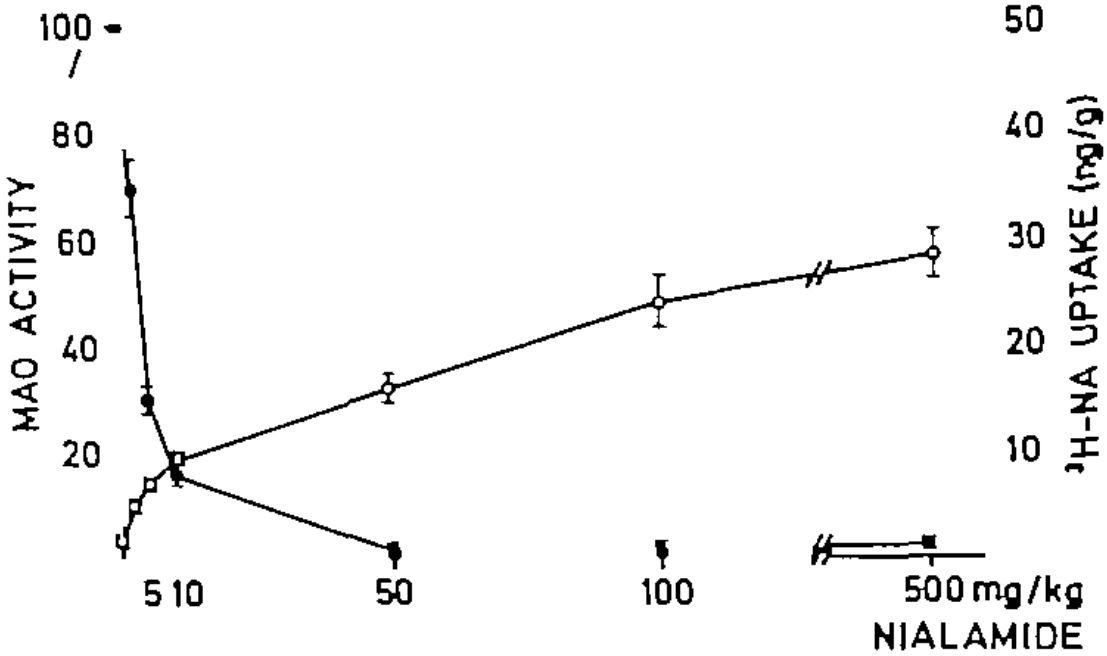


Fig. 24. Effects of various doses of nialamide (injected i.p.) on the MAO activity (● ——— ● ——— ●) and on the uptake of H NA (10^{-7} M, 30 min and a 10 min wash-out) (○ ——— ○ ——— ○) in tria from reserpine (10 mg/kg i.p. 16 hr) pretreated mice. The animals were sacrificed 2 hr after the nialamid injection. H NA was isol ted and deter mined specifically The MAO activity is expressed as per cent of control. Each point represents the mean ± S.E.M. from 4—8 determinations.

Uptake of ^{3}H NA was determined in atria from mice pretreated with reserpine + varying doses of nialamide (Fig 24) After reserpine alone, the uptake and accumulation of ^{3}H NA was very low and was still low after 2 mg/kg nialamide, a dose which caused a 30 % inhibition of MAO activity With increasing dose of nialamide, however the MAO inhibition and the ^{3}H NA uptake increased.

The radioactivity recovered after reserpine alone, consisted of 43 % unchanged ^{3}H NA 17 % ^{3}H NM and 40 % ^{3}H-deaminated metabolites. With increasing dose of nialamide, the relative amount of ^{3}H NA increased being about 81 % after 100 mg/kg nialamide, 2 hr

In control experiments it was observed that nialamide in the incubation medium (50 or 100 μg/ml) increased the uptake and accumulation of ^{3}H NA (10^{-7} M 30 min) 20—30 % of control, both in atria from nialamide pretreated (100 mg/kg i.p. 2 hr) and untreated mice In another experiment it was found that nialamide in the buffer used for wash-out (50 or 100 μg/ml) had no effect on the disappearance of ^{3}H NA previously taken up (10^{-7} M 30 min)

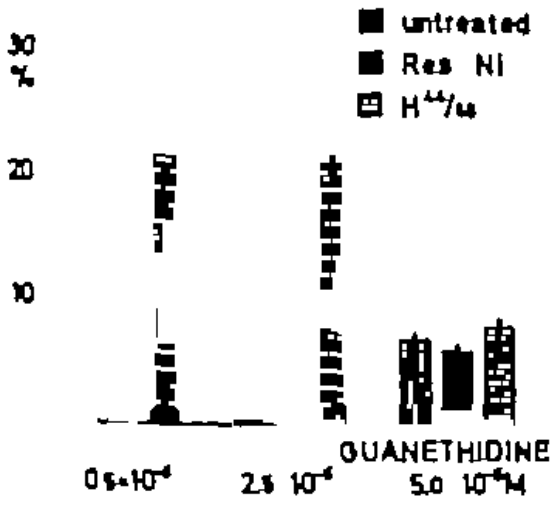


Fig. 25

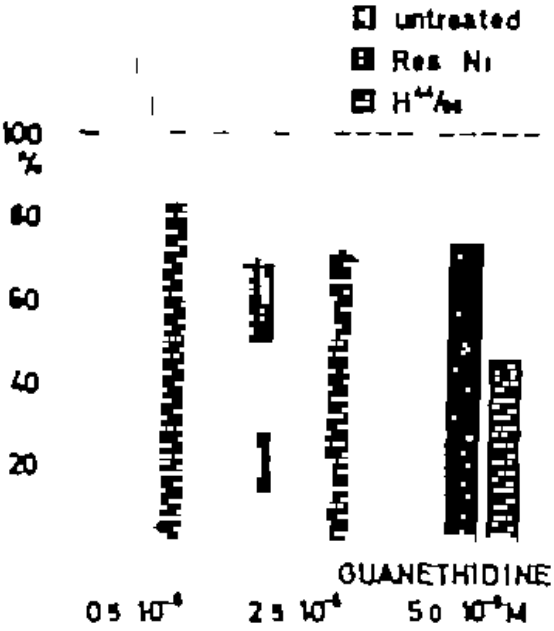


Fig. 26

Fig. 25. Effect of various concentrations of guanethidine *in vitro* on the uptake of H NA (10^{-7} M) in atria of untreated, reserpine (10 mg/kg i.p. 16 hr) + nialamid (100 mg/kg i.p. 2 hr) or H44/68 (500 mg/kg i.p. 16 hr) pretreated mice. The incubation time was 30 min, followed by 10 ml reincubation in fresh buffer. The values are expressed as per cent of the control value for each *in vitro* treatment. Each column represents the mean from 2 determinations.

Fig. 26. Effect of various concentrations of guanethidine *in vitro* on the uptake of H NA in atria of untreated, reserpine (10 mg/kg i.p. 16 hr) + nialamide (100 mg/kg i.p. 2 hr) or H44/68 (500 mg/kg i.p. 16 hr) pretreated mice. The tissue was preincubated with guanethidine for 30 min, followed by 5 min wash-out and hereafter incubated in H NA, 10^{-7} M for 30 min. The values are expressed as per cent of the control value for each *in vitro* treatment. Each column represents the mean ± S.E.M. of 8 determinations.

Effect of guanethidine on the uptake and accumulation of H NA in vitro

Guanethidine *in vitro* caused a reduction of ^{3}H NA uptake, and the inhibitory effect increased with increasing concentration of the drug in the medium. There was, however, no marked difference in the effect of guanethidine on the ^{3}H NA uptake between untreated, reserpine + nialamide or H44/68 pretreated animals (Fig. 25).

In other experiments atria were preincubated with guanethidine (0.5×10^{-6}, 2.5×10^{-6} or 5×10^{-6} M) for 30 min, washed in fresh buffer for 5 min and then incubated in H NA (10^{-7} M, 30 min) (Fig. 26). There was a gradual decrease in ^{3}H NA uptake with increasing concentration of guanethidine in

atria from untreated and H44/68 pretreated mice, whereas in atria from reserpine + nialamide pretreated mice, the inhibitory effect was not observed until the tissue was preincubated in 5×10^{-3} M guanethidine. When using a 15 min wash-out time after the preincubation in guanethidine (5×10^{-3} M) the inhibitory effect observed in the reserpine + nialamide case was, however abolished. The uptake inhibition caused by guanethidine was quantitatively the same in atria from untreated and H44/68 pretreated mice.

No decrease in the NA fluorescence intensity of the adrenergic nerves in atria was observed after incubation for 30 min in 0.5×10^{-6} 2.5×10^{-6} or 5×10^{-3} M guanethidine.

Discussion

The atria of mice consist mainly of the auricles, which are richly adrenergically innervated. A large number of adrenergic nerves is located in the endocardium where they form a two-dimensional network of characteristic varicose nerve terminals (see Jonsson and Sachs 1969 1970a) some of which leave the endocardium and penetrate in between the heart muscle cells. A large number of adrenergic nerves in atria do not seem to be directly related to those innervating the blood vessels (*cf* Nielsen and Owman 1968) A few superficially located clusters of yellow green fluorescent catecholamine containing cells, often with processes, are occasionally present (Jonsson and Sachs 1969) Fluorescence microspectrographic measurements indicate that the dominating catecholamine in these cells is NA (A. Björklund personal communication) and they are probably of a chromaffin" celltype (*cf* Jacobowitz 1967 Nielsen and Owman 1968)

The *in vitro* technique utilized in this study has been successfully used in earlier uptake studies of NA and other amines (see *i.a.* Dengler *et al.* 1962, Giachetti and Shore 1966, Hamberger 1967 and Jonsson *et al.* 1969) It has the advantage of providing controlled experimental conditions, *e.g.* the composition of the medium, amine and drug concentration, temperature and pH. Furthermore, existing differences in circulatory conditions *in vivo* are circumvented. It has been shown that the membrane potentials of neurons in slices of cerebral cortex are maintained for several hours of incubation provided the metabolic requirements, such as good oxygenation and sufficient glucose, are fulfilled (McIlwain and Rodnight 1962) In most of the *in vitro* studies on catecholamine uptake mechanisms referred to above tissue slices have been used. In the present study however whole auricles have been used instead of heart slices, since it was considered that the former preparation would give more intact cells and therefore some disturbances which might be produced by the slicing procedure are eliminated (*cf* Arvill and Ahrén 1966) Mouse atria are also very thin, having a wall thickness of about 0 2—0.5 mm, which is well comparable to that of tissue slices (see Hamberger 1967) Another advantage of mouse atria is that they can be easily prepared as whole mounts for flourescence histochemical studies, which is a simple and highly reproducable technique for the visualization of the adrenergic nerves in their full extent. In addition, mouse atria are as already mentioned richly innervated by adrenergic

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nerves and no change in fluorescence morphology of the nerves have been observed even after a two hour incubation *in vitro*. Mouse atrium can thus be considered as a very suitable preparation for catecholamine uptake studies *in vitro*.

Studies on the metabolism of ^{3}H NA during the *in vitro* incubation showed that the amount of metabolites of ^{3}H NA recovered from the tissue was in most cases rather small. This is probably due to the fact that mouse atrium is a very richly innervated organ, and a large part of amine is accumulated in the adrenergic nerves where it is protected from enzymatic degradation. Furthermore, metabolites do not accumulate in the tissue and are easily washed out (see Iversen 1965). Therefore it has been possible to avoid time-consuming analytical procedures and only determine total radioactivity which in most cases mainly represents unchanged ^{3}H NA. There was observed, however, that the proportion of metabolites increased after pretreatment with reserpine or 6-OH DA (see below).

A factor that would interfere with the determination of the tissue uptake of ^{3}H NA, is the amount of ^{3}H NA located in the extracellular space (≈ sorbitol space) *when the tissue is taken for radioactivity analysis (see Iversen 1963)*. The sorbitol space of mouse atrium was estimated to be about 23 % and this space could be efficiently cleared by incubation in isotope free buffer for 10 min, a procedure which has generally been used in this study. The amount of amine present in the sorbitol space must be considered of significance when using short incubation times or high medium concentrations of amine. This follows from the fact that incubation in 10^{-7} M ^{3}H NA for 5 min, the amount present in the sorbitol space constitutes about 14 % of that present in the tissue. This figure is about 34 % when using 10^{-8} M ^{3}H NA and incubation for 30 min.

Extraneuronal uptake

The extraneuronal uptake of NA has attracted great interest since it might be a significant inactivation mechanism for NA. It is also a factor of importance for the evaluation of the neuronal uptake of exogenously administered NA. Extraneuronal uptake has been shown to occur in several organs *e.g.* in pericytes in brain capillaries (Hamberger 1967), certain cells of salivary glands (Hamberger *et al.* 1967), aortic smooth muscle cells (Avakian and Gillespie 1968), special connective tissue cells and muscle cells of rat heart (Farnebo and Malmfors 1969) and muscle cells of rat iris (Jonsson *et al.* 1969).

In this study the extraneuronal uptake was investigated in atria and whole hearts from mice pretreated twice with a high dose of 6-OH DA, a drug which

has been shown to cause within a short time a severe damage of the NA uptake-storage mechanisms (Jonsson and Sachs 1970a) and specific degeneration of the adrenergic nerves (Tranzer and Thoenen 1967, 1968; Malmfors and Sachs 1968b, Thoenen and Tranzer 1968). The ^{3}H NA uptake in atria from mice pretreated with 6-OH DA represents almost exclusively extraneuronal uptake since it was the same as that of atria from immunosympathectomized mice and to judge from histochemical results (Jonsson and Sachs 1970a). After 6-OH DA pretreatment there was a relative increase in the amount of metabolites, recovered from the tissue, especially of NM but also of deaminated ones, although the absolute amounts were practically the same as in normal atria.

The extraneuronal uptake in atria was found to increase with increasing concentration of ^{3}H NA in the medium and with incubation time, although the amount present extraneuronally calculated as per cent of that present in innervated atria identically incubated, was fairly constant using different incubation times.

There was no true accumulation of NA in the atrial tissue, since the uptake ratios were below one. This does not exclude a local accumulation of NA, however but this possibility seems very unlikely since no special fluorescent structures were observed histochemically after administration of NA. A large part of ^{3}H NA taken up extraneuronally is loosely bound since it is easily washed out. The values for extraneuronal uptake presented in this study are obtained after a 10 min wash-out period. During this wash-out period there is, no doubt, an efficient clearing of the sorbitol space thus eliminating this error when evaluating tissue uptake mechanisms, but also a wash-out of NA very loosely bound to the tissue. The present values are evidently too low but when correcting for the NA washed out only small changes of the extraneuronal uptake values are obtained. The value for 10^{-7} M ^{3}H NA, 30 min should thus be 16.5 % instead of 12 % and for 10^{-6} M ^{3}H NA 30 min 37 % instead of 32 %.

The extraneuronal uptake found in the present study *in vitro* was in the same order of magnitude as that found in *in vivo* infusion experiments (*cf* Thoenen and Tranzer 1968).

Results obtained from the perfused rat heart have demonstrated the existence of two different uptake mechanisms for catecholamines: one, Uptake$_1$ working at low (0.01—1 μg/ml) and the other Uptake$_2$ at high amine concentrations (1—40 μg/ml) (Iversen 1965). The former process is efficiently inhibited by metaraminol, while the latter by NM. Both uptake processes have been claimed to be associated with the adrenergic nerves, but recent findings have given evidence for the view that Uptake$_2$ is mainly extraneuronal (Clarke *et al* 1969, Farnebo and Malmfors 1969). The extraneuronal uptake of mouse atria seemed to be of another character than Uptake$_2$ described by Iversen (1965).

since it was not inhibited by NM in a concentration of 5×10^{-8} M and 5×10^{-6} M caused only a slight inhibition of the uptake, both in atria from untreated and 6-OH DA pretreated mice. In the perfused rat heart (Iversen 1965) there was an 85 % inhibition of $Uptake_2$ and 31 % of $Uptake_1$ by 5×10^{-6} M NM. It has to be considered that in Iversen's study no wash-out had been performed, which might in part explain the differences. It might also in part be due to differences in the activity of the catabolic enzymes MAO and COMT (see Lightman and Iversen 1969)

The exact nature of the extraneuronal uptake is still a matter of uncertainty and its physiological relevance is difficult to evaluate. In the present study it was observed that fairly high concentrations of NA were required to obtain a pronounced extraneuronal uptake of NA and furthermore, no true accumulation of NA took place. It has been suggested that high NA concentrations, about 6×10^{-6} M (see Gillespie 1968 see also Hamberger 1967) exist in the adrenergic nerve-effector cell junctions and the extraneuronal uptake might thus locally have a physiological significance.

The importance of extraneuronal uptake as an inactivation mechanism for circulating catecholamines, is difficult to judge, since the exact quantitative aspects of the metabolism of NA extraneuronally is not completely known. This is mainly due to the fact that neither NA nor its metabolites are firmly retained extraneuronally (see Iversen 1965) It has recently been shown by Lightman and Iversen (1969) that $Uptake_2$ is operating also at low concentrations of NA, but the NA taken up is then rapidly metabolized. At higher concentrations the catabolic enzymes are saturated, providing possibilities for uptake of unchanged NA. These authors claim that $Uptake_2$ may prove to have physiological importance in termination of the actions of NA, both circulating catecholamines and after its release from adrenergic nerve terminals.

Uptake and accumulation of NA — effect of reserpine, MAO and tyrosine hydroxylase inhibition

The uptake processes for catecholamines in the adrenergic nerves are considered to be of greatest importance for the function of these nerves. The general opinion regarding catecholamine uptake mechanisms is that there is one located to the axonal membrane of the adrenergic neuron, the so-called membrane pump and one located intraneuronally in the amine storage granules (see *i.a* Kirshner 1962a, b, Carlsson *et al.* 1962, 1963 Euler and Lishajko 1963a, Carlsson 1966a, b Malmfors 1965 1967 Hamberger 1967 Iversen 1967 Lundborg 1967 Hokfelt 1968 1969) Exogenously administered NA is actively taken up by the membrane pump and is thereafter incorporated

into the amine storage granules, mainly by a Mg^{++}-ATP dependent mechanism. The membrane pump is effectively blocked by *e.g.* cocaine and desipramine (Hillarp and Malmfors 1964 Hamberger 1967) while the uptake into amine storage granules is blocked by reserpine (Carlsson *et al.* 1963 Euler and Lishajko 1963c Stjärne 1964 Iversen *et al.* 1965 Lundborg 1967). It has also been shown that there is a small but significant uptake of NA into the granules, which is reserpine-resistant (Stitzel and Lundborg 1967 Jonsson and Sachs 1969). NA is in addition incorporated into the granules via β-hydroxylation of dopamine (Stjärne 1966 Häggendal and Malmfors 1969 Jonsson and Sachs 1970b).

In the present study four different approaches were used in order to elucidate the neuronal uptake mechanisms: untreated, nialamide, reserpine + nialamide and H44/68 pretreatment. As can be concluded from the results on studies on the extraneuronal uptake, most of the ^{3}H NA taken up and accumulated in mouse atria is neuronal, using medium concentration of 10^{-8}—10^{-6} M. The neuronal represents 70—90 % of the total uptake under these conditions. The uptake of ^{3}H NA in atria increased with time and concentration of ^{3}H NA in the medium. The uptake process obeyed Michaelis-Menten kinetics in accordance with the results of several investigators (Dengler *et al.* 1962, Iversen 1963 Jonsson *et al.* 1969). There is a true accumulation of NA from the external environment. The over-all accumulation of ^{3}H NA in atria from *untreated mice* after incubation in 10^{-7} M ^{3}H NA for 30 min was 6-fold a figure which is about 3 times lower than that obtained in rat iris. The over-all accumulation of ^{3}H NA under these conditions in the rat iris varicosity the structure specialized for the synthesis, storage and release of the adrenergic transmitter has been calculated to be as great as 30 000 times (Jonsson *et al.* 1969). This figure is in the same order of magnitude for the varicosity of the adrenergic nerves in mouse atrium, if it is assumed that the NA concentration of an organ reflects the number of nerves and that the number of varicosities per nerve unit' is constant, since rat iris contains about 3—4 times more NA per weight unit than mouse atrium (see Jonsson *et al.* 1969 Jonsson 1969). In view of the extreme efficiency of the neuronal uptake, this process certainly plays a very important role in termination of the actions of NA released at the sympathetic nerve endings, thereby subserving transmitter economy. This is furthermore supported by results obtained from studies on cat nictitating membrane, where it was found that the response to *l*-NA was potentiated about 40-fold when th neuronal uptake was blocked by cocaine (Trendelenburg *et al.* 1962, Langer and Trendelenburg 1969). The neuronal uptake process is in all probability also of great importance for the inactivation of circulating catecholamines, especially in densely innervated tissues and organs.

Nialamide a potent and irreversible inhibitor of MAO (see Pletscher *et al* 1966) caused in a dose of 100 mg/kg i.p 2 hr an almost 100 % inhibition of the MAO activity in mouse atrium, leading to markedly decreased formation of deaminated metabolites and an increase in the endogenous NA level. The uptake and accumulation of ^{3}H NA was somewhat higher than in untreated animals, which is certainly a consequence of the MAO inhibition decreasing the intraneuronal catabolism of the amine taken up in the nerves. The transport of NA over the axon membrane after nialamide is, however the same as that of untreated.

Reserpine is known to be a strong inhibitor of the Mg^{++} ATP dependent uptake mechanism of the amine storage granules, and this effect results in a depletion of the amine stores (Carlsson *et al.* 1962 1963 Kuruhner 1962a, b Euler and Lishajko 1963c, Stjärne 1964 Rutledge and Weiner 1967 Roth and Stone 1968 Sugrue and Shore 1969) The dose of reserpine used in this study reduced the NA content of the heart to about 8 % of untreated and induced a complete disappearance of the fluorescence of the adrenergic nerves in mouse atria, as observed histochemically The ^{3}H NA recovered from atria incubated in ^{3}H NA was very low after reserpine pretreatment and MAO had to be inhibited in order to retain at least some of the NA taken up intraneuronally The low recovery of ^{3}H NA after reserpine alone is a result of the granular blockade caused by reserpine, thereby making ^{3}H NA taken up in the nerves available for degradation by MAO This is also supported by the finding that there was an increased amount of deaminated H-metabolites recovered from the tissue after incubation in ^{3}H NA (Carlsson 1960 Kopin *et al* 196?)

Although many reports on the site of action of reserpine have appeared dur ing the last decade, the question whether reserpine affects the membrane pump is still a matter of controversy Many authors have reported that reserpine leaves the membrane pump unaffected (see i.a. Hamberger *et al* 1964 Lind mair and Muscholl 1964 Iversen *et al.* 1965 Malmfors 1965, Carlsson 1966b Hamberger 1967 Snyder *et al.* 1968 Jonsson *et al.*) although others have concluded that reserpine interferes with the N the axonal membrane (Dengler *et al.* 1961 Brodie and Beaven 1 964 Costa *et al* 1967) The results presented in this paper s *e+niala* mide pretreatment gives a linear initial uptake of which quantitatively the same After 5 min, h ion fr linearity starts and the sured is lower th This wa

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also the case for longer incubation times and for higher concentrations of ^{3}H NA in the medium, even when the values were corrected for an increased wash-out of ^{3}H NA during the 10 min of reincubation in isotope-free medium (see below). The extraneuronal uptake was not affected by reserpine + nialamide pretreatment. The results presented thus, indicate that a dose of 10 *mg/kg i.p.* of reserpine does not cause any inhibition of NA uptake by the membrane pump since the initial rate of uptake is the same as that after no treatment and the reduced uptake values later observed, certainly reflect a decreased retention of the NA taken up, caused by the inhibitory effect of reserpine on the granular uptake storage mechanism (*cf* Kopin *et al.* 1962). The reduced accumulation of ^{3}H NA after reserpine + nialamide may in part also be due to a not complete MAO inhibition (see page 39).

In addition this hypothesis of reserpine not affecting the membrane pump is supported by the observation that reserpine in a concentration of 0.05—5 *μg*/ml in the incubation medium caused no apparent inhibition of ^{3}H NA uptake in atria from reserpine + nialamide pretreated mice. A slight reduction of ^{3}H NA uptake was observed using a reserpine concentration of 25 *μg/ml*. It has to be pointed out, however that this concentration is very high and the effect of reserpine might be unspecific at this concentration.

Tyrosine hydroxylase catalyzes the formation of 3,4-dihydroxyphenylalanine (DOPA) from tyrosine which is considered to be the rate-limiting step in the NA biosynthesis (Levitt *et al.* 1965). A large dose of the *tyrosine hydroxylase inhibitor H44/68* causes a marked depletion of the endogenous NA stores (Andén *et al.* 1966, Corrodi and Hamson 1966). In mouse heart the NA content was about 40 % of normal. This depletion is dependent on nerve impulse activity of the neuron (Andén *et al.* 1966, Corrodi and Malmfors 1966). It was observed histochemically that there was some variation in the degree of NA depletion in atria from different animals and in different neuron systems from the same animal, which might reflect differences in nerve impulse activity. The results presented in this study show that the ^{3}H NA uptake characteristics in atria from H44/68 pretreated animals are practically the same as normal. Subcellular distribution studies have revealed that there is a granular uptake of ^{3}H NA after H44/68 pretreatment which in quality and quantity was approximately the same as normal, after *i.v.* injection of ^{3}H NA and after *in vitro* incubation in ^{3}H NA (Jonsson and Sachs 1969).

Results from fluorescence morphology studies have indicated a granular uptake, since the appearance of the nerves was normal, *i.e.* there were distinct varicosities and weakly fluorescent non-terminal axons. This is in contrast to the reserpine + nialamide case, where the nerves have a smooth appearance and prominent non-terminal axons (Malmfors 1965, Malmfors and Sach

1968a) It has also been shown in iris from H44/68 pretreated rats that ^{3}H NA previously taken up *in vitro* can be released by field stimulation indicating a granular uptake and storage of the administered amine (Farnebo and Hamberger 1970) I s seems that after tyrosine hydroxylase inhibition, both the membrane a e granular uptake mechanisms are operating, although the endogenous res are considerably reduced. H44/68 itself is not a MAO-inhibitor a l ot interfere with the membrane pump mechanism (Jonsson *et al.* 1969 and Hamberger 1970 Jonsson and Sachs 1970b)

The to and accumulation of ^{3}H NA, both *in vitro* and *in vivo* after H4 ıtment was slightly higher than normal (*cf* Laverty and Roberts n ^{3}H NA infusion experiments it was observed, however that the r as 62 % higher after H44/68-pretreatment than the net uptake hereas the exchange with the endogenous NA store was the sam ulated as a percentage. Thus, a larger amount of the ^{3}H NA is incorporated in the granules without being involved in a displacement The NA taken up after H44/68 pretreatment could in no case account for a complete replenishment of the reduced endogenous NA stores, which *consistent with the results of Laverty and Robertson (1967)* Hokfelt (1968) and Jonsson *et al.* (1969) However Spector *et al.* (1965) obtained a more than complete replenishment in guinea pig spleen and heart after i.p injection of 2 mg/kg NA, in animals pretreated with α-methyl-β-tyrosine In the present study however no complete replenishment was obtained in H44/68 pretreated mice with a high dose of NA (1.5 mg/kg i.p.) Some 30 % was lacking compared to untreated when considering the NA present extraneuronally After incubation in 10^{-8} M ^{3}H NA for 2 hours some 25 % was also lacking. An explanation for these results may be a reduced storage capacity of the adrenergic nerves when the endogenous NA has been released. The reduced storage capacity may be caused by losses of enzymes and other protein-complexes of importance for the uptake and storage of amine. It has indeed been shown that β hydroxylase, ATP and some proteins are released by acetylcholine stimulation of the isolated bovine adrenal medulla (Kirshner *et al.* 1966) and by electrical stimulation *in vivo* of the splanchnic nerve (Blaschko *et al.* 1967 de Potter *et al.* 1969 Geffen *et al.* 1969) The incapacity to replenish the emptied stores might also be due to the possibility that the NA taken up may not easily mix with the NA pool or pools (*cf* Iversen 1963 Crout 1964) It has been found however that in rat brain the intraneuronal catecholamine levels reach normal values after a L-dihydroxy phenylalanine injection in animals previously depleted of their catecholamines by H44/68 (Corrodi *et al* 1966) This difference in results may be explained by assuming that a storage pool of the granules are filled in the ordinary

synthetic pathway via a route (*e g* the β-hydroxylation step) which is reached slowly by NA taken up from the external environment (*cf* Stjärne 1966, Haggendal and Malmfors 1969)

It was thought of interest to investigate the uptake of ^{3}H metaraminol in mouse atria, since this compound is a structural analogue to NA, and has been shown to use the membrane pump for transport into the neuron, and is incorporated into amine storage granules (see Lundborg 1967) Furthermore, it is not metabolized by MAO or COMT The ^{3}H metaraminol uptake for each concentration was quite similar in atria from the differently pretreated groups of mice. This is in contrast to the results obtained with ^{3}H NA, where a lower ^{3}H NA uptake and accumulation was observed after reserpine + nialamide pretreatment. The relatively high uptake of ^{3}H metaraminol after reserpine cannot be explained by a higher extraneuronal uptake compared with ^{3}H NA, but rather reflects a high affinity of metaraminol to the reserpine resistant uptake mechanism in the granules (Stitzel and Lundborg 1967)

Stereospecificity of NA uptake

The uptake and retention of *d* NA and *l* NA has been the subject of many investigations. A pronounced stereospecificity for *l* NA in splenic nerve granules (Stjärne and Euler 1965) and in adrenal medullary granules (Carlsson *et al* 1963) has been proposed. An even more pronounced preference for the *l* isomer has been reported for the axon membrane pump (Maickel *et al* 1963) In histochemical fluorescence studies by Malmfors (1965) no difference in the fluorescence of the adrenergic nerves of rat iris was observed after *d* or *l* NA administration in reserpine + nialamide pretreated animals. This treatment would reveal the uptake by the membrane pump alone. When, on the other hand, the endogenous NA was depleted by H44/68 there was a more marked restitution of th nerve fluorescence by *l* NA (Malmfors, personal communication) These data support the view that the stereospecificity of NA uptake by adrenergic nerves is localized to the amine storage granules. Iversen (1968) however could not find any stereospecificity of the overall uptake in the iris cillary body preparation, while the perfused rat heart showed a marked preference for *l*-NA (Iversen 1963)

The results obtained in this study show that there is a small preference for the uptake of the *l* isomer in intact nerves of both mouse atria and rat irides, while blockade of the Mg^{++} ATP dependent uptake mechanism in the storage granules by reserpine seem to abolish this difference. These data taken together would also indicat that the stereospecificity of the NA uptake of adrenergic nerves is associated with the amine storage granules (*cf* Shore *et al.* 1964)

Disappearance of ^{3}H NA

Studies on the disappearance of ^{3}H NA previously taken up and accumulated in atria, disclosed a rapid initial wash-out of ^{3}H NA which was more pronounced when the incubation was performed in higher concentrations of ^{3}H NA. The rapid initial wash-out certainly reflects clearance of the sorbitol space and wash-out of ^{3}H NA from extraneuronal sites, as shown by results from the ^{14}C-sorbitol studies and the disappearance of ^{3}H NA from atria of 6-OH DA pretreated mice. A 10 min wash-out caused a disappearance of ^{3}H NA previously taken up from a medium containing 10^{-7} M ^{3}H NA (30 min) which constituted 10 % of that initially present in the tissue. Of the amount washed out, 87 % was calculated to originate from extraneuronal sites whereas the rest (13 %) represented ^{3}H NA spontaneously released from the adrenergic nerves, corresponding to about 1.5 % of the amount which had been taken up and accumulated in the nerves. When 10^{-6} M ^{3}H NA was used instead 60 % of that present in the tissue after 30 min, disappeared during a 10 min wash-out. In this case, the spontaneous release from the nerves was calculated to be about 40 % of that taken up in the nerves. It has to be pointed out, however that the last figure is relatively uncertain since a small change in the 10 min wash-out value of the denervated atria, will cause a considerable change in the amount of NA calculated to have been released from the nerves. It was also observed that the wash-out of ^{3}H NA taken up during a short time of incubation (5 min) was more pronounced than after a longer incubation time (30 or 60 min) This cannot be solely explained by the fact that the amount of ^{3}H NA in the sorbitol space constitutes a relatively larger proportion when using a short incubation time. The retention is thus in this case poorer which might be due to a less complete incorporation of amine into the granules.

The time-course of wash-out of ^{3}H NA taken up in atria from untreated nialamide and H44/68 pretreated mice was very similar while reserpine+ nialamide pretreatment caused a more rapid disappearance of the amine. These results show that the granular uptake mechanism is of great importance for the retention of the amine intraneuronally

The wash-out at 0 C of ^{3}H NA was rapid during the first 5 min, and probably represents a clearing of ^{3}H NA present in the sorbitol space, since there was no significant loss of ^{3}H NA between 10 and 120 min. The amount of ^{3}H NA washed out in 10 min approximately equals that initially present in the sorbitol space Thus incubation at 0° C seems to inhibit a spontaneous release of neuronal amine

Effect of temperature and pH on the uptake and accumulation of ^{3}H NA

Exogenously administered NA is actively taken up by the membrane pump, which is considered to be an energy dependent carrier mediated process (Dengler *et al.* 1962, Bertl and Shore 1967 Hamberger 1967 Wakade and Furchgott 1968) The NA uptake is considerably reduced at 0 C, which may be due to inhibition of the energy-yielding metabolic processes at this temperature, although it cannot be excluded that this inhibition is also in part due to changes in physical-chemical properties of the membranes.

Studies on H NA uptake at various temperatures and pHs disclosed optima at 37—40 C and pH 7 4 mainly due to effects on the adrenergic nerves, since the extraneuronal uptake was not considerably affected by changes in temperature or pH of the medium. Different temperatures of the incubation medium did not affect the fluorescence morphology of the adrenergic nerves, while it was observed that a low pH (5.8) induced a decreased fluorescence intensity of the nerves which disclosed a somewhat blurred appearance. The latter finding may indicate a damage of the adrenergic nerves, which would explain the low uptake and retention of ^{3}H NA at low pHs. The uptake of ^{3}H NA in atria was lower at pH 8.0 than at pH 7 4 in contrast to results obtained from studies on isolated bovine splenic nerve granules, which had a higher uptake at pH 8.5 than at pH 7 0 (Euler and Lishajko 1963b) This may indicate that the lower uptake in atrial nerves at pH 8.0 is due to an interference with the membrane pump mechanism. The retention of ^{3}H NA taken up at pH 7 4 or pH 8 0 was practically the same after a 30 min wash-out, while a longer wash-out time resulted in an increased disappearance of ^{3}H NA at pH 8.0 pointing to a possible interference also of the amine storage granules. In view of the results presented it is obvious that pH and temperature have to be carefully controlled in studies on NA uptake-storage mechanisms.

Effect of MAO activity on uptake and accumulation of ^{3}H NA

MAO is an enzyme which is located both intra and extraneuronally and in mouse atria the main part is located outside the adrenergic nerves (Jonsson and Sachs 1970a, *cf* Iversen *et al.* 1966) The enzyme is considered to be present in the mitochondria (Laduron and Belpaire 1968 Jarrott and Iversen 1968, Hörtnagl *et al.* 1969) Under normal conditions, when NA is taken up into the nerves very little NA is deaminated by MAO since the amine is rapidly incorporated into the granules. This is supported by the finding that

MAO inhibition leads only to a minor increase of accumulation of endogenous NA. The present studies were made on atria from reserpine pretreated mice, since it provides possibilities to follow experimentally induced changes of intraneuronal MAO activity. After a granular blockade by reserpine the amine accumulates extragranularly where it is readily available for degradation by MAO; therefore the amount of ^{3}H NA recovered from the tissue after reserpine pretreatment is very small and MAO has thus to be inhibited in order to retain NA intraneuronally. The effect of differentiated MAO inhibition, produced by nialamide, on the ^{3}H NA uptake and accumulation in atria from reserpinized mice was investigated in some detail.

The uptake of ^{3}H NA gradually increased with increasing dose of nialamide, whereas the MAO activity was already inhibited almost completely after 50 mg/kg (*i.p.* 2 hr). This may be because a very small fraction of intraneuronal MAO, too small to be measured with accuracy, is sufficient to account for a considerable deamination of NA. If this is correct, then the effect of a higher dose of nialamide than 50 mg/kg should be a reduction of the activity of the small MAO fraction, leading to a subsequent increased accumulation of NA.

Nialamide, 10 or 100 mg/kg, had no effect on the uptake of ^{3}H metaraminol in atria from reserpinized mice. The uptake values were about the same for the two doses used and for all the time periods studied. This can be explained by the fact that metaraminol is not metabolized by MAO.

It has been reported by Carlsson and Waldeck (1967), however, that the optimal dose of nialamide for the *in vivo* uptake of ^{3}H NA in hearts from reserpinized mice was 10 mg/kg. This observation has been confirmed in this laboratory (Sachs, unpublished observations). The findings *in vivo* are thus in contrast to the present results obtained *in vitro*, when the ^{3}H NA uptake and accumulation was in all cases higher after 100 mg/kg than after 10 mg/kg nialamide. Carlsson and Waldeck (1966, 1968) have furthermore reported that nialamide causes an increased disappearance of metaraminol and α-methyl NA taken up *in vivo*. These effects of nialamide obtained *in vivo* were suggested to be mediated by endogenous monoamines accumulating in the adrenergic nerves as a consequence of the MAO-inhibition, leading to a displacement of the amine taken up. The increased disappearance could be abolished by decarboxylase inhibition (Carlsson *et al.* 1968). The discrepancies between the results obtained in this study and those of Carlsson and coworkers, might be due to effects of nialamide observed *in vivo* not being maintained *in vitro*. A possible explanation could be that nialamide in addition to its MAO inhibitory effect also may interefere directly with the uptake and/or releasing processes of NA *in vivo* (*cf.* Besson *et al.* 1969). This is not

likely however since nialamide in a high concentration *in vitro* did not caus any uptake inhibition or any increase in spontaneously released ^{3}H NA pre viously taken up These data taken together indicate that the differences be tween the *in vivo* and the *in vitro* results might be due to a decreased accumu lation of endogenous monoamines *in vitro* after MAO inhibition. Considering these viewpoints it may be suggested that studies using NA uptake for the examination of drugs affecting MAO activity in the adrenergic nerves are preferably performed *in vitro*

Effect of guanethidine on the H NA uptake

The antihypertensive drug, guanethidine has been shown to be taken up by the peripheral adrenergic nerves and is incorporated into the amine storage granules, thereby depleting the tissues of their NA stores. The uptake and accumulation of guanethidine is inhibited by denervation or reserpine pre treatment (Sheppard and Zimmerman 1959 Obianwu *et al.* 1968) It has furthermore been shown that guanethidine can be released upon nerve stimu lation and act as a false transmitter (Boullin *et al.* 1966) Guanethidine in hibits both the membrane pump and the granular uptake-storage mechanisms (Shore and Giachetti 1966 Lundborg and Stutzel 1968, Malmfors and Sachs 1968a)

In the present investigation it was found that the ^{3}H NA uptake decreased with increasing concentration of guanethidine in the medium. When using a concentration of 5×10^{-8} M guanethidine together with ^{3}H NA (10^{-7} M) in the medium the inhibition of ^{3}H NA uptake into the nerves was almost com plete since it was approximately the same as the extraneuronal. The inhibitory effect observed in these experiments, is certainly due to interference with the membrane pump since no difference was observed between reserpine + nialamide pretreated on one hand and untreated or H44/68 pretreated on the other

Preincubation of atria in guanethidine, however followed by a short wash out period (5 min) before the incubation in ^{3}H NA, caused an inhibition of the ^{3}H NA uptake which was considerably less than that caused by incubation with ^{3}H NA and the drug together This was especially pronounced when using a low concentration of guanethidine. Also in these experiments the inhibitory effect of guanethidin increased with increasing medium concentration of the drug, but after reserpine + nialamide pretreatment no uptake inhibition wa observed until a high guanethidine concentration was used. These results are thus consistent with those of Shore and Giachetti (1966) showing that pre incubation in a relatively low concentration of guanethidine results in a prefer

ential inhibition of the granular uptake, while a high concentration leads to an inhibition of both uptake processes.

The results obtained may be explained by guanethidine competing with NA for uptake and storage sites in the adrenergic nerves. When the tissue is incubated with guanethidine and ^{3}H NA together in the medium, the inhibition of NA uptake reflects the action of the drug on the membrane pump. Pre incubation with a low concentration of the drug, however and subsequent washing led to an inhibition of ^{3}H NA uptake in atria from untreated and H44/68 pretreated mice. This is certainly due to a granular blockade caused by a previous uptake and incorporation of guanethidine in the granules. This follows from the observation that no effect on ^{3}H NA uptake was noticed after reserpine + nialamide pretreatment. After preincubation in a high medium concentration of guanethidine an effect on the membrane pump was also obtained, probably in this case due to an insufficient clearing of the drug from *extraneuronal sites*.

Conclusions

In isolated mouse atria there is a rapid and efficient uptake and accumulation of NA *in vitro*. Using NA concentrations of 10^{-8}—10^{-5} M relatively small amounts of NA were recovered from adrenergically denervated atria, corresponding to 9—30 % of the uptake in innervated atria. Thus extraneuronal uptake increases with an increasing medium concentration up to 75 % of innervated atria when the incubation is performed in 10^{-4} M NA. There was no true extraneuronal accumulation of NA compared with the medium. The amine taken up and retained extraneuronally was not observed to be localized in any specific structures, but was evenly distributed in the heart muscle cells and was found to be easily washed out from the tissue.

The neuronal uptake accumulation capacity is very efficient, and the ratio between amine concentration in the vicinity of the adrenergic nerve and the medium has been estimated to be in the order of 30 000 : 1 after incubation in 10^{-7} M NA for 30 min. The neuronal NA uptake is efficiently blocked at 0° C, and exhibits temperature and pH optima of 37—40° C and pH 7.4 respectively. There is a stereospecificity with a small preference for the *l* isomer probably localized to the amine storage granules. It is postulated that the axon membrane pump of the adrenergic neuron is the most important mechanism for the initial uptake of NA. This is supported by the findings that pharmacological modifications of intraneuronal mechanisms, such as the inhibition of MAO or tyrosine hydroxylase or blockade of the Mg^{++} ATP dependent granular uptake did not alter the transport of NA into the neuron. The membrane uptake process obeys Michaelis-Menten kinetics.

To prevent rapid deamination of the NA taken up intraneuronally when the granular uptake is blocked by reserpine MAO has to be inhibited. This inhibition must be very efficient, since a relatively small enzyme activity can cause a substantial decrease in the accumulation of NA.

The reduced accumulation of exogenous NA found after reserpine and MAO inhibition undoubtedly reflects an impaired retention of amine taken up, rather than a direct action of reserpine on the membrane pump. A granular blockade also leads to an increased disappearance of the NA taken up intraneuronally indicating that the amine storage granules are the most important structures for the total uptake and retention of NA.

The uptake and accumulation of ^{3}H NA after tyrosine hydroxylase inhibi-

tion when the endogenous NA content was considerably lowered, were the same as in controls, while the net uptake was markedly increased. Less of the NA taken up was thus involved in a displacement process. In no case could the NA taken up account for a complete replenishment of the NA store. This might be due an incomplete mixing of exogenously administered NA with the endogenous NA pool or pools. It may in part also be due to a reduced storage capacity

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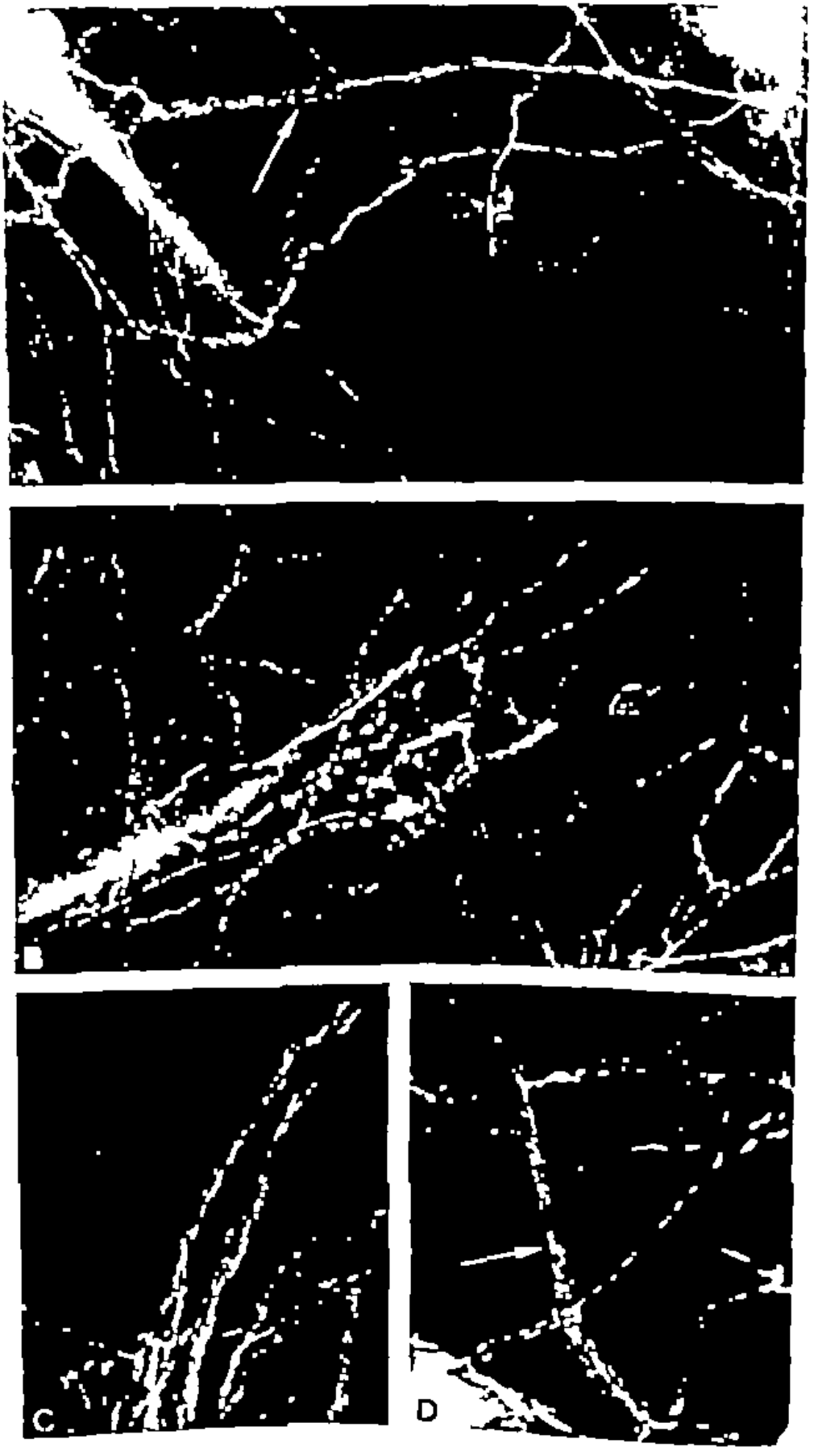
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Micrographs

Micrograph 1 Histochemical demonstration of NA in stretch preparations of atria from untreated mice. The adrenergic nerves form a ground-plexus in the endocardium. Several nerve terminals are seen in each strand (see micrographs 1A and 1D). Single varicose terminals lea the strands.

Micrograph 1C shows a densely innerva ed blood vessel.

Magnifications: A, 190; B-D, 300.

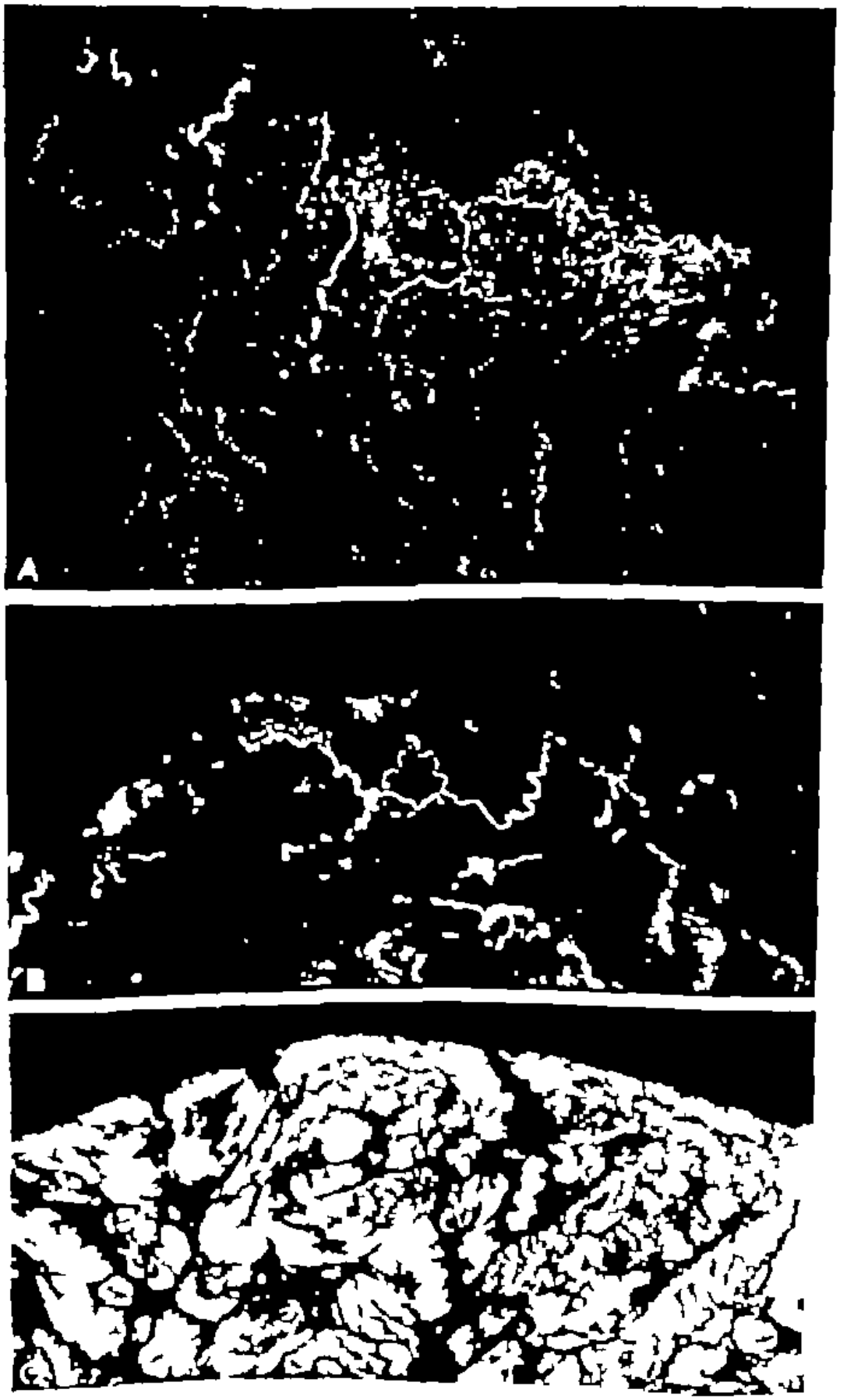

A
B
C
D

Micrograph 2 Histochemical demonstration of NA in sections (8μ) of mouse atria.

Micrograph 2A shows a large amount of adrenergic nerves lying in the endocardium. Some single nerve terminals penetrate in between the muscle cells of the myocardium. ×190.

Micrograph 2B shows arcose adrenergic nerves in bundles, just under the endothelium. ×300.

Micrograph 2C shows a bernsically denervated atrium from a mouse pretreated with 6-OH DA (2 × 50 mg/kg i.p. 10 and 2 hr). The tissue had been incubated in 10^{-5} M adr. NA for 30 min. NA adrenergic nerves are visible but only a faint even distributed fluorescence throughout the muscle cells can be seen. ×300


A
B
C

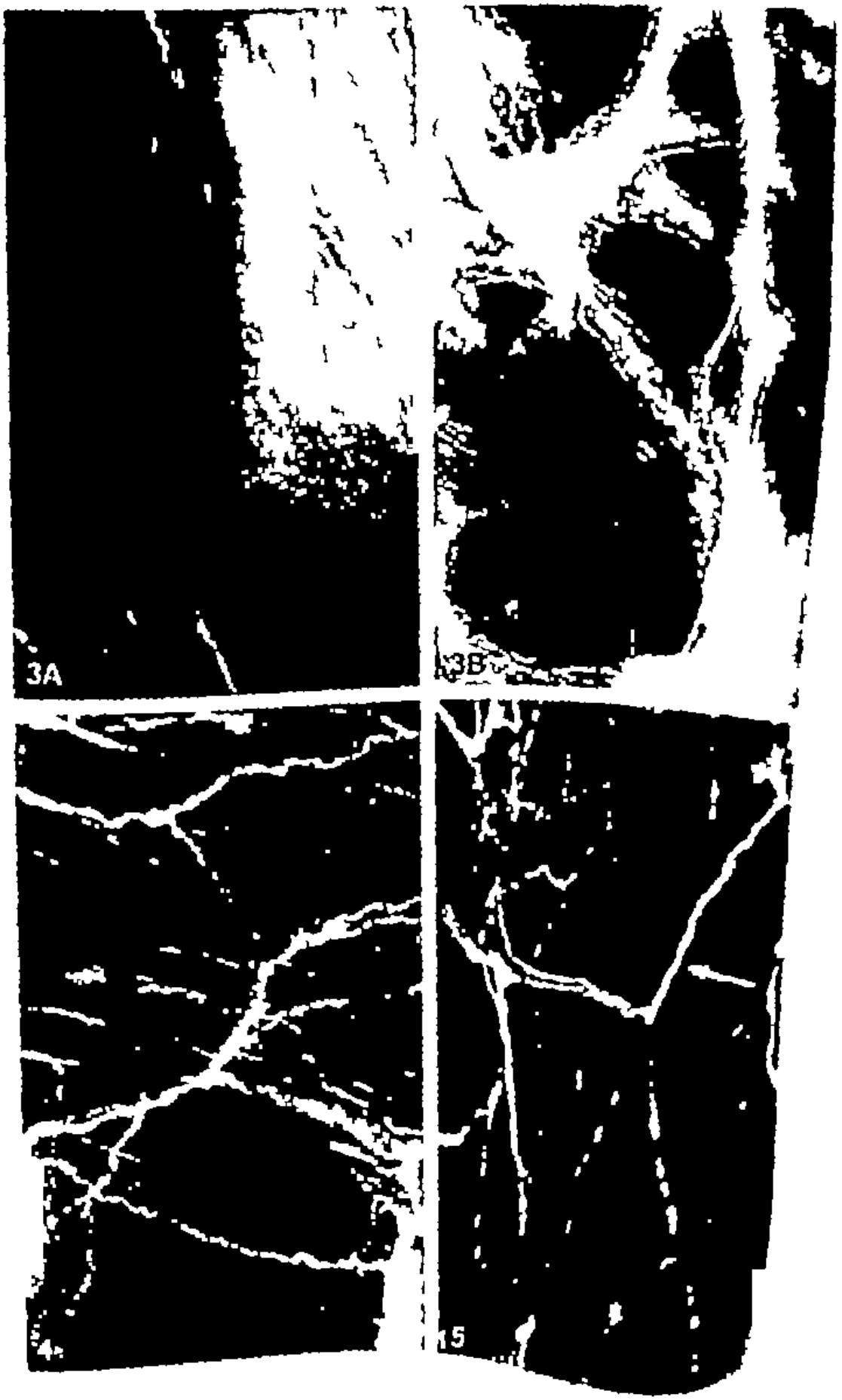
Micrograph 3. Stretch preparation of atrium from mice pretreated with 6-OH DA (2 × 50 mg/kg i.p. 16 and 2 hr).

Micrograph 3A. Many adrenergic nerves can be observed. The white lines are due to diffracted light. Atrium from immunosympathectomized mice showed the same appearance. × 300.

Micrograph 3B. The atrium had been incubated in 10^{-4} M of NA for 30 min. No adrenergic nerves but an intense fluorescence of the heart muscle fibres can be seen. × 300.

Micrograph 4. Stretch preparation of atrium from untreated mouse. The atrium had been incubated in Krebs-Ringer phosphate buffer pH 5.8 for 30 min. The adrenergic nerves display a fuzzy appearance. × 300.

Micrograph 5. Atrium from mouse pretreated with nialamide (100 mg/kg i.p. 2 hr). The atrium had been incubated in Krebs-Ringer bicarbonate buffer for 30 min. The non-terminal axons (→) display a slightly increased fluorescence intensity compared to those in the treated. × 300.


3A
3B
4
5

Micrograph 6. Stretch preparations of atria from mice pretreated with reserpine (10 mg/kg i.p. 16 hr) and nialamide (100 mg/kg i.p. 2 hr)

Micrograph 6A. No adrenergic nerves are visible. The white lines are caused by diffraction. × 190.

Micrograph 6B. The atrium had been incubated in *dl*-NA 10^{-6} M for 30 min. The nonterminal varicosities are more prominent than in untreated and the terminals have a smooth appearance. × 190.

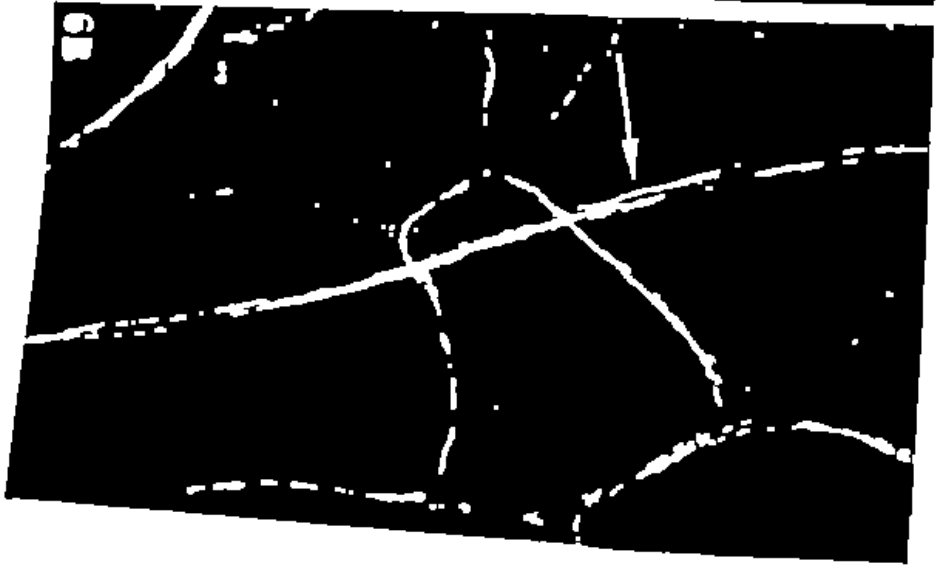
Micrograph 7. Stretch preparations of atria from mice pretreated with H44/68 (500 mg/kg i.p. 16 hr)

Micrograph 7A. Only a few faint nerve terminals (arrows) can be observed. × 300.

Micrograph 7B. The atrium had been incubated in 10^{-6} M *dl*-NA for 30 min. The adrenergic nerves show the same varicose appearance as untreated. × 300.







STOCKHOLM 1970. KUNGL. BOKTRYCKERIET P.A. NORSTEDT & SÖNER

ACTA PHYSIOLOGICA SCANDINAVICA
SUPPLEMENTUM 342

CARDIOVASCULAR ADJUSTMENT TO SOMATOMOTOR ACTIVATION

The elicitation of increments in heart rate, aortic pressure and venomotor tone with the initiation of muscle contraction

By

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AKADEMISK AVHANDLING

som med vederbörligt tillstånd
för erhållande av medicine doktorsgrad
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försvaras i Aulan Karolinska sjukhuset
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CONTENTS

I	*Introduction*	5
	Aim of the present study	7
II	*General Procedures and Methods*	8
III	*Cardiovascular Effects of Voluntary Isometric Muscle Contractions*	12
	A Subjects	12
	B Procedure	12
	C Results	13
	Heart rate and aortic pressures	13
	Right atrial and central venous pressures	15
	Venomotor reactions	15
	D Effect of body position	17
	E Effect of developed muscle force	17
	F Comments	18
IV	*Autonomic Nervous Mediation of the Heart Rate and Blood Pressure Increase on an Isometric Muscle Contraction*	20
	A Subjects	20
	B Procedure	20
	C Drug influence at rest	22
	D Results	22
	Effect of atropine	22
	Effect of propranolol	23
	Effect of phentolamine	23
	Effect of atropine and phentolamine	25
	Effect of orthostatic reaction	25
	E Comments	25
V	*Autonomic Nervous Mediation of the Heart Rate Acceleration on an Isometric Muscle Contraction in Tetraplegic Men*	28
	A Subjects	28
	B Procedure	28
	C Results	29
	D Comments	31
VI	*Cardiovascular Response to Intended Muscle Contraction during Neuromuscular Blockade*	34
	A Subjects	34

B Procedure 34
C Results 36
Effect of succinylcholine at rest 36
Cardiovascular response to intended muscle contraction 37
D Comments 40
VII *The Relationship between Cardiovascular Response and Extent of Muscle Activation* 43
A Subjects 43
B Procedure 43
C Results 43
D Comments 44
VIII *Cardiovascular Response and Augmentation of Spinal Reflexes on Performed and Intended Muscle Contraction* 46
A Subjects 46
B Procedure 46
C Results 48
D Comments 50
IX *General Discussion* 53
X *Summary and Conclusions* 56
Acknowledgements 59
References 60

The author has had the privilege to cooperate with M D Evert Knutsson and some of the results of Chapters VI and VIII will appear in a common, more extensive study

CHAPTER I

Introduction

The maintenance of circulatory homeostasis in the organism on the transition from rest to muscular work implies rapid adjustments of cardiac activity and vascular tone. The instantaneous onset of the circulatory events suggests a neurogenic mechanism, which has been described both as a reflex, originating in the active muscles (Alam and Smirk 1937, 1938, Asmussen *et al.* 1943 b, Tuttle and Horvath 1957, Asmussen *et al.* 1963, Humphreys and Lind 1963, Asmussen and Nielsen 1964, Lind *et al.* 1964, Donald *et al.* 1967, Lind *et al.* 1968) as well as a centrally evoked "cortical irradiation" to the vasomotor centers (Krogh and Lindhard 1913). Topical excitation of selected hypothalamic and subthalamic structures in awake animals, which simulated the cardiovascular response to exercise (Rushmer and Smith 1959) supported the concept of a centrally induced cardiovascular drive on the initiation of muscular exercise. Investigations of the "defence reaction" in animals (Hess 1949, Abrahams *et al.* 1964) and other studies of induced behavioral reaction patterns have indicated that somatomotor activity is accompanied by "efferent discharges from the corticohypothalamic centers to lower nerve centers, which affect the cardiovascular system" (Folkow *et al.* 1965). Whether this centrally induced circulatory response is appropriate for the behavioral changes has, however, not been established. Recent experiments suggest that the cardiovascular changes during naturally elicited preparation for fighting are different from those induced by electrical stimulation of the hypothalamus (Adam *et al.* 1968).

In man, circulatory adaptations to muscular exercise have been suggested to occur in anticipation of muscle activation. It has been felt that the magnitude of this mentally induced cardiovascular response should be related to the extent of mental engagement (Folkow *et al.* 1965) and to the amount of intended muscle activity. This circulatory phenomenon is apt to decrease with habituation to the appropriate muscle activation. Thus it has been thought unlikely that voluntary habituated muscle contractions and, especially within restricted muscle groups, should be accompanied by any

significant centrally initiated cardiovascular drive (Folkow *et al* 1965)

From comparisons of voluntary and electrically induced work in humans (Asmussen *et al* 1943 a, b) the similar circulatory and respiratory adjustments to oxygen uptake in the two kinds of work were supposed to be of peripheral nervous rather than of cortical origin The decreased cardiovascular response to a sustained handgrip performed by the affected limb of a patient with syringomyelia (Lind *et al* 1968) was thought to illustrate the earlier documented hypothesis (Lind *et al* 1964 1966) that the circulatory events were initiated by some peripherally evoked reflex.

The concepts of the mechanism initiating the cardiovascular response to muscular work may be summarized as follows

1 Stimuli from motor cortex are conducted to the vasomotor centers, provoking an activation of the cardiovascular system parallelly to that of the somatomotor system The cardiovascular response will be appropriately modulated to the metabolic demands through feedback mechanisms.

2 Receptors in the activated muscles incite afferent discharges to the vasomotor centers which adapt the cardiovascular response. A part of the autonomic response might also be elicited at the level of the spinal cord.

Thus the proposed regulatory mechanisms are seemingly contradictory as was commented on by Horvath and Howell (1964) who wrote "most confusing has been the failure of any theories of regulation to explain adequately the initial events of exercise

The efferent autonomic nervous mediation of the initial circulatory changes at the onset of a voluntary muscle contraction has not been assessed in healthy man. In an attempt to analyze the underlying mechanism it was found that after the administration of a beta adrenergic blocking drug propranolol Macdonald *et al* 1966) a sustained handgrip elicited similar elevations in mean aortic pressure and a greater percentage increase in heart rate *The immediate heart rate acceleration at the onset of mild, dynamic exercise in dogs under beta adrenergic blockade (Smulyan and Eich 1968)* was taken to be largely independent of the beta adrenergic system

In man the effect on heart rate of beta adrenergic, vagal and a combined sympathetic and parasympathetic blockade has been observed during the 5th and 7th minute of dynamic leg exercise (Robinson *et al* 1966) It was concluded that the increase in heart rate with mild exercise was mediated predominantly by a decrease in parasympathetic activity while at higher levels of work sympathetic stimulation also contributed to cardiac acceleration. Corresponding studies of the efferent autonomic activity at the initiation of muscle work have not been found in the literature.

Aim of the present study

As will be evident from this introductory account, the origin of the cardiovascular drive at the onset of muscle activity has not been clearly demonstrated. Furthermore, a differentiated function of the outflow from the autonomic nervous system evoking the initial circulatory response to isometric muscle work has not been established.

In the present study an attempt was therefore made

to study cardiovascular effects at the initiation of voluntary isometric muscle contractions,

to demonstrate the readjustment of the autonomic nervous system provoking the initial circulatory response, and

to establish whether the origin of the cardiovascular drive is central or peripheral.

Dynamic exercise with small muscle groups provokes a higher heart rate and peripheral vascular resistance than exercise with large muscle groups at similar working intensities (Bevegård *et al.* 1966 Freyschuss and Strandell 1968) Isometric work induces more rapid and marked increases of aortic pressures than dynamic work (Tuttle and Horvath 1957) Thus isometric work within small muscle groups was thought to be a preferable stimulus for the cardiovascular effects to be studied.

Through administration of drugs with inhibitory effects on the parasympathetic and the alpha-adrenergic and beta-adrenergic sympathetic nervous system an attempt was made to define the efferent pathways mediating the initial cardiovascular response.

Finally it was supposed that the circulatory changes accompanying an intended contraction in muscle groups with a blocked neuromuscular transmission would elucidate the origin of the cardiovascular drive.

CHAPTER II

General Procedures and Methods

Isometric muscle contraction The various types of isometric contractions will be designated under the attempted movements. The subjects were trained to perform the appropriate, isometric muscle contractions while relaxing non working parts of the body and breathing quietly When a tendency to Valsalva manœuvres was observed, it was mostly prevented by asking the subject to count aloud. Voluntary isometric muscle contractions were performed as flexion of the elbow and as handgrip. During elbow flexion the upper arm was held parallel to the longitudinal axis of the body The forearm was held in a position between pronation and supination and the angle between the upper arm and forearm was 90° The force developed in the contraction was displayed on a dual beam oscilloscope (Model OS 25A, Advance Instrum.) Three to four determinations were made of the maximal force of contraction exerted during 5 sec. Then the position of the other beam on the oscilloscope screen was adjusted to correspond to 50 up to 100 per cent of the highest observed value (100% MVC) and the subjects were told to exert the indicated force of contraction They were informed of both the start and the duration (in general 5—10 sec) of the contractions verbally (Chapter III IV V) or through a visual signal (Chapter III VI—VIII) and they were also trained to monitor their own performances. The muscle contractions were followed by resting periods, which allowed circulatory variables to attain precontraction values. All subjects were investigated in supine position unless otherwise stated in the respective chapters.

Further details concerning the performance of various isometric muscle contractions will be given in the appropriate chapters.

Catheterizations A teflon catheter was inserted percutaneously (Seldinger 1953) into the left brachial artery and under fluoroscopic guidance the tip was positioned in the aortic arch. The catheter was made visible on the X ray screen by introduction of a metal spiral guide, which was withdrawn when the correct position had been obtained. Via a medial cubital vein of

the right arm and by the same technique a teflon catheter was placed in the right atrium or in the superior vena cava. In the study of venomotor reactions a teflon catheter was percutaneously introduced 2—3 cm either upstream or downstream in a forearm vein

For the administration of succinylcholine the right brachial artery was punctured below or at the intercondylar line and a teflon catheter was inserted approximately 10 cm in the proximal direction.

All catheters were made of teflon tubing with external and internal diameters of 1 45 mm and 1 05 mm, respectively The tips of the catheters were adapted during heating to the dimensions of guides with a diameter of 0 8 mm.

Before arterial catheterization the skin and subcutaneous tissue were infiltrated with ca. 1 ml 0.5 per cent mepivacaine solution (Carbocain®)

Venomotor reactions in a resting arm were detected by the occluded limb technique, as described by Wallace (1956) and used by others (Samueloff *et al.* 1966 a, b Zitnik and Lorenz 1969) according to the following procedure.

A teflon catheter within a forearm vein in the resting arm was connected to a pressure transducer (strain gauge) The zero reference level for the venous pressure was the tip of the catheter A pneumatic cuff 12 cm wide was placed around the limb just above the elbow The pressure in the cuff was first raised until the venous pressure in the distal segment was approximately 25 mm Hg The cuff pressure was then rapidly increased to 250—300 mm Hg Around 2 min after the arrest of circulation the venous pressure stabilized and the observations were commenced. The occlusion was maintained for periods up to 10—12 min, during which time only small changes of P_{O_2} P_{CO_2} and pH of the venous blood in the occluded segment have been reported (Samueloff *et al.* 1966 b) A deep inspiration was used as a standard procedure for testing both free pressure transmission and the reactivity of the system. As suggested in a previous report (Samueloff *et al* 1966 b) 5—10 min were allowed for recovery Artefacts due to changes in tension of the muscles in the occluded limb were easily detected as abrupt and irregular pressure oscillations in the otherwise smooth venous pressure record.

Drugs and their administration. Atropine, 1 mg/ml, phentolamine (Regitine®) 10 mg/ml, or propranolol (Inderal®) 1 mg/ml, was rapidly injected intravenously (iv) The initial doses were roughly adapted to body

weight and were for atropine 0 5—1 0 mg, for phentolamine 7.5—10 mg and for propranolol 10 mg The total amount of drug given was dependent on the resulting heart rate and aortic pressures and, for a combined dose of drugs, also on preceding administrations. Further details are given in appropriate chapters.

Succinylcholine (Celocurin klorid®) was diluted in physiologic saline solution to give a concentration of 0 5 mg/ml of the drug The solution was injected intra arterially (ia) in the brachial artery of the limb which had performed hand contractions in the preceding control study As an initial test dose 0 5 mg of succinylcholine was used. The subsequent doses, 0 7—4 mg, were adjusted to the degree and duration of the neuromuscular blockade.

Blood pressures were recorded with Elema differential transformer transducers (EMT 490 A) in the earlier part of the study (Chapters III—V) in the latter part (Chapters VI—VIII) Medec strain gauge transducers (30—145) were used. Both types of transducers had a pressure range of 0—300 mm Hg Mean pressures were obtained after electrical integration (time constant 1 0 sec) The reference point was taken at the midthoracic point of the anteroposterior plane at the insertion of the fourth rib of the sternum.

Respiratory movements were indicated by means of a strain gauge, mounted in a clasp by which the two ends of a piece of rubber tape were held around the thorax. The start and end of the thoracic excursions during respiration were thus indicated and the records allowed a semiquantitative evaluation of the amphtude of the respiratory movements.

The force developed by the contracting muscles was recorded by dynamometers of different designs.

Dynamometer 1 was used during flexion of the elbow and consisted of a rigidly mounted strain gauge transducer to which the exerted force of contraction was transmitted through a strap around the wrist at the level of the styloid process of the radius.

Dynamometer 2 was used during handgrip and had the shape of a rod. It was made up of two metal plates separated by a strain gauge transducer The plates were covered with plexiglass to minimize the effect of temperature of the hand.

The strain gauges were coupled as one arm of a bridge at the input of a chopper amplifier The linearity was ±0 02 per cent within the operative

ranges and the frequency response was estimated to be at least 1 Hz. The forces measured were read directly in kiloponds according to prior calibration.

Muscle action potentials (EMG) were recorded with Ag AgCl surface electrodes (Beckman) and the myopotentials were fed to a differential amplifier (linear frequency response 4 Hz to 10 kHz) In general they were rectified and time averaged to facilitate the estimation of the degree of electromyographic activity

Heart rate was determined from an electrocardiogram (ECG) and was presented both as an ECG and as the inverted value of each R R interval recorded as an analogue signal on the y-axis (see, *e g* Fig 1) on a UV writer The heart rate signal was triggered by the preceding R R interval.

Recording instruments Four to eight variables were simultaneously and continuously recorded on a 8-channel UV writer (0—550 Hz—3dB Oscillofil, Siemens) and four of them were preserved on a FM tape recorder (0—620 Hz±0.5 db model T 3000 Thermionic) allowing further reproductions and changes of scales to facilitate further analyses. ECG and actual blood pressures were also continuously monitored on a 4-channel oscilloscope (model KP-404 ITT) The developed muscular force was displayed as previously described on a double-beam oscilloscope. At times some of the variables were also recorded on a Grass Polygraph DC Driver Amplifier (Grass, Instrument Co)

Statistical methods Statistical calculations were performed according to Snedecor (1959) The following probability (P) levels of significance were used

P <0 001	highly significant	***
P <0 01	significant	**
P <0 05	probably significant	*

Definitions of cardiovascular effects on muscle contractions The heart and aortic pressure response to isometric contractions were measured highest values attained within 3—8 sec from the start of the muscle tion, and so were eventual changes in right atrial or central venous p res. In each individual the measurements have been made prior to and within the same time interval during the contraction F details will be given in the following chapters.

CHAPTER III

Cardiovascular Effects of Voluntary Isometric Muscle Contractions

Subjects

The subjects were 18 healthy male blood donors, aged 22—28 years, and 4 healthy women, aged 26—37 years, belonging to the laboratory staff. Right atrial pressures were recorded in 9 of the subjects, central venous pressure in one. In the study of venomotor reactions two of the females from the laboratory staff and five of the blood donors participated.

Procedure

The procedures for catheterization, for detection of venomotor reactions and for continuous recording of the observed variables (*viz.* heart rate, aortic, right atrial, central and peripheral venous pressures, respiratory movements, integrated EMG and muscular force) are described under General Procedures and Methods.

Voluntary isometric muscle contractions were performed as flexion of the elbow in 6 of the subjects and as handgrip in 21 according to the procedure described in Chapter II.

EMG recorded with surface electrodes over different muscle groups of the shoulder revealed some activity during handgrips of maximal strength, but almost none was observed during contractions of the commonly used lower intensities (*viz.* $\leqq$70 % of MVC). In some instances the EMG output was led through an audio-amplifier and a loudspeaker to make even small amounts of electrical activity from the muscles audible. The subject and the observers were thereby informed of any muscular activity. An attempt was made to remove or minimize such stray activity.

The observed force of contraction during handgrip does not reflect the total muscle activity as stabilizing muscles in the forearm are activated. However, by comparing the recorded force with EMGs from both the flexor and the extensor muscles of the forearm at varying percentage of MVC, a reasonable proportionality was shown to be present between the recorded tension and the myopotentials of both types of muscles.

After determination of the maximal force of contraction (see General Procedures and Methods) the 50—100 per cent of the observed force during MVC was indicated on the oscilloscope.

The changes in heart rate and aortic pressures accompanying the muscle contractions were measured as the highest values attained 3—8 sec from the start of muscle activation. In each individual the measurements have been made within the same time interval after the start of contraction. Contraction periods less than 7 sec were only used in five of the subjects, who performed muscle contractions of intensities higher than 70 % of MVC.

The changes in peripheral venous pressure evoked by muscle contractions or deep inspirations represent data recorded immediately prior to the respective stimuli and at the height of the effects.

All investigations were performed in the supine position, and in five of the subjects also during 60° head-up tilting for a period of 5 to 10 min.

Results

Heart rate and aortic pressures Increments of heart rate and aortic pressures initiated by voluntary isometric muscle contractions in supine position were always obtained. The circulatory effects were similar when the con-



Fig. 1 The circulatory response of subject K. G. on isometric muscle contractions at 70 % of MVC in supine position. Small downward deflections of the respiration record denote expiration. Baseline shifts are due to artefacts. P_{RA} = right atrial mean pressure, mm Hg. (U. Freyschuss, J. appl. Physiol. 1970, in press)

tractions were performed as elbow flexion or handgrip. The cardiovascular response is illustrated by individual cases in Figs 1 2 and 4

At the onset of muscle activity the next R R interval was shortened the pulse pressure of the subsequent beat had decreased, and the systolic and

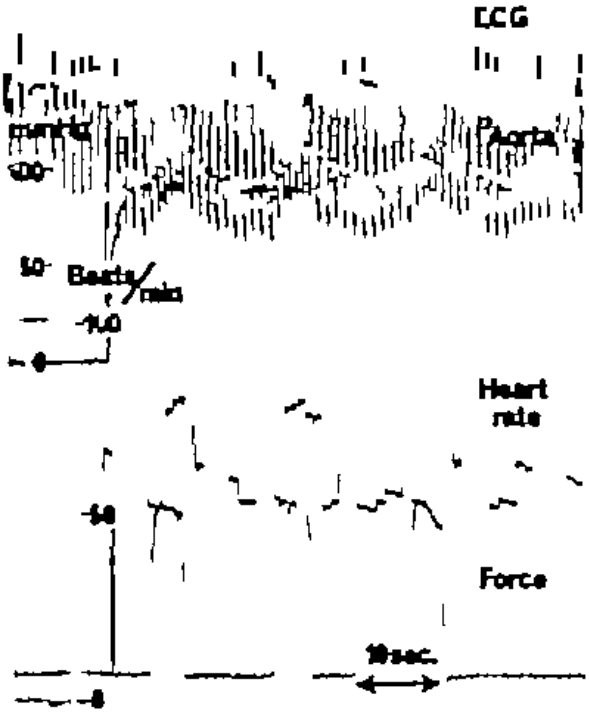


Fig 2. Increases in heart rate and in aortic pressures (subject U F) initiated by muscle contractions at 70 % of MVC in supine position.

diastolic aortic pressures started to rise. For another few beats the pulse pressure remained reduced but thereafter it mostly attained the precontraction amplitude. In general the heart rate increase attained a peak during the contraction while the aortic mean pressure levelled off or demonstrated a continuous rise. The mean increase in heart rate of the group (see Fig 3) was 18 beats/min (SE ±1 6 range 8—35 beats/min, n = 20) and the rise in aortic mean pressure averaged 16 mm Hg (SE ±1 1 range 6—26 mm Hg n = 20) The individual mean values are summarized in Fig 3 and each thin arrow represents data obtained during 3—6 determinations which were made 3—8 sec after the start of contractions and at 50—95% of MVC (average value 73 % of MVC) in each individual the circulatory effect of muscular contraction has been measured at the same muscular force and within the same time interval after the start of contraction. Within the denoted intervals of the relative muscle force (% of MVC) the mean values are ranked, starting with the low values to the left. The SE of the heart rate increase observed within the individuals varied from ±0 7 to ±4 4 beats/min corresponding values for the rise in aortic pressure were ±0 8 and ±6 3 mm Hg

The regression of the mean increments in aortic mean pressure on heart rate was $y = 0\,4890\,x + 6\,9224$ SD $y = 3\,7982$, $r = 0\,69^{***}$

The reproducibility of the cardiovascular effects within an individual is illustrated in Figs 1 and 2. In Fig 1 the SE of the increments in heart rate and aortic pressure were ±1 0 beats/min and 3 0 mm Hg respectively Corresponding values in Fig 2 were ±1 2 beats/min and ±0.3 mm Hg

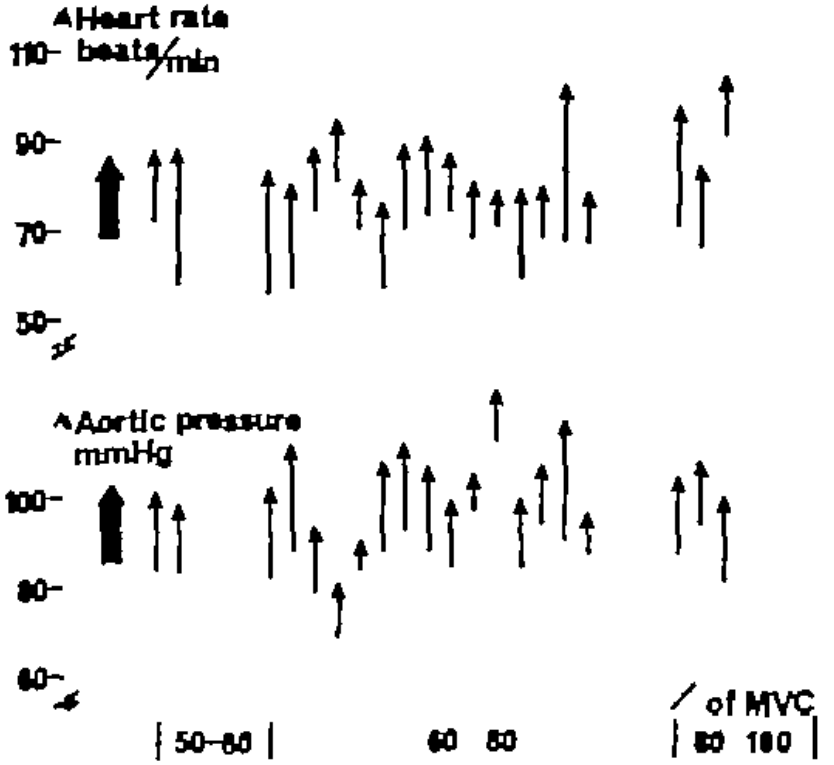


Fig. 3 The effect on heart rate and aortic mean pressure of isometric muscle contraction in supine position. Thin arrows represent individual mean values, while heavy arrows indicate the mean values of th group (n=20) For further information see text.

Right atrial and central venous pressures. The records in Fig 1 indicate that respiration continues during the contractions. The increase in right atrial pressure varied between 4 and 2 mm Hg in Fig 1 in Fig 4 the pressure of the right atrium was 2 mm Hg prior to the contraction and changed to 2.5 and 1 mm Hg during muscle activation. In Fig. 5 right atrial pressure is the same prior to and during the contraction. Changes in right atrial or central venous pressures were not a consistent finding

Venomotor reactions in a resting limb Isometric contractions of the hand initiated an increase in venous pressure in the occluded segment of the contralateral, resting forearm. A typical pattern of response is illustrated in

Fig 5 With the onset of a handgrip at 70 per cent of MVC, venous pressure increased smoothly from 34 mm Hg and reached the peak value, 38 mm Hg 5 sec after the release of contraction. During the following 15 sec the pressure subsequently attained the precontraction level. The pressures of the right atrium, 7 mm Hg and of the artery in the occluded segment 18 mm Hg, were unchanged during the contraction, and the

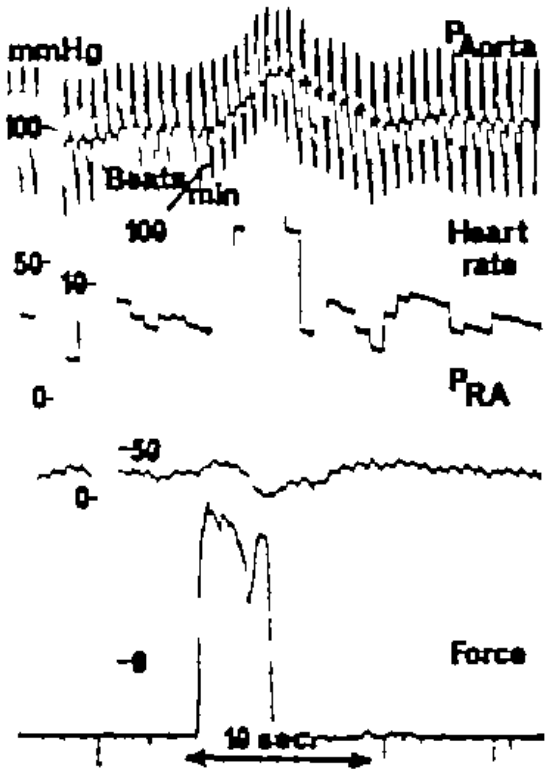


Fig. 4 The circulatory response of subject U A. to an isometric muscle contraction (100 % of MVC) in supine position. Symbols as in Fig. 1 (U Freyschuss, J appl. Physiol. 1970 in press)

depth and frequency of breathing were uninfluenced In the same figure a deep inspiration elicited a similar smooth but greater pressure rise, from 36 to 47 mm Hg

By venous congestion before complete circulatory arrest the equilibrium pressure in the veins was always maintained above that of the arteries in the occluded segment.

Although the rise in venous pressure on muscle activation appeared consistently the amplitude of the pressor response was rather varying In the same subject contractions of the same per cent of MVC induced increases in venous pressure from 3—9 mm Hg during the same period of occlusion Similar variations in response were also noted during deep inspirations.

The average values of venous pressure response to isometric handgrip and to deep inspiration were 3 mm Hg (range 1—6) and 5 mm Hg (range 2—10) respectively

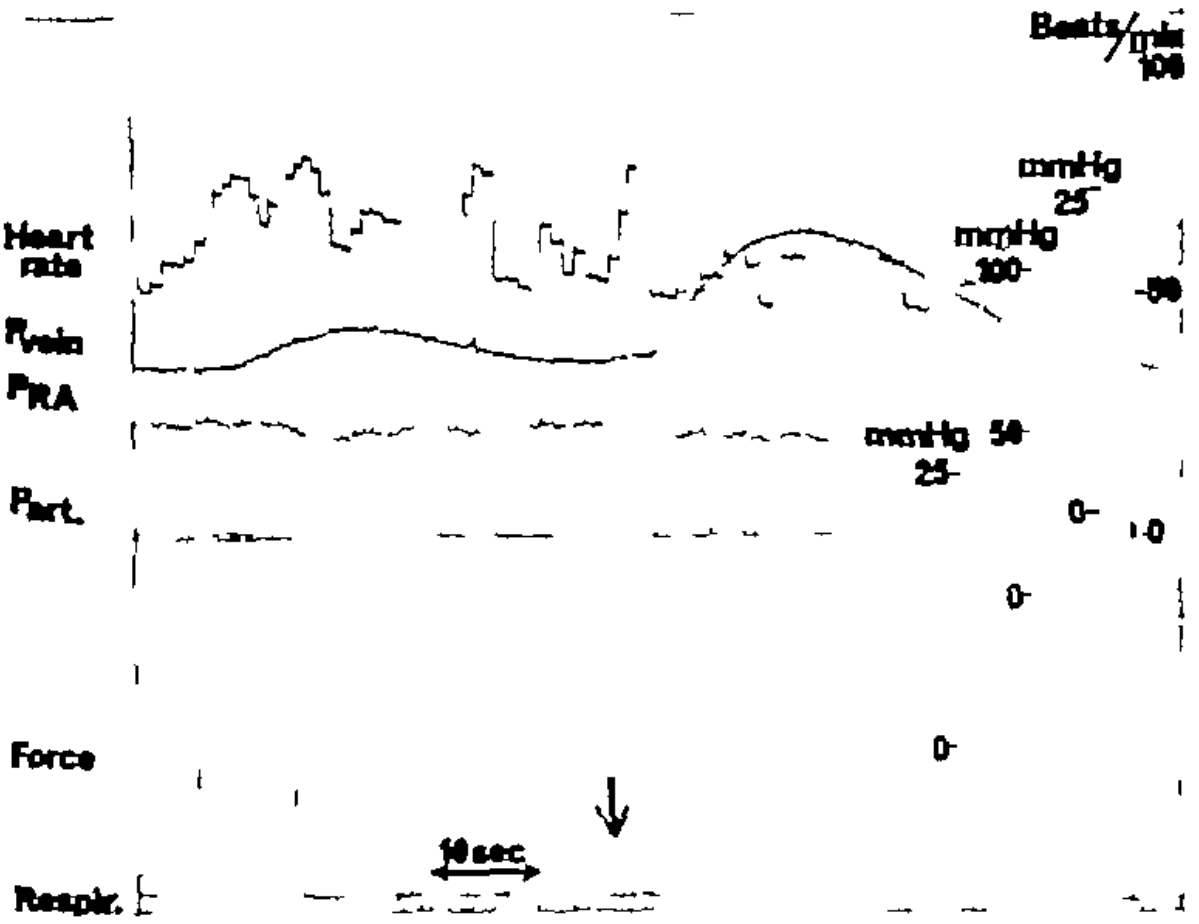


Fig. 5 The cardiovascular effects of an isometric muscle contraction (70 % of MVC) and of a deep inspiration (a downward deflection of the respiration record) denoted by an arrow In subject H. M. P_{v} and P_{art} = venous and brachial artery mean pressures in the occluded limb segment. The reference points for zero and 25 mm Hg refer to (from the left) peripheral venous and right trial pressures (P_{RA}) respec tively

Effect of body position

The 60 head-up tilting did not significantly affect the amplitude of the heart rate blood pressure response to muscle activation in three of the subjects. However in the two others, A.B and L.B. with an orthostatic heart rate reaction upon head-up tilting (heart rate increases from 73 to 115 beats/min and 78 to 114 beats/min, aortic mean pressure elevations from 69 to 72 mm Hg, 78 to 81 mm Hg) a muscle contraction elicited a rise in aortic mean pressure similar to that in the horizontal position although the heart rate response was reduced to some few beats (Fig 6)

Effect of developed muscle force

The rate at which the aortic pressure rose during contraction was roughly related to the exerted force of contraction, i e the difference in time course was discernible between 90 or 100 % of MVC and 50 or 60 % of MVC.

However within the actual range no significant correlation could be assessed between the developed muscle force in per cent of MVC or in absolute values (kilopond) and the magnitude of the heart rate and aortic pressure effects. Both intraindividual and interindividual data demonstrated this discordance.

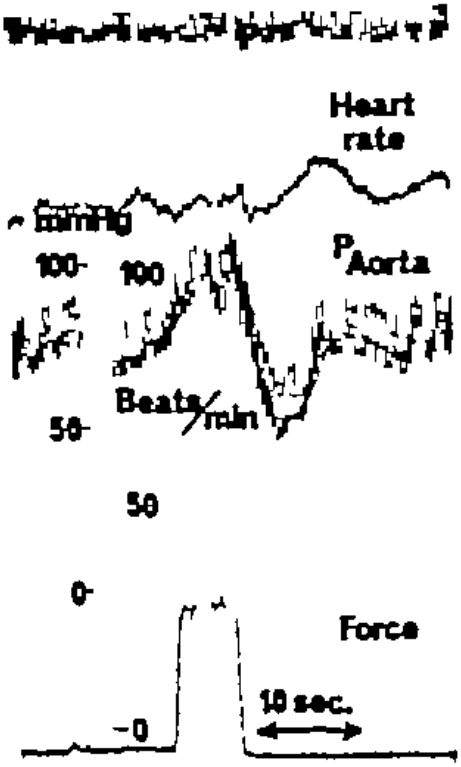


Fig. 6. The effect on heart rate and aortic pressures in subject A. B. of a muscle contraction at 70 % of MVC during 60 head-up tilting. (U Freyschuss, J appl. Physiol 1970, in press)

The upward slope of the EMG record is presumably due to a synchronization of the myo-electric potentials a recruitment of new motor-units might also be involved. (See Fig 1)

Comments

The magnitudes of the heart rate and aortic pressure increments evoked during a voluntary isometric muscle contraction in the present study are in accordance with earlier reported data obtained 10—15 sec after the start of contraction (Tuttle and Horvath 1957 Humphreys and Lind 1963 Lind *et al* 1964 Donald *et al* 1967) However the instantaneous onset of the circulatory events and the fact that their elicitation is independent of changes in respiratory movements and of right atrial pressures have not been previously demonstrated.

The lack of correlation between the magnitude of the pressor response and the relative muscle force developed was also observed by Humphreys

and Lind (1963) during sustained handgrips at tensions varying from 30 to 100 % of MVC and within 10—15 sec from the start of contraction. When the flexion of the hand muscles was sustained for longer periods, *e g* 1—5 min the cardiovascular response appeared to be determined by the relative intensity of the contraction (Lind and McNicol 1967)

A reflex venoconstriction in a resting forearm with arrested circulation has been reported to occur during strong sustained isometric contractions of small muscle groups (Bevegård and Shepherd 1966) but simultaneous respiratory movements or right atrial pressures have not been recorded. Due to the reactivity of venous tone to extraneous stimuli (Merritt and Wessler 1959 Epstein *et al* 1968) such as irregularities in the depth of breathing, special care must be taken to eliminate them. The elicitation by isometric muscle contraction of increases in peripheral venous tone together with an acceleration of the heart and a rise in aortic pressures has not been previously demonstrated From the intra-arterial pressures in the occluded segment it can be concluded that the changes in venous pressure reflect venomotor reactions and not a shift of blood into the veins secondary to a constriction of the precapillary vessels (see Fig 5)

The time course of the central cardiovascular effects and the peripheral venous adjustments to muscle contraction suggests a nervously mediated mechanism. The dissociation of the heart rate and aortic pressure response in the presence of an orthostatic reaction might indicate that the efferent pathways adjusting the heart rate and aortic pressure response emanate from different divisions of the autonomic nervous system. In an attempt to analyze the underlying mechanism the study of Chapter IV was undertaken.

CHAPTER IV

Autonomic Nervous Mediation of the Heart Rate and Blood Pressure Increase on an Isometric Muscle Contraction

The *circulatory effects evoked by a short lasting isometric muscle contraction* suggested a mediation through the autonomic nervous system. In an attempt to investigate the autonomic nervous outflows initiating the cardiovascular response to muscle activation drugs with inhibitory effects on the parasympathetic, on the alpha adrenergic and on the beta adrenergic sympathetic nervous system were administered and their influence on the circulatory response to muscle contraction was studied

Some of the results described in this chapter are obtained from U Freyschuss, J appl. Physiol 1970 in press.

Subjects

Ten healthy male blood donors, aged 22—28 years (average weight 70 1 kg) volunteered for the study One of the subjects was investigated twice

Procedure

Details about catheterizations, drugs and recordings of the observed variables *viz.* heart rate aortic right atrial or central venous pressures, respiratory movements, integrated EMG and muscular force) are given under General Procedures and Methods. Isometric muscle contractions were performed *in supine position as described in Chapters II and III*

When appropriate control data had been obtained, the circulatory response to similar muscle contractions was observed after iv drug administration. Two series of observations were made the first pertained to the effect of a single drug in the second one the effect, in the same subjects, of a combined dose of drugs was studied. Thus atropine (1 0—1 5 mg) was given to five subjects, a beta blocking agent, propranolol (Inderal® 10 mg) to one subject, phentolamine (Regitine® 7.5—10 mg) to six, and to seven of the subjects both atropine (0 5—2.0 mg) and phentolamine (5 0—10 mg were administered This composite dose was injected on an

average 9 min after the injection of the first drug In four investigations of three subjects the combined dose of drugs could not be given due to technical defects of the recording systems. The circulatory effect of muscular contraction has in each individual been measured as the highest value attained within the same time interval from the start of the muscle activa

TABLE 1 Effect on heart rate (HR) beats/min, and on aortic mean pressure (P_{Ao}) mm Hg, of iv administration of atropine (A) or phentolamine (Ph) and of both atropine and phentolamine (A+Ph) C refers to predrug data. Mean values ($\bar{X}$) ± standard error (SE) number of observations (n) and probability levels (P) are given.
(U Freyschuss, J appl. Physiol. 1970 in press)

Subject			C	A	C	Ph	C	A+Ph
A. B.	HR		68	93				
	P_{Ao}		80	96				
L. B.	HR		82	100	73	103		
	P_{Ao}		63	66	81	0		
U. R.	HR		79	95				
	P_{Ao}		82	86				
S. G.	HR		66	90			81	124
	P_{Ao}		85	103			98	92
P. L.	HR		73	116			102	154
	P_{Ao}		90	102			96	75
B. L.	HR				72	103	75	123
	P_{Ao}				96	85	89	105
J. F.	HR						68	129
	P_{Ao}						93	90
A. H.	HR				74	108	87	154
	P_{Ao}				94	86	92	79
K. G.	HR				49	74	61	119
	P_{Ao}				89	72	89	79
U. A.	HR				71	110	97	153
	P_{Ao}				96	88	99	86
		n	5	5	5	5	7	7
	HR	$\bar{x}$	74	99	68	100	82	137
		SE±	3.1	4.6	4.7	6.5	5.6	6.1
			P <0.01		P <0.001		P <0.001	
	P_{Ao}	$\bar{x}$	80	91	91	80	94	87
		SE±	4.6	6.9	4.9	3.8	1.5	3.9
			P <0.05		P <0.05		P	n. s.

[illegible] whereby each individual serves as his

[illegible] of atropine (1 mg) or phentolamine (7.5—[illegible] and dose of both atropine (0 5—1 5 mg) and phen[illegible] are presented in Table 1 These data were obtained within [illegible] of the iv administration when the circulatory variables on the continuous records demonstrated a temporary stationary level. No figures are available of the phentolamine effect in one subject due to technical error The changes in right atrial pressures are not listed, since the number of observations is incomplete. Atropine as well as phentolamine induced decreases in right atrial pressure which averaged 1.5 (n=3) and 3 0 mm Hg (n=4) respectively When both drugs were given together the mean decrease was 2 mm Hg (n=5) The effects of atropine are in accordance with previous reports (Wessler *et al* 1957 Berry *et al* 1959) and so are the cardiovascular adjustments to phentolamine (Taylor *et al* 1965 a, b) The predrug observations prior to the second injection do not fulfil control criteria, as an influence of the previously given drug might be or was obviously present.

Propranolol 10 mg, was given iv 65 min after the administration of 1 mg of atropine and induced a decrease of heart rate from 73 to 62 beats/min aortic mean pressure did not significantly change.

Results

Common to the single-dose injections of drugs in the present study are their relatively short lasting effects as judged from the observed circulatory variables. In general the magnitude of the initial circulatory response to muscle contraction successively started to attain predrug values approximately 5 min after the administration. A second dose of the same compound was at times necessary The data presented below were obtained within a period when the precontraction circulatory values were similar to the drug effects at rest.

Effect of atropine Fig 7 section A, and data in Table 2 demonstrate the anticholinergic effect on the change of heart rate and aortic pressure elicited on muscle activation. The mean heart rate increase of the group 104 to 107 beats/min was 17 beats/min less ($P<0.01$) than in the predrug study A concomitant but less marked ($P<0.05$) decrease (6 mm Hg) in the pressor response was recorded.

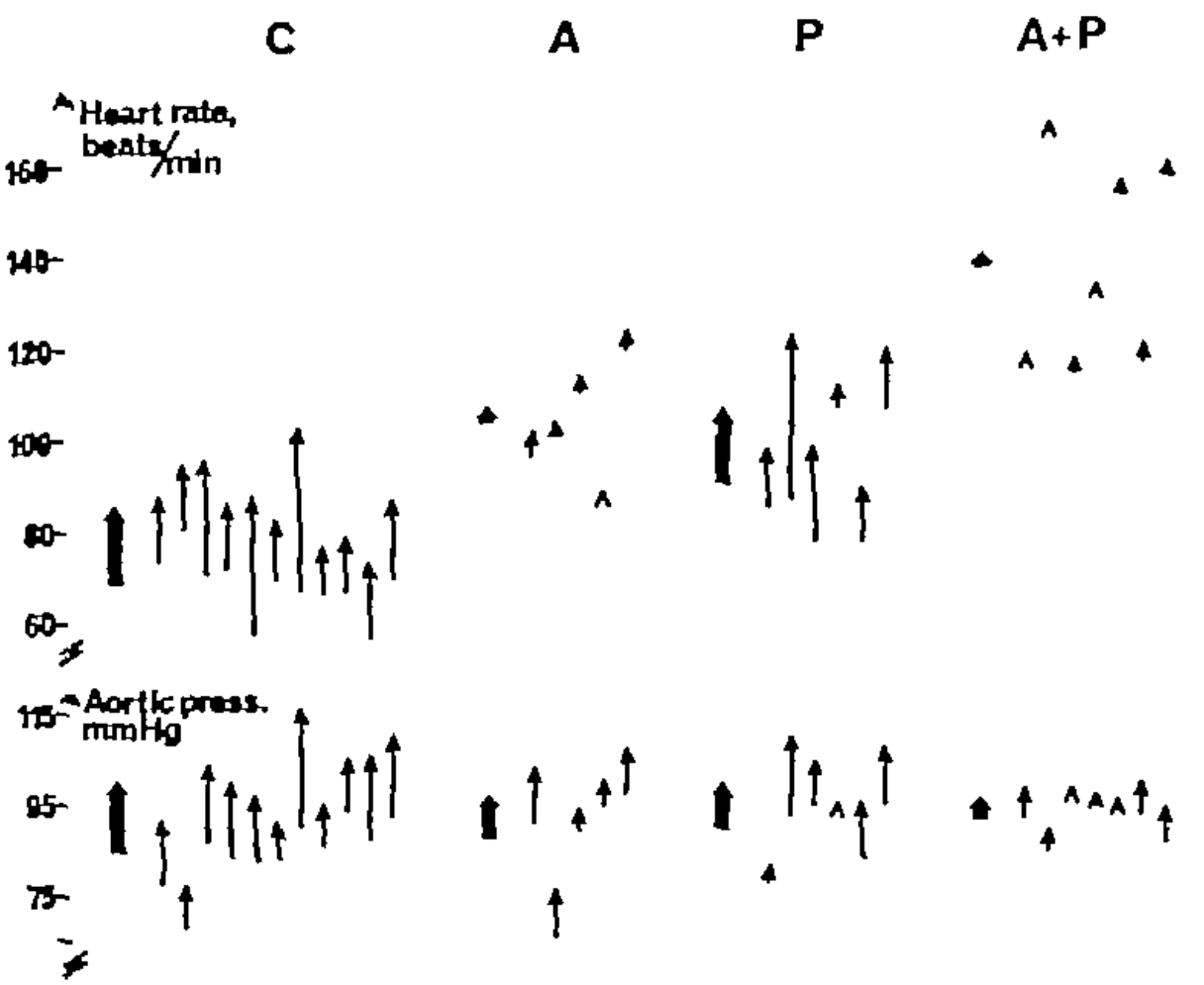


Fig. 7 The effect on heart rate and aortic mean pressure of isometric muscle contraction in C) control studies, and under the influence of A) atropine, P) phentolamine, A+P) atropine and phentolamine. Thin arrows represent individual mean values while heavy arrows indicate the mean values of the group. The first five mean values in section C represent control data for the atropine study and the remaining six mean values the control data for the phentolamine study. Of the mean values in section A+P nos. 1 and 2, should be compared with nos. 4 and 5 in section C the remaining five in section A+P should be compared with the last five in section C. (U. Freyschuss, J. appl. Physiol. 1970, in press)

Effect of propranolol. Under the influence of a beta-blocking agent the circulatory effects initiated by a muscle contraction were similar to those in the predrug, control period. The mean increments in heart rate and in aortic mean pressure were 15 beats/min and 16 mm Hg.

Effect of phentolamine. When phentolamine was given as the first drug, the mean increase in heart rate of the group, 17 beats/min, was similar to that in the control studies (Figs. 7 and 8 and Table 2) although the individual mean heart rates at the initiation of muscle activation after drug

TABLE 2. Difference ($\bar{d}$) in the heart rate increase (ΔHR) beats/min, and the aortic mean pressure rise (ΔP_{Ao}) mm Hg, elicited on a muscle contraction prior to and after drug administration. C—A, C—Ph, C—A+Ph predrug data minus those attained after atropine, phentolamine, and atropine plus phentolamine, respectively. Degrees of freedom (df) and t-test values (t) are given. P values are for paired comparisons between individual means.

	C — A		C — Ph		C — A+Ph	
	ΔHR	ΔP_{Ao}	ΔHR	ΔP_{Ao}	ΔHR	ΔP_{Ao}
$\bar{d}$	16.7	6.0	0.6	4.7	18.0	11.8
df	4	4	5	5	6	6
t	5.3252	2.8476	0.2147	3.0349	5.1228	4.9862
P	<0.01	<0.05	>0.40	<0.05	<0.01	<0.01

administration were on an average 20 beats/min higher (Fig. 7). The rise in aortic pressure was less marked, the mean value of the group being 5 mm Hg ($P < 0.05$) lower than in the control situation (Fig. 7 and Table 2).

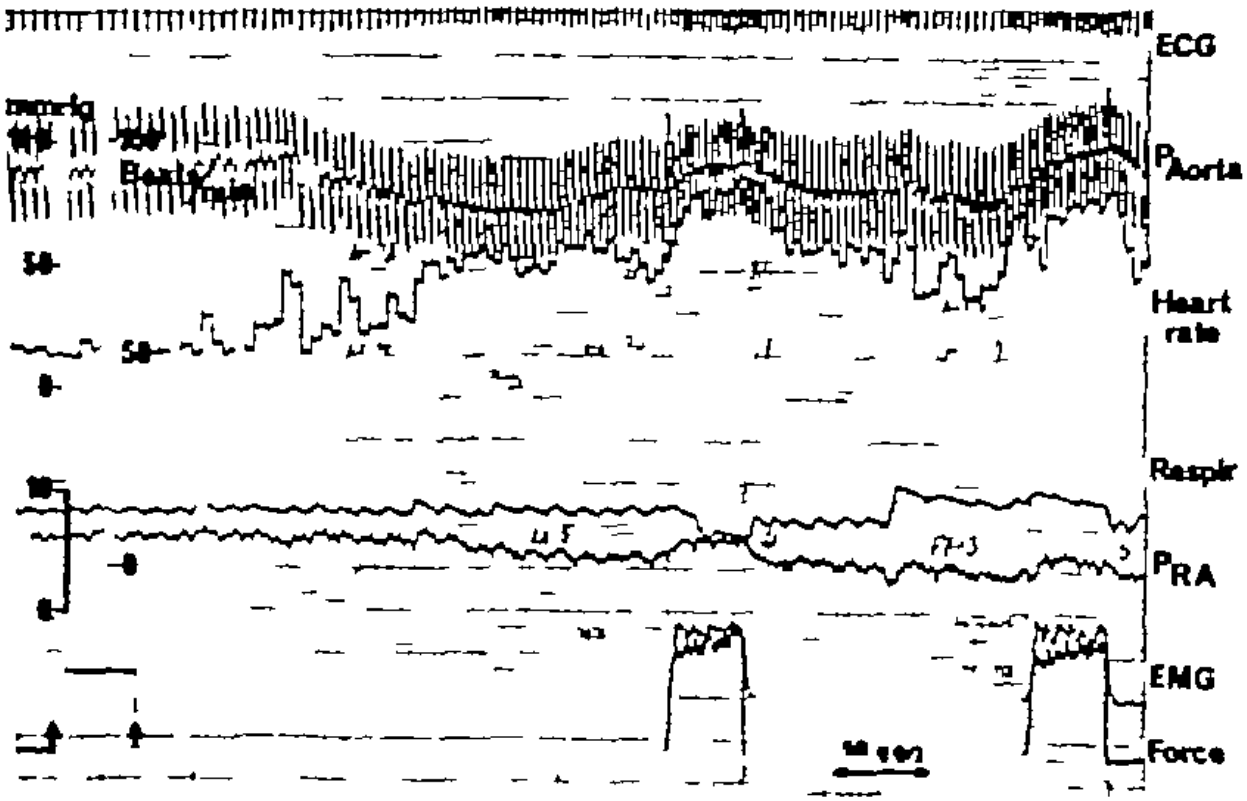


Fig. 8. The circulatory effects in subject K. G. of iv injection of phentolamine (10 mg) denoted by arrows, and of muscle contractions at 70 % of MVC. Subject and symbols as in Fig. 1, Chapter III. (U. Freyschuss, J. appl. Physiol. 1970, in press)

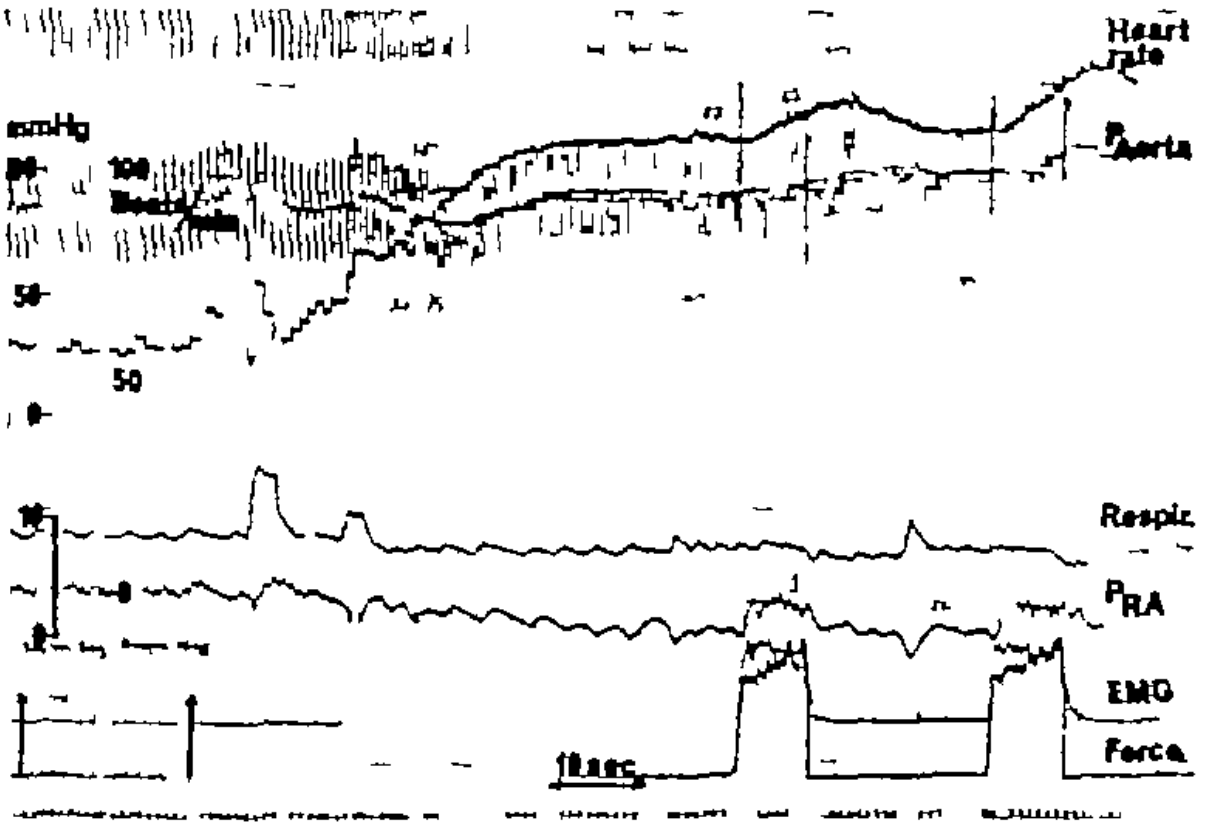


Fig 9 The circulatory effects of v injection of 5 mg phentolamine and 1.5 mg atropine (within the arrows) and of subsequent muscle contractions at 70 % of MVC. Symbols and subject are the same as in Figs 1 and 8.

Effect of atropine and phentolamine Under the influence of both drugs (Figs 7 9 10 and Table 2) the acceleration of heart rate evoked by muscle contraction was significantly ($P < 0.01$) inhibited and in many instances completely abolished The average rise in aortic pressure, 5 mm Hg, was 12 mm Hg lower ($P < 0.01$) than the corresponding control value

Effect of orthostatic reaction As commented on in Chapter III an orthostatic reaction upon head-up tilting greatly reduced the heart rate acceleration evoked by the muscle activity but did not affect the amplitude of the pressor response.

Comments

According to the design of the study an attempt was made to abolish the heart rate acceleration initiated by the muscle contraction, thus permitting observations of changes in aortic pressure response. In the earlier study by Macdonald *et al* (1966) on the cardiovascular effects of handgrips, 30 % of MVC, sustained for 5 min, it appeared that under the influence of



Fig 10. The cardiovascular response to muscle contractions (70 % of MVC) at 1 min 20 sec, 2 min and 4 min after a second iv injection of the combined dose of 5 mg phentolamine + 0.5 mg atropine in the same experiment as in Fig. 9 (U Freyschuss, J appl. Physiol. 1970 in press)

10 mg iv of a beta adrenergic blocking agent propranolol, the percentage increase of heart rate was greater but the rise in aortic pressure unchanged. In their records of the individual heart rate accelerations it can be observed that the rate of increase during the first minute of contraction is higher than in the control study. In keeping with this observation are the data of Smulyan and Eich (1968) concerning the effect of beta-sympathetic blockade on the initial hemodynamic response of dogs to treadmill exercise.

In the single investigation in the present study of the effect of propranolol 10 mg iv the circulatory response to isometric muscle contractions was not affected. The deviation from the findings of Macdonald *et al* (1966) might be explained by the fact that the data emanate from different time intervals from the start of contraction, 7 sec and 60 sec, respectively.

With the administration of atropine the heart rate acceleration evoked by muscle activation was inhibited to a degree which was dependent on the amount given. During head-up tilting of the orthostatic subjects in the control study the heart rate reaction on muscle contraction was likewise diminished. These findings suggest that the heart rate modulation during

the initial part of voluntary isometric muscle contraction is predominantly elicited by a withdrawal of vagal tone.

A significant pressure response was maintained both during the cholinergic block and during tilting. The pressure rise evoked by muscle activation can consequently not be ascribed solely to a heart rate acceleration. The time course of this circulatory reaction seems to preclude a humoral mechanism.

When phentolamine was given as the first drug muscle contraction elicited a less marked rise in aortic pressure although the increment in heart rate was the same as in the control study. Phentolamine is known as a moderate effective adrenergic blocking drug (Goodman and Gilman 1965) which prevents responses to circulating adrenergic mediators more readily than those to sympathetic nerve impulses. Recently the drug has been ascribed a beta adrenergic stimulating action (Zahir and Gould 1968, Gould 1969, Gould *et al* 1969). However the latter effect seems rather to be indirect, due to a neurotransmitter overflow (Iversen 1967). In the present study no effects of an indirect beta adrenergic stimulation are discernible when phentolamine is given alone. It might be hypothesized that, under the influence of both atropine and phentolamine, the high heart rates above the level of vagal dominance and the small but remaining heart rate acceleration during muscle contraction would reflect a lowering of the threshold for beta receptor stimulation.

The maintained pressure effect on the withdrawal of vagal tone presupposes an increased peripheral vascular resistance. The inhibition of the blood pressure response under the influence of phentolamine suggests that the pressure rise is partly mediated by alpha-adrenergic receptors.

The pressor response to an isometric muscle contraction could thus be ascribed both to the heart rate acceleration and to an effect on the systemic vascular resistance mediated by sympathetic constrictor nerves.

Further if the observed increase in venomotor tone reflects a more general effect on the capacity vessels, an increase of venous return would be provoked, which should compensate for the greater outflow and maintain the stroke volume. Thus, the heart rate acceleration, the increase of the systemic vascular resistance and the venomotor effect would contribute to an enhanced perfusion of the activated muscle groups.

CHAPTER V

Autonomic Nervous Mediation of the Heart Rate Acceleration on an Isometric Muscle Contraction in Tetraplegic Men

Some data from a study of the cardiovascular control in men with transverse cervical cord lesions (a preliminary report has appeared, see Freyschuss and Knutsson 1969) might further elucidate the autonomic nervous mediation of the circulatory response to isometric muscle contractions.

Subjects

The subjects were eight tetraplegic men, 21—42 years of age, with a complete transverse spinal syndrome below segments C_5—C_7 one of the subjects (no. 4) was investigated twice. Clinical tests of sensory and motor functions had indicated a complete loss of functional continuity at the appropriate cervical levels. All lesions were traumatic and 1—5 years had elapsed since the accident. None of the subjects gave any history of previous neurological or cardiovascular diseases. They had passed courses of physical training and rehabilitation

Procedure

The catheterizations and recordings of the observed variables (*viz.* heart rate aortic right atrial or central venous pressures, respiratory movements, EMG and muscular force) are described under General Procedures and Methods. Isometric muscle contractions were performed according to the descriptions in Chapters II and III The circulatory reaction upon head-up tilting 30—60 above the horizontal plane during 2—10 min was tested in all subjects. The drug influence on the blood pressure control during tilting was investigated after the administration of 1 mg of atropine in two of the subjects, of 7 5 mg f phentolamine in two other subjects and finally after the injection of the composite dose (0 5—1 mg atropine plus 10 mg phentolamine) in another two ubjects. Observations of the cardiovascular response to isometric muscle contractions were also made after the iv administration of atropine 1 0 mg in three subjects, of phentolamine (5 mg) in

one subject and in three subjects once again after an iv dose containing both drugs (atropine 0.5—1.5 mg and phentolamine 5—10 mg) The circulatory effect of muscle contraction has in each individual been measured as the highest value attained within the same interval from the start of the muscle activation and at the same muscular force, whereby each individual serves as his own control.

Results

The increments of heart rate and aortic pressures evoked by isometric muscle contractions demonstrated the same rapid onset as in healthy individuals (see Fig 11) The individual mean values of the increments in heart

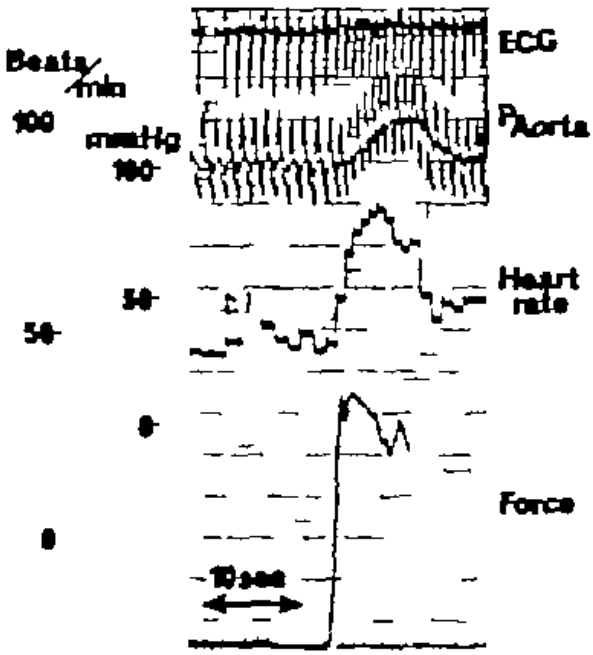


Fig. 11 Increments in heart rate, beats/min, and aortic pressures, mm Hg, initiated by an isometric muscle contraction at 90 % of MVC in a tetraplegic patient (subject 4)

rate and aortic pressures are summarized in Fig 12, where each thin arrow represents data obtained during 2—7 determinations at the same muscular force. More numerous observations of the heart rate blood pressure response were made within the same individual but at various per cent of MVC. The circulatory effect was a consistent finding The mean value of the heart rate increase among the tetraplegic men did not differ significantly from that among healthy individuals ($t = 0.2521$ degrees of freedom 22) Several data of the heart rate blood pressure response to muscle contraction, especially in subjects 4 and 6 had to be excluded due to the well-known phenomenon in patients with high spinal lesions associated with profuse sweating, a marked rise in blood pressure and bradycardia. The mean value of the heart rate increment of the group (see Fig 12) was 17 beats/min (SE ±3.8 range 8—23 beats/min $n = 4$) in supine position. The in

dividual mean heart rate at the initiation of muscle contraction was 63 beats/min. The rise in aortic pressures in supine position averaged 12 mm Hg (SE ± 2.3 range 7—18 mm Hg, n=4)

The circulatory effects of head up tilting (see Table 3) 30—60° above the horizontal plane revealed an incapacity to maintain an effective blood

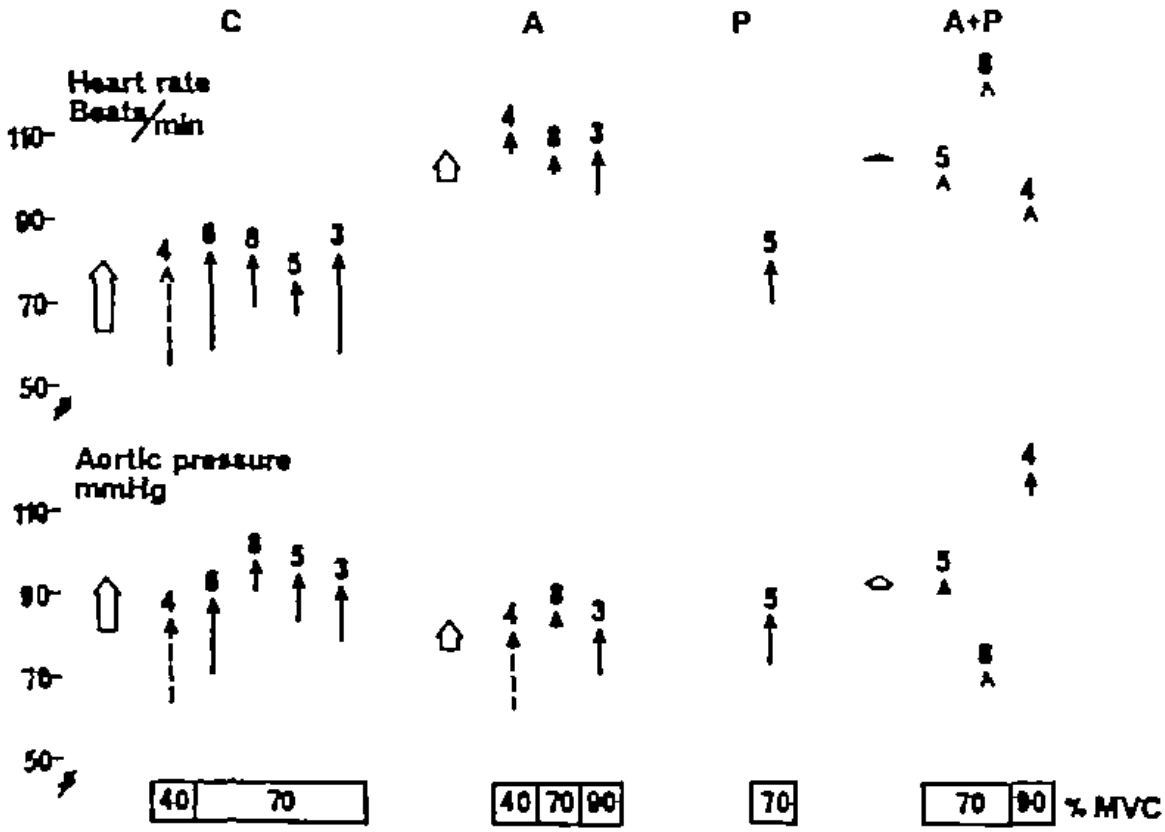


Fig 12 The increments in heart rate, beats/min, and in aortic mean pressure, mm Hg, evoked by isometric muscle contractions in supine position. Thin arrows represent individual mean values, while heavy arrows denote the mean values of the group. A broken arrow indicates that the observations were made during 60° head-up tilting The data were obtained prior to drug administration (C) after iv injections of atropine (A) or phentolamine (P) and of both drugs (A+P)

pressure. In one subject the tilting had to be interrupted due to a continuous decrease in aortic pressure In two other subjects a similar tendency was stopped by decreasing the angle of tilting

Under the influence of atropine or phentolamine a rapid decline in aortic pressure necessitated an interruption of the tilt test in five of six investigations.

The heart rate acceleration initiated by muscle contraction was inhibited (see Fig 12) or in some instances completely abolished after the iv injec

TABLE 3 Effect on heart rate (HR) beats/min, and on aortic mean pressure (P_{Ao}) mm Hg, of head-up tilting prior to (C) and after the administration of atropine (A) or phentolamine (Ph) and of both atropine and phentolamine (A+Ph). Values attained during tilting and their deviation (+ —) from preceding supine values are presented as individual observations and as mean values ($\bar{X}$) ± standard error (SE) of the group. n = number of observations.

Subject No.		C	A	Ph	A+Ph
1	HR	96+44[1]			
	P_{Ao}	54—42[2]			
2	HR	67+11[1]			
	P_A	58—11			
3	HR	102+49	120+92[2]		
	P_{Ao}	54—23	32—47		
4	HR	69+ 1	109+17		
	P_{Ao}	56—13	58—50		
5	HR	86+ 3			110+22[2]
	P_{Ao}	52—27			32—56
6	HR	76+21		92+30[2]	
	P_{Ao}	6 —19		49—29	
7	HR	85+29		91+24[2]	
	P_{Ao}	55—23		44—19	
8	HR				129+5[2]
	P_{Ao}				56—21
HR	n	7 7	2	2 2	2 2
	$\bar{X}$	83+28	115+20	92+27	120+14
	SE ±	5.0 5.1			
P_{Ao}	n	7 7	2	2 2	2 2
	$\bar{X}$	53—23	45—49	47—24	44—39
	SE ±	5.4 3.9			

[1] Intraarterial pressure and upper body tilting, while sitting in wheelchair
[2] Tilting had to be interrupted.

tion of atropine. The inhibition was more marked in tilted position (see Fig 13) and under the influence of both atropine and phentolamine (Figs 12 and 14)

Comments

There is no physiological evidence for a sympathetic nervous outflow from the cervical region of the spinal cord (Hillarp 1960) It might thus be

expected that patients with a transverse cord lesion at cervical levels should be deprived of centrally controlled sympathetic nervous discharges to the heart. The cardiac adjustment elicited in the vasomotor centers would consequently be mediated by changes of the vagal tone only. The maximal

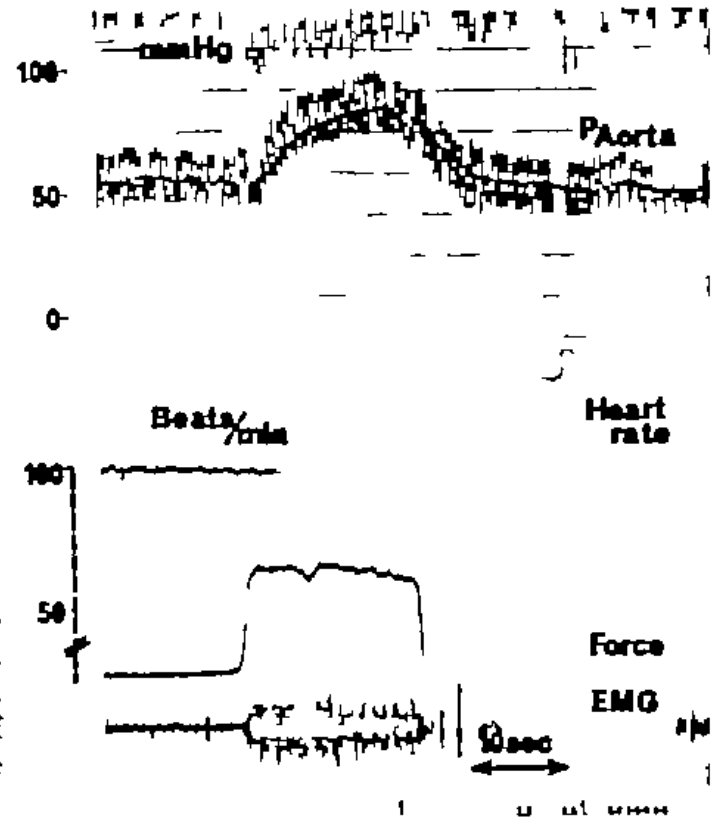


Fig. 13. Effect of 1 mg of atropine i. and of head-up tilting on the circulatory response to a muscle contraction at 40% of MVC, exerted during 20 sec. The same subject as in Fig. 11.

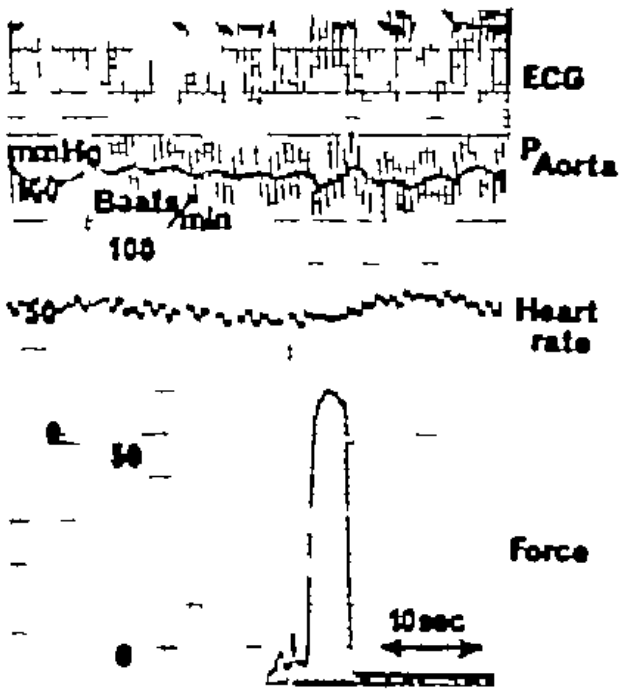


Fig. 14. Effect of a muscle contraction at 90 % of MVC after the administration of a combined dose of drugs (0.5 mg of atropine and 5 mg of phentolamine). The same subject as in Figs. 11 and 13.

heart rates confined to the range of vagal influence and the insufficient circulatory adaptation to postural changes are in accordance with the proposed mechanism

An inadequate blood pressure control during head-up tilt has been reported in earlier studies of patients with cervical spinal syndromes (*e g Guttman et al 1963 Gilliat et al. 1948 Cole et al. 1967*)

When vagal tone was diminished as after the administration of atropine or phentolamine, five of six subjects were unable to maintain the blood pressure in tilted position. This seems to indicate a loss of the integrative cardioaccelerating function of the efferent sympathetic nervous part of the baroreceptor reflex loop.

The magnitude of the heart rate response to isometric muscle contractions, mean 17 beats/min, was similar to the value (mean 18 beats/min) observed in healthy individuals. The highest value measured during a contraction under the influence of atropine in the present study 113 beats/min, was never attained by the healthy subjects prior to drug administration. As in healthy controls, atropine diminished or abolished the heart rate response to muscle contraction. When both atropine and phentolamine were given the heart rate during an isometric contraction was maximally 127 beats/min among the tetraplegics, while higher values were observed among the healthy volunteers in corresponding drug study (see Fig 7 Chapter IV)

The pathways mediating the pressor response to muscle contraction in tetraplegic men remain obscure and possible mechanisms will not be discussed here. It should only be noted briefly that the muscles activated during the contractions were innervated from segments of the spinal cord located both above and below the level of the functional discontinuity

The similar heart rate response to isometric muscle contractions observed both in healthy individuals, and in tetraplegic men with an apparent loss of the cardioaccelerator function mediated through the sympathetic nerves of the baroreceptor reflex loop indicates a common mechanism. The inhibited heart rate response under the influence of atropine in both materials supports the earlier (Chapter IV) proposed interpretation that the heart rate modulation during the initial part of a voluntary isometric muscle contraction is elicited by a withdrawal of vagal tone.

It might also be assumed that the high heart rates observed during contraction in healty individuals but not in tetraplegic men after the administration of the combined dose of drugs might indicate that, depending on the adjustment of the prevailing autonomic tone, a cardioacceleration due to muscle activation could also be initiated by a stimulation of beta adrenergic receptors.

CHAPTER VI

Cardiovascular Response to Intended Muscle Contraction during Neuromuscular Blockade

The origin of the nervous activity provoking the circulatory response at the onset of muscular work has been disputed. The basis for the main concepts of a "centrally" or peripherally" initiated neurogenic drive provoking the cardiovascular changes might be exemplified as follows.

The cardiovascular effects induced in animal experiments, whether behavioural or concerned with electrical stimulation of diencephalic structures in the fields of Forel (Rushmer and Smith 1959) have provided deductive evidence of a "centrally" initiated cardiovascular reaction in parallel to somatomotor activation

In human subjects the similarity of the circulatory adjustments to voluntional and electrically induced exercise (Asmussen *et al* 1943 b) has been taken to be the result of a neurogenic drive emanating from the periphery

In the present study an attempt was made to test whether the hypothesis of a centrally initiated autonomic drive might be valid in man. It was thought that the mechanism might be elucidated by observations of the cardiovascular response to intended muscle contractions within restricted paralyzed muscle groups. Hence stimulation due to mechanical and peripheral metabolic events could be eliminated.

Subjects

Six healthy male blood donors (24—29 years of age) volunteered for the study The right atrium was catheterized in three of the subjects.

Procedure

The procedures for catheterization are described under General Procedures and Methods. Methods of recording of the observed variables, *viz.* heart rate aortic and right atrial pressures and muscular force are presented in the same chapter

A local neuromuscular blockade in the forearm and hand was induced

by an intraarterial injection of a depolarizing agent, succinylcholine (Celocurin klorid®) via a catheter in the brachial artery. The tip of the catheter was localized ca. 10 cm above the elbow. Successively increasing doses (0.5 to 4 mg) were given until an apparently total neuromuscular blockade was obtained. For indication of the degree of blockade as well as the time of its onset and the time when it starts to disappear the muscle action potentials of the M. flexor carpi ulnaris in response to electrical stimulation of the ulnar nerve were recorded. The stimulation was made with felt skin electrodes placed over the nerve at the elbow. A Grass stimulator (type S 8) delivered the electrical stimuli which were square wave impulses of 0.1 sec duration at a frequency of 1 imp/sec and of just supramaximal strength. The muscle action potentials were recorded with two skin electrodes (Beckman) placed over the distal end of M. flexor carpi ulnaris and the signals were fed to a differential a.c. amplifier (Grass type P 9). The amplification was chosen so that even a minimal activity during the neuromuscular blockade would be detected. The action potentials were monitored on an oscilloscope (type 502 Tektronix Inc.) for direct observations of alternations in the response thus facilitating timing of the intended contractions to periods of blockade. The signals were also fed to the UV writer used for concomitant recordings of the cardiovascular variables, but were then passed through a high-frequency filter to avoid recording of a capacitive stimulus artefact that could not be differentiated from the myopotentials at the time scale used.

The criteria of neuromuscular blockade were as follows:

no discernible muscular movement or muscle action potentials in response to electrical stimulation of N. ulnaris,

no discernable muscle activation or EMG activity in response to intended handgrip.

Control data of the cardiovascular response to voluntary isometric handgrips were obtained according to the description in Chapter II. During neuromuscular blockade the subjects were told to act volitionally within the periods of intended contraction as during the previously performed handgrips. In order to reduce extraneous stimuli, the surroundings were kept quiet and the subjects were always informed by a visual signal of the start and duration of the performed or intended contraction. Indications of lighting and extinction of the visual signal were made by a test signal in the dynamometer record. These signals thus denote the duration of the intended handgrip with a slight fault due to the response time of the individuals.

Activation of muscles proximal to the blockade cannot be totally excluded, but EMG records from various positions over the shoulder revealed no

activity and inspections and palpations were made to check that other parts of the body were relaxed.

The effect on heart rate and aortic mean pressure has in each individual been measured as the highest value attained within 7 sec from the start of an isometric handgrip performed at 70 % of MVC and within the same time interval from the start of an intended handgrip in the same, paralyzed arm.

Results

Effect of succinylcholine at rest In some of the subjects the injection of the drug induced a short-lasting feeling of increased pressure in the vessel causing discomfort or pain during the subsequent 5—15 seconds. In two of the subjects the effect was reproduced with every injection of succinylcholine

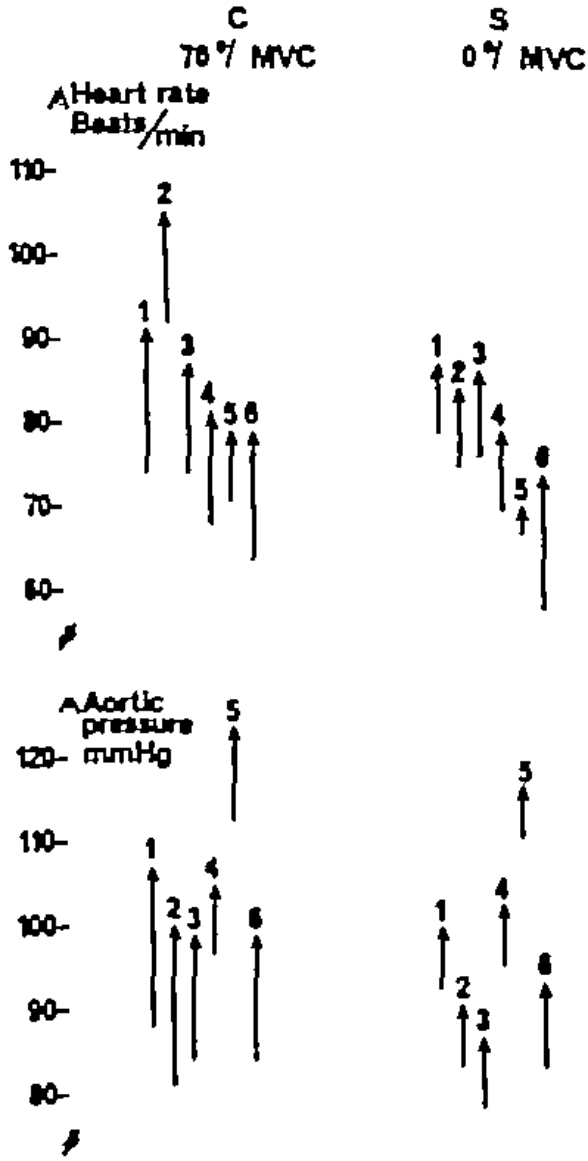


Fig 15 Individual mean values of the increments in heart rate, beats/min, and in aortic mean pressure, mm Hg initiated by performed (C, at 70 % of MVC) and intended (S) muscle contractions.

in the others it was not a consistent finding When pain was induced a short-lasting increase in heart rate and aortic pressures was recorded otherwise no circulatory changes were initiated by the drug administration.

With the initial test dose, 0.5 mg complete neuromuscular blockade was usually not obtained. With higher doses, 1—4 mg of succinylcholine, criteria of blocked neuromuscular transmission were fulfilled within 5 sec from the start of the injection. Muscle fasciculation was never observed during the onset of the blockade. The duration of the depolarization block was dependent on the amount of drug given, but interindividual variations were noted. In general, single doses of 1—4 mg of the drug induced a neuromuscular blockade during 1—4 min. The rapid restoration of motor function relieved the volunteer from anxiety about the unfamiliar situation of paralysis.

No symptoms or signs appeared of drug influence on other muscle groups than those perfused by the arterial blood distally to the catheter tip

Cardiovascular response to intended muscle contraction Individual mean values of the increments in heart rate and aortic mean pressure during performed and intended isometric handgrips are summarized in Fig. 15 and represent data obtained during 3 to 19 determinations in each subject.

The mean heart rate increase of the group (n=6) during performed handgrip was 14 beats/min (SE ±1 8 range 8—21 beats/min) The SE observed within the individuals varied from ±0 7 to ±1 7 beats/min. The mean value for the rise in aortic mean pressure was 15 mm Hg (SE ±1 8, range 8—19 mm Hg) and corresponding intraindividual values of SE varied from ±0 8 to ±3 8 mm Hg

Intended handgrips in the muscle groups with a blocked neuromuscular transmission elicited increments in heart rate and aortic mean pressure which averaged 9 beats/min (SE ±1 6 range 4—16 beats/min) and 8 mm Hg (SE ±0 6 range 6—10 mm Hg) among the individuals (n=6) The SE range of the intraindividual mean values for the heart rate increment was ±0.3 to ±1 0 beats/mm and corresponding values for the aortic pressure rise varied from ±0 6 to ±1 7 mm Hg

The heart rate increase initiated by intended handgrips among the individuals (n=6) was on the average 64 per cent (range 47—76 %) of the value observed during performed muscle contractions. The corresponding mean value of the aortic pressure rise was 55 per cent (range 37—75 %) of control data.

The cardiovascular response to performed and intended muscle contractions is illustrated in Figs 16 and 17 During the isometric handgrip in

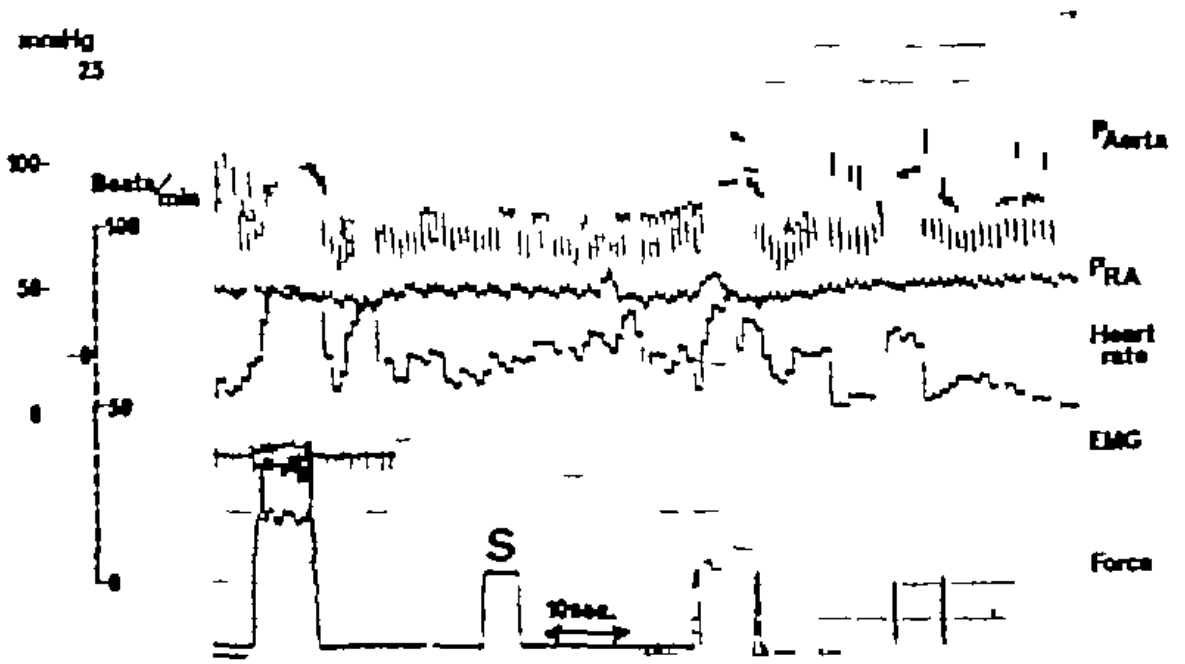


Fig. 16. The cardiovascular effects in subject no. 6 of an isometric muscle contraction at 0 ° of MVC and of intended muscle contractions after an i.a. administration of 2 mg succinylcholine (denoted by S). Periods for intended muscle contractions are indicated by test signals on the force record. The EMG demonstrates myopotentials evoked both by volitional muscle activity and by electrical stimulation (1 imp/sec) of the ulnar nerve. P_{RA} = right atrial mean pressure. Further information is given in the text.

Fig. 16 the heart rate increased from 64 to 84 beats/min, the aortic mean pressure rose from 83 to 101 mm Hg while the right atrial pressure remained unchanged, 7 mm Hg. A subsequent intraarterial injection of 2 mg of succinylcholine denoted by S induced a short-lasting pain and a heart rate acceleration during the subsequent 20 sec up to maximally 76 beats/min. The contractions of the hand and fingers induced by the ulnar nerve stimulation were abolished within a few seconds. During the two subsequent visually ordered contractions with start and end denoted by the test signals the heart rate increased by 20 and 15 beats/min, respectively and aortic mean pressures by 11 and 17 mm Hg respectively. In the account of the experiment the technician had noted a delay of the second "on-signal". During the first intended contraction the right atrial pressure rose by 1.6 mm Hg probably due to a slight Valsalva manoeuvre. During the second intended contraction the right atrial pressure was unchanged and the amplitude of the aortic pressure elevation was greater. Fig. 17 illustrates the cardiovascular response in the same subject to a performed handgrip with increases in heart rate from 68 to 87 beats/min and in aortic mean pressure from 82 to 107 mm Hg and the effect of a second intraarterial injection of 2 mg succinylcholine. Within the subsequent periods of intended contrac

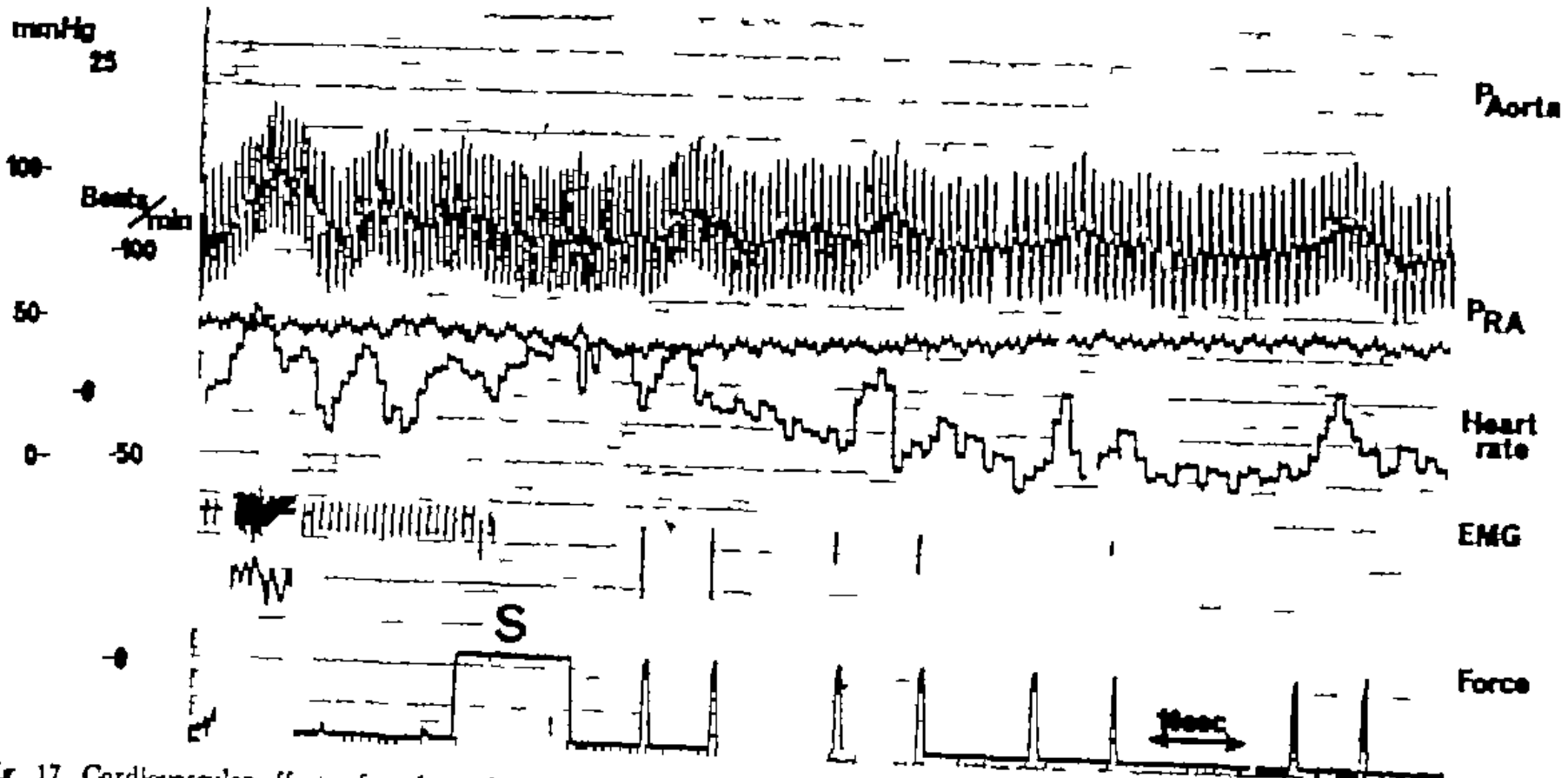


Fig. 17 Cardiovascular effects of perf rmed and intended muscle contractions during neuromuscular blockade. Symbols and subject as in Fig 16.

tions, consistent increments in heart rate and aortic mean pressures appear The aortic pressure elevation within the third intended contraction, 6 mm Hg, was the lowest value observed in the investigation of that volunteer (subject no 6)

Fig 18 illustrates the effects on heart rate and aortic mean pressure at the initiation of handgrips in the period of successive restitution of the neuromuscular transmission. In the same individual (no. 6) similar changes in heart rate and blood pressure were observed during total neuromuscular blockade and no correlation could be obtained between the circulatory variables and the exerted muscular force.

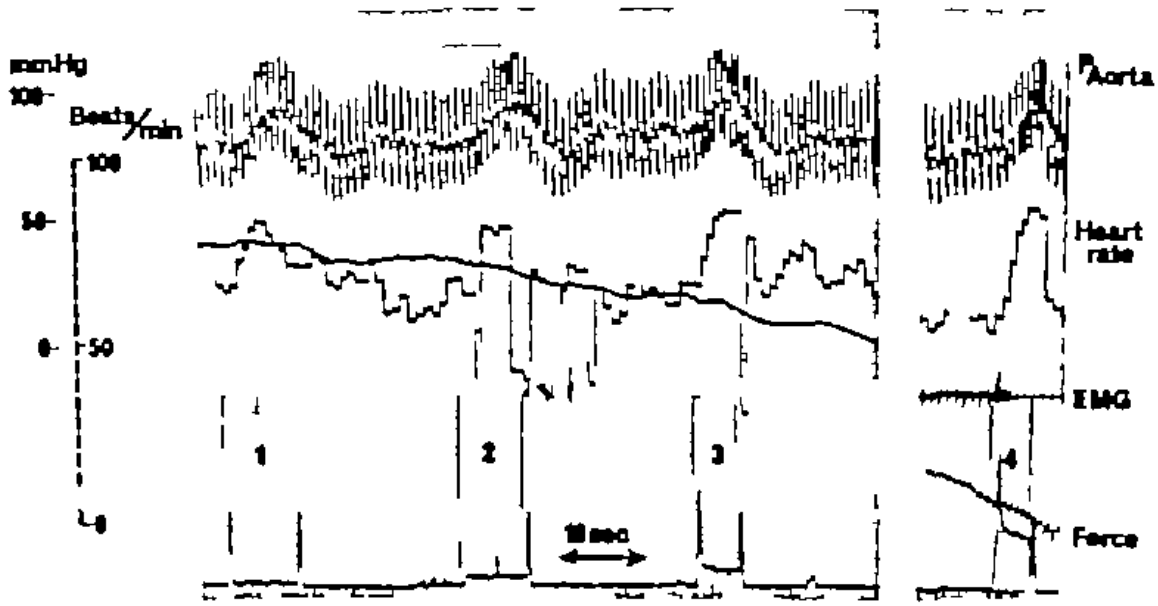


Fig 18 Effects on heart rate and aortic pressures of muscle contractions in the phase of successive restitution of the neuromuscular transmission (after blockade) The eighth trial record is disturbed by a technical fault. During the contractions (nos. 1—4) the last force exerted was ca. 2 %, 5 %, 10 % and 30 % of MVC, respectively Symbols and subjects as in Figs. 16 and 17

When the same subject performed isometric handgrips at similar or higher per cent of MVC, when not under the influence of succinylcholine, the observed circulatory effects were definitely less marked (see Fig 19) This finding was pertinent to all subjects.

Comments

Observations of the heart rate—aortic pressure response to intended contractions in muscle groups with a complete depolarization block have not been found in the literature Earlier investigations (Ochwadt *et al* 1959

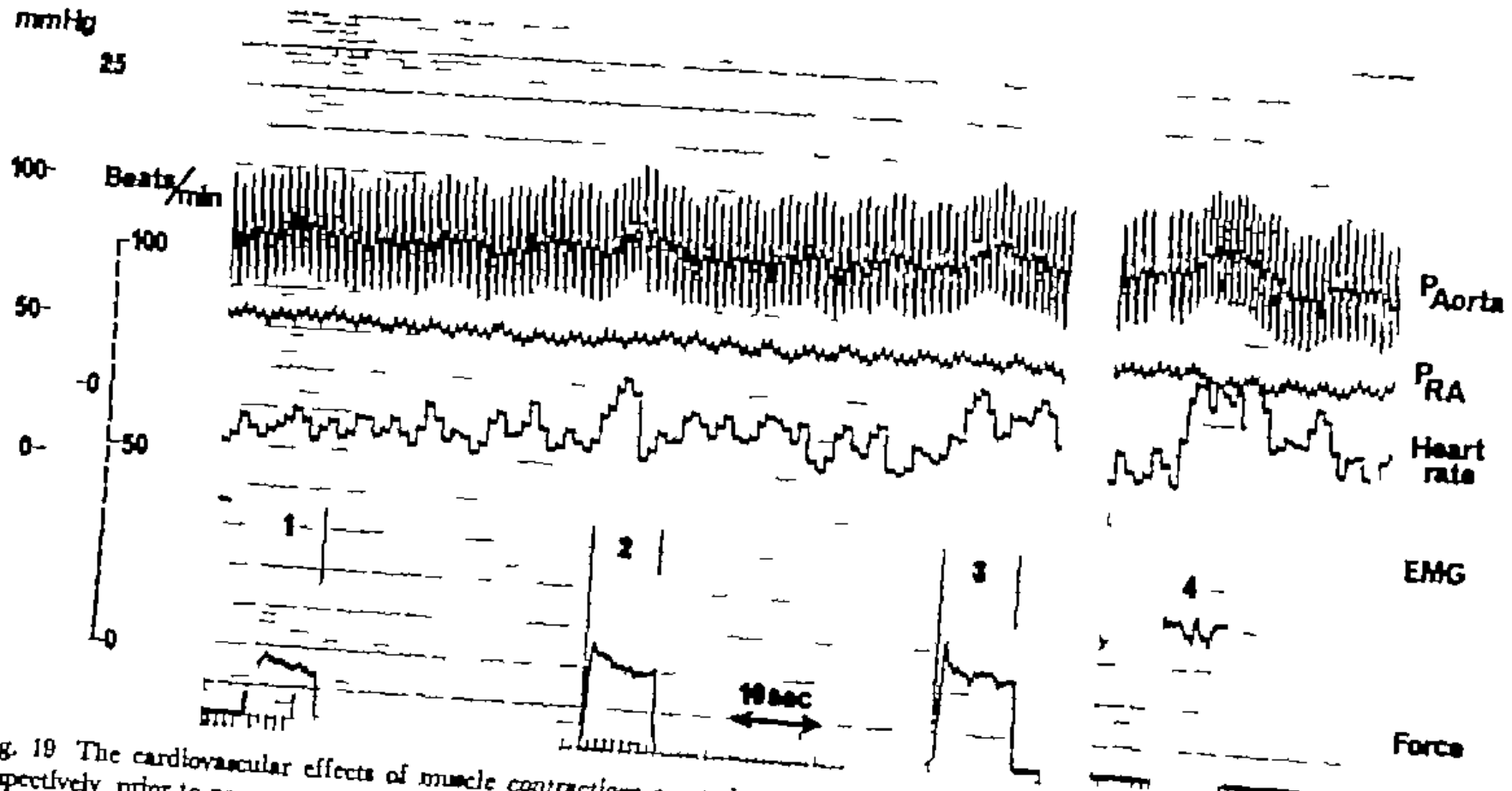


Fig. 19 The cardiovascular effects of muscle contractions exerted at (nos. 1 to 4) 25 % 35 %, 40 % and 70 % of MVC, respectively prior to neuromuscular blockade. Symbols and subjects as in Figs 16—18. EMG record is shut off.

Asmussen *et al* 1963 and 1965) have been concerned with respiratory and circulatory effects of exercise performed after iv injections of (+) tubocurarine, a non-depolarizing neuromuscular blocking agent. Owing to the route of administration the neuromuscular transmission within the dynamically working leg muscles could only be partially blocked, and effects on other muscle groups were noted. Finally the induced hyperventilation could not provide basal conditions at the observations of the circulatory changes evoked by muscle exercise in the state of partial curarization. Thus the cited data do not allow comparisons with the present results.

In the actual experiments, possible extraneous cardiovascular stimuli, occurring simultaneously with the intended muscle activation were scrutinized. From experience it was learnt that two kinds of artefacts might appear *viz* breathholding with a more or less marked tendency to a Valsalva manœuvre and activation of muscles proximal to the paralyzed limb segment. Inspections were therefore made to check that breathing was unrestricted, which was confirmed by records of the right atrial pressure. EMG s from various muscles of the shoulder revealed no appreciable activity although irrelevant muscle activation could never be totally excluded. However in order to provoke circulatory effects of the magnitude observed during the depolarization block, the exerted muscular force must attain so high a fraction of MVC (see Fig 19) that such activity cannot possibly remain undetected. This will further be elucidated by the investigation presented in Chapter VII

Thus the cardiovascular effects on intended muscle contraction indicate that the increments of heart rate and blood pressure at the initiation of somatomotor activity are elicited by an autonomic nervous drive of central origin.

The lower amplitude of the cardiovascular response to intended muscle contraction might be the result of a less marked central excitatory drive. Alternatively provided this drive was of the same intensity as in the control study the lower values attained during an intended handgrip would indicate that peripheral feedback mechanisms operate within 7 sec from the start of a developed contraction Comparisons of heart rate increments from rest to voluntary muscular exercise and from rest to passive movements of the same amplitude and speed have been made The results have indicated that a proprioceptive stimulus accounts for a part of the cardiac acceleration Dejours *et al* 1956)

CHAPTER VII

The Relationship between Cardiovascular Response and Extent of Muscle Activation

The present study was undertaken to evaluate whether the size of the activated muscle groups might influence the heart rate and the aortic pressure response to muscle contraction. Further the cardiovascular effects of contraction intensities within a wide range of exerted relative force will be discussed.

Subjects

Ten of the male blood donors, 24—29 years of age, participated in the investigation.

Procedure

Details of the catheterization and recording of the observed variables, *viz.* heart rate, aortic pressure and muscle force are given in Chapter II Six of the subjects performed isometric contractions in the form of handgrips and elbow flexions at the same per cent of MVC and according to descriptions in Chapters II and III In four of the subjects isometric handgrips were performed at 70 per cent of the maximal force observed for the right hand as described in the same chapters. In randomized order isometric handgrips were exerted with the left hand, the right hand and both. When handgrip was performed simultaneously with both hands, the left hand contracted over a rod of the same diameter as the dynamometer The subjects were told to exert the muscle force indicated (on the oscilloscope) and to contract both hands at similar intensities.

Results

Paired comparisons of the individual increases in heart rate and aortic mean pressure revealed no differences in the circulatory response to any of the three types of handgrip The differences calculated from paired comparisons of the individual mean increments of heart rate and aortic mean pressure were likewise non-significant (see Table 4) As commented

TABLE 4 Difference ($\bar{d}$) in the heart rate increase (Δ HR) beats/min, and the aortic mean pressure rise (ΔP_{Ao}) mm Hg, elicited on a muscle contraction performed with right hand (R) left hand (L) or with both (B) Degrees of freedom (df) and t-test values (t) are given. P values are for paired comparisons between individual means. (ns = not significant)

	R — L		R — B		L — B	
	ΔHR	ΔP_{Ao}	ΔHR	ΔP_{Ao}	ΔHR	ΔP_{Ao}
$\bar{d}$	1.5	3.8	0.5	1.8	—1.0	—2.0
df	3	3	3	3	3	3
t	0.5508	2.7699	0.3216	0.8197	—0.3572	—1.4771
P	ns	ns	ns	ns	ns	ns

on in Chapter III the individual circulatory responses to isometric hand grips and to elbow flexions were similar The mean differences ($\bar{d}$) between the increments in heart rate and blood pressure observed during elbow flexion and the values observed during handgrip were $\bar{d}$ -0 7 beats/min t = -0 4822 df = 4 and $\bar{d}$ 1 2 mm Hg, t = 1 2335 df = 4 respectively

As demonstrated in Fig 19 Chapter VI and in accordance with the observations in other subjects during the procedure for selecting a suitable per cent of MVC, the circulatory response to muscle contractions less than one fourth of the value observed during maximal contraction was non-significant. According to Fig 19 there seems to be a rough direct correlation between the exerted relative muscle force and the circulatory effects. However in the study of Chapter III no correlation could be obtained between the increments in heart rate and aortic pressure and the fractions of maximal force of contraction from 50 to 100 per cent of MVC. Thus a relation between the force of contraction and the initial cardiovascular effect seems to be discernible only within ranges of 25 and 70 per cent of MVC It has been beyond the scope of the investigation to make a more thorough analysis of any quantitative relationship

Comments

The lack of additive effect on the circulatory response when one or two muscle groups contracted at the same relative tension is in keeping with the findings of Lind and McNicol (1967) They observed similar increments in heart rate and blood pressure when two or more muscle groups contracted separately or together Yet the latter data were obtained during contractions sustained for 1 to 3 minutes.

No correlation could be obtained between the magnitude of the heart

rate — blood pressure response and the degree of somatomotor activity expressed as relative muscle force developed within the range of 50—100 per cent of MVC. Similar results concerning the pressor response within the first 10—15 sec of contractions at varying intensities were observed by Humphreys and Lind (1963) The circulatory response to rather small fractions of MVC (see Fig 19) was of lower magnitude than the circulatory effects evoked by even lower total developed strength of contraction (intended to be higher) in the period immediately following total neuromuscular blockade (see Fig 18) This is in accordance with the assumption that the evoked cardiovascular effects are related to the degree of intended muscle activation rather than to the total muscular activity developed.

Thus the initial circulatory effects of isometric contractions are not determined by the size of the activated muscle groups considered. The circulatory response seems to be related to the intensity of voluntary motor innervation.

taps, the foot being kept in position by the tension recording device and thus involuntary tension changes in the Achilles tendon were also recorded for control purposes (Knutsson and Mattsson 1970) The elicited muscle contractions were recorded by this device, i e a rigidly mounted strain gauge transducer displaying the pressure exerted by the sole of the foot during the plantar flexion. The strain gauge constituted one arm of a bridge at the input of a chopper amplifier The amplitude of the contrac tions was recorded on a Grass Polygraph ink writer with a linear fre quency response at frequencies below 100 Hz. In the female subject the contractions were simultaneously recorded on the UV writer

Recordings of the muscle action potentials over M triceps surae were made with Ag AgCl surface electrodes (Beckman) After amplification the potentials were displayed on an oscilloscope screen (type 502, Tektronix Inc) and photographed. In the female subject (see Figs 20 and 21)

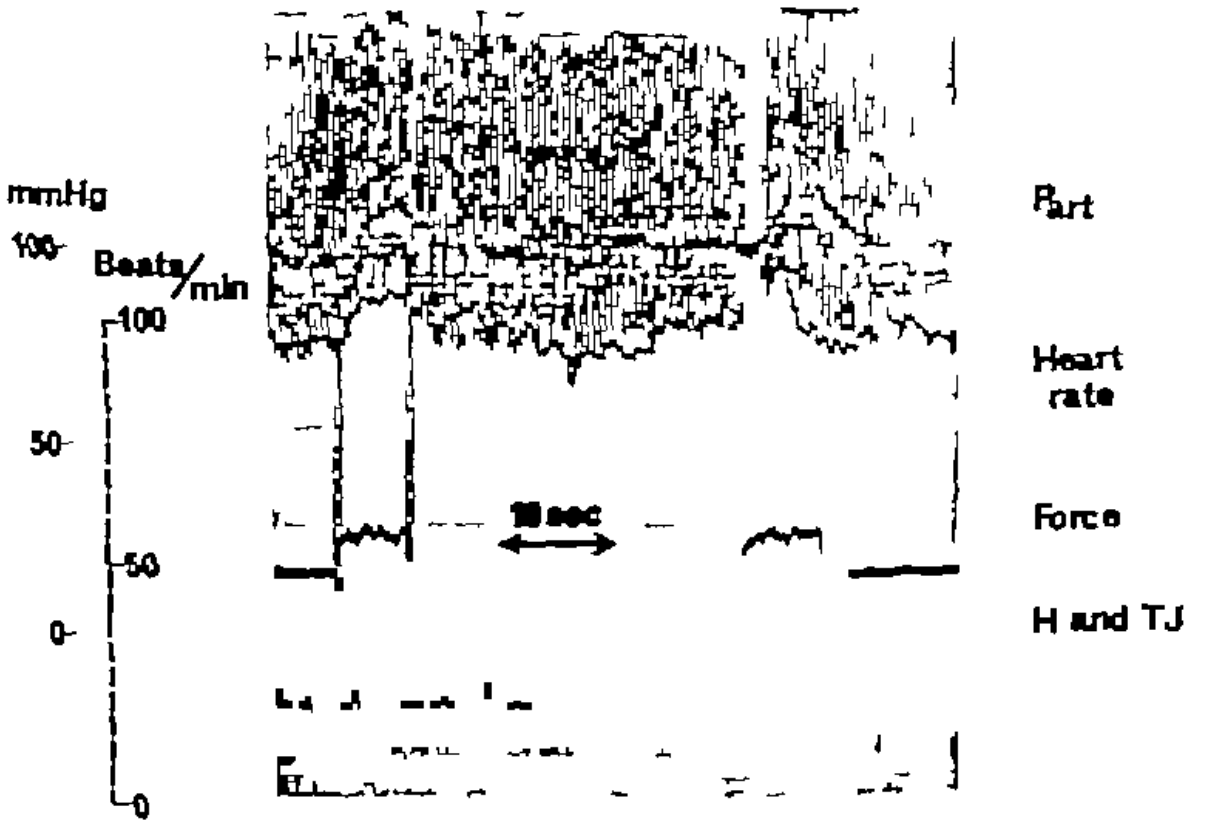


Fig. 20. Subject M. B.. Effects on heart rate, arterial pressures (P_{art}) and on triceps surae tendon jerk (H and TJ) of isometric handgrips performed at 70 per cent of MVC. In the record of H and TJ each deflection gives th amplitude of the force exerted during a plantar flexion. Triceps surae jerks were elicited by alternate tendon (TJ) taps (the smaller deflections) and electrical (H) stimulation (the bigger deflections) of the posterior tibial nerve. In the EMG records from M.carpi ulnaris and M.triceps sura electrical stimuli artef cts were packed p fo the sake of clearness the simultaneous tracings were omitted in Figs 20 and 21

H reflexes, *i e* monosynaptic reflex responses elicited by stimulation of Group I afferent fibres proximal to the muscle spindles, were elicited by electrical stimulation of the posterior tibial nerve, but the results will not be discussed in the present investigation.

Due to technical faults, arterial pressure and heart rate could be recorded in only two of the subjects.

Results

Isometric handgrips at 70 per cent of MVC initiated increases in heart rate of 12 (SE ±1 8 n = 10) beats/min in subject M B. and 23 (SE ±2.3 n=5) beats/min in subject J R. Corresponding values for the rise in arterial mean pressure were 13 (SE ±0 8 n=10) mm Hg and 19 (SE ±2.4 n=5) mm Hg respectively During handgrip all subjects demonstrated the well-known reinforcement of the tendon jerk associated with a Jendrassik's manœuvre (see Figs 20 22a and 23a)

Intended handgrips in the muscles with total neuromuscular blockade initiated increments in heart rate of 16 (SE ±2 0 n = 9) beats/min in subject M B and 11 (SE ±1 8 n=3) beats/min in subject J R. Arterial mean pressure rose by 10 (SE ±1 7 n=7) mm Hg and 9 (SE ±1 2 n=3) mm Hg, respectively

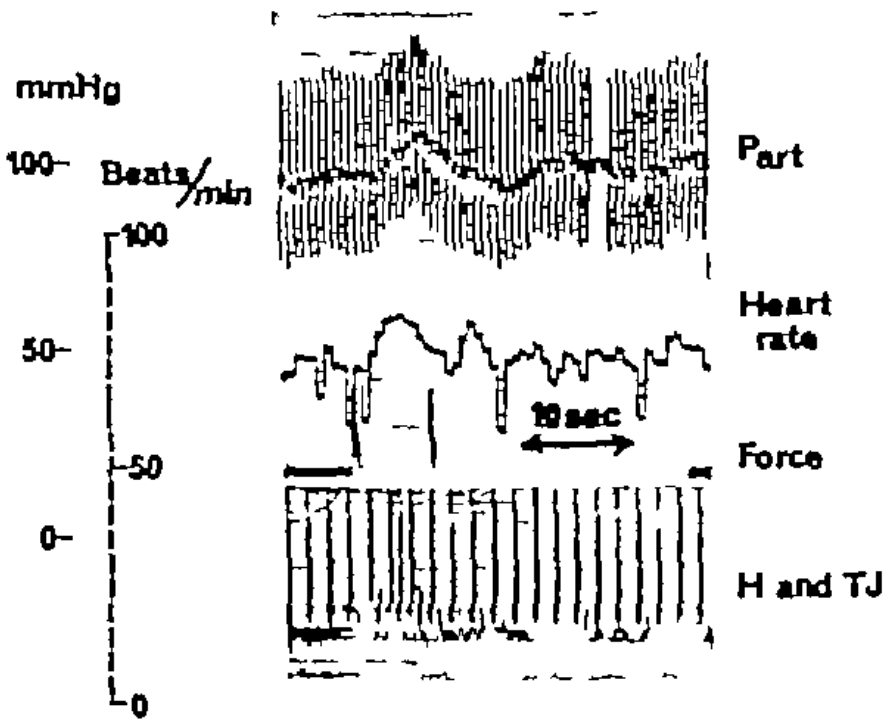
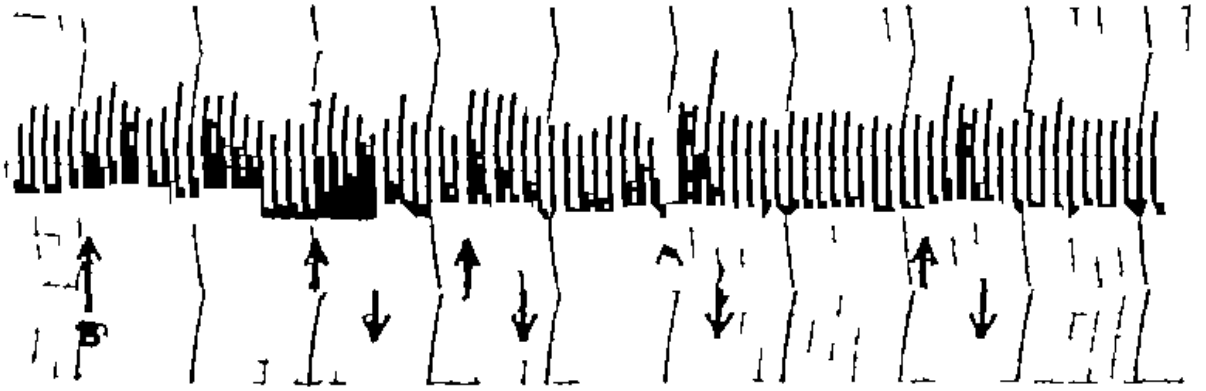


Fig. 21 Effect on heart rate arterial pressures and on triceps sura jerk of intended handgrip during neuromuscular blockade Symbols and subjects are the same as in Fig 20.



Fig. 22. a) Effects of isometric handgrips at 70 per cent of MVC (within the arrows) on triceps surae jerks elicited by alternate tendon taps (the smaller deflections) and electrical stimulation (the bigger deflections) of the posterior tibial nerve. Each square = 3 sec. Same subject as in Figs 20 and 21



b) Effects of succinylcholine (S) and intended handgrips (within the arrows) during neuromuscular blockade. Same recording procedure and subject as in a)

At the time of and during the subsequent ca. 20 sec after the injection of succinylcholine a consistent finding was an enhancement of the tendon jerks. Similar changes were observed during instruction of or other communications with the subjects. However prior to the first intended contraction during blockade, the reinforcement of the reflex response had been deleted (see Figs 22 b and 23b)

During intended handgrips in the periods of neuromuscular blockade the tendon jerks were augmented in all subjects. This finding pertained both to the electromyographically recorded responses (see Fig 24) and to the recorded plantar flexions (see Figs 21 22b and 23b) elicited by the tendon taps. In Fig 23b the mean maximal force of plantar flexion increased by ca. 45 and 30 per cent when compared with an equal number of tendon jerks preceding the intended contraction. In Fig 23a the same subject demonstrated a corresponding increase of 55 and 37 per cent, respectively

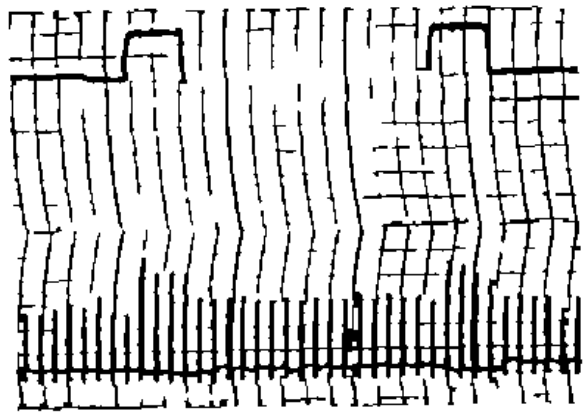
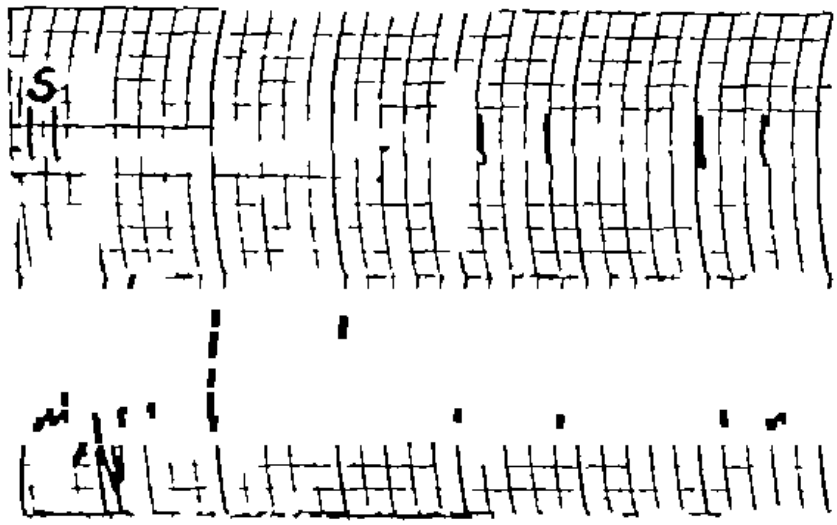


Fig. 23. a) The effect of isometric handgrip (upper record) at 70 per cent of MVC on the force of plantar reflexion (lower record) elicited by Achilles tendon tap in subject J. R. Each square = 3 sec.



b) Effects of succinylcholine (S) and intended handgrips (upper record) during neuromuscular blockad on the force of plantar reflexion (lower record) elicited by tendon tap. Recording and subject as in a)

Comments

The increments in heart rate and arterial mean pressure both on performed and intended muscle contraction during blockade were similar to those observed in Chapters III and VI where blood pressure was measured in the aorta.

It has been reported that the augmentation of the quadriceps jerk is lost if the Jendrassik's manoeuvre is continued for more than 1 sec (Clarke 1967) but others (Buller and Dornhorst 1957) have found that reinforcement has persisted at least up to 6 sec. The present data are in accordance with the latter results.

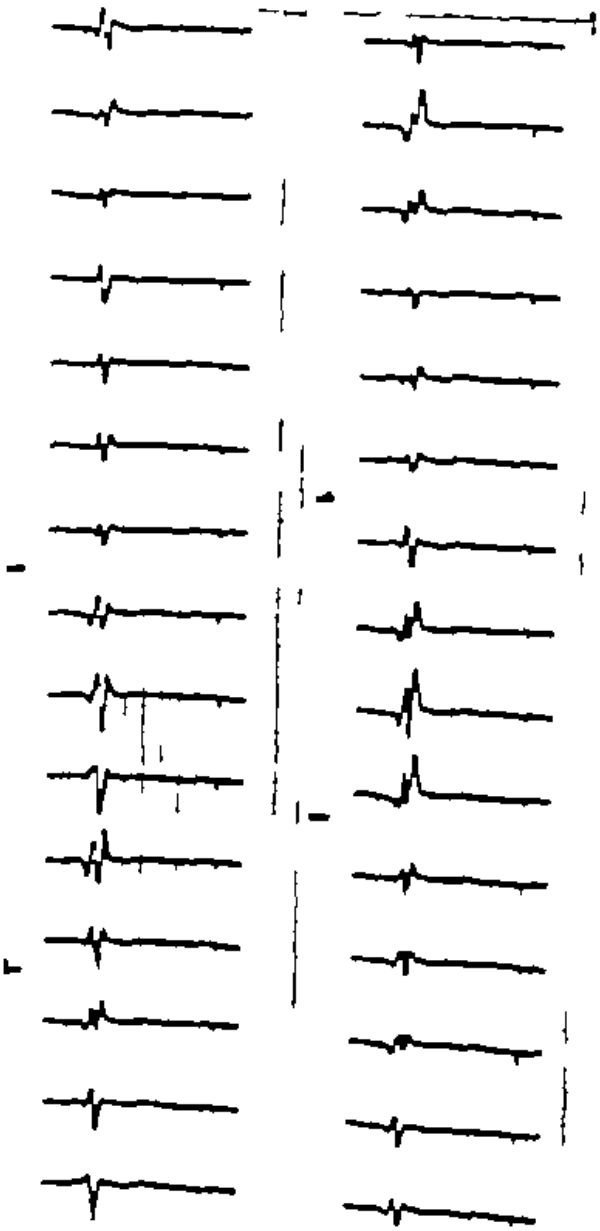


Fig. 24. Muscle action potentials of M.triceps surae elicited by tendon taps. The tracings within brackets were obtained during the same periods of intended handgrips as illustrated in Fig 23.b)

The enhancing effect on the tendon jerk during performed or intended handgrips cannot be quantified, since at the stimulation frequency used a slight high-frequency depression (Magladery *et al* 1952) of the tendon jerk might be present. Due to the short lasting periods of the neuromuscular blockade and also to the short-lasting duration of intended handgrips it would have been inconvenient to use stimulations at lower frequencies.

Granit and Kaada (1952) demonstrated that gamma efferent activity (to the muscle spindles) was facilitated by reticular stimulation at strengths considerably below those which would elicit a discharge of the large skeletal-muscle (alpha) motoneurons. It might be hypothesized that the reinforcement of a spinal reflex on intended somatomotor activity is an analo-

gous phenomenon.

A non-hypothalamic route for influencing somatomotor and autonomic function was defined by Rossi and Brodal (1956) They found corticofugal fibres passing from cortex to the reticular formation of the pons and medulla, with the motor area as the chief contributor Thus the mechanism proposed by Landau (1953) concerning the facilitation of activity patterns, which are essentially determined at spinal and peripheral levels, might be substantiated.

However the purpose of the presentation was merely to demonstrate the parallel appearance of a cardiovascular response and an enhancement of a spinal reflex on intended somatomotor activity

CHAPTER IX

General Discussion

The present study was incited by observations on the central and peripheral circulatory adjustment to exercise with small and large muscle groups. A further analysis demanded a differentiation of the regulatory factors involved and prompted a simplified model of work. Therefore muscular exercise was substituted for a single, isometric muscle contraction and studies of steady state circulatory conditions were replaced by continuous observations during the first 3 to 8 sec of the initial cardiovascular response on muscle activation. In the selected period a modulation of the circulatory effects to mechanical and peripheral metabolic factors would be less marked.

In Chapters IV and V it was demonstrated that the heart rate acceleration at the onset of muscle contraction was predominantly elicited by a withdrawal of vagal tone, but depending on the prevailing autonomic balance a stimulation of beta-adrenergic receptors might also contribute. The mediation of the heart rate acceleration through a decrease of parasympathetic activity at the onset of muscular work has been anticipated in studies demonstrating that, in the presence of a beta-adrenergic blockade, rapid and marked increments in heart rate occurred during isometric handgrips (Macdonald *et al* 1966) or treadmill exercise (Smulyan and Eich 1968)

The effects on heart rate during supine leg exercise of either atropine, propranolol or both drugs were studied in human subjects by Robinson *et al* (1966) They observed that the heart rate increase during mild exercise (oxygen uptake $\leq$ 500 ml/min) was primarily associated with a decrease in parasympathetic activity while at higher working intensities sympathetic stimulation also contributed to cardiac acceleration.

The aortic pressure response to muscle activation, demonstrated in Chapters III and IV was ascribed both to the heart rate acceleration and to an effect on systemic vascular resistance mediated by alpha-adrenergic receptors. In addition, an increase of venomotor tone was initiated on muscle contraction. All three circulatory effects contribute to an enhanced perfusion of activated muscle groups.

In Chapter VI cardiovascular effects were revealed during intended contractions within restricted paralyzed muscle groups. An accompanying facilitation of spinal reflexes was demonstrated in Chapter VIII Thus the conditions may exist for a central nervous excitatory drive evoking the observed cardiovascular effects and the facilitation of somatomotor activity as proposed by Landau (1953) The influence on somatomotor and autonomic function might be exerted both via a non-hypothalamic (Landau 1953 Rosa and Brodal 1956) and a hypothalamic route.

The relation between the cardiovascular response and the baroreceptor mechanism might be considered as follows

Is a counterregulation via the baroreceptors elicited by the cardiovascular response to somatomotor activity or is a simultaneous centrifugal inhibition of the baroreceptor function induced?

The tetraplegic men (Chapter V) were deprived of the integrative cardioaccelerating function of the efferent sympathetic nervous part of the baroreceptor reflex loop Still, the heart rate response to muscle activation was the same as that in healthy subjects. This indicates that normally the baroreceptor function is superseded during muscle contraction. This is furthermore confirmed by the highly significant direct relation found between the increments in blood pressure and heart rate (see Chapter III) An interference between the studied mechanism and the baroreceptor function would have resulted in an inverted relation between the two circulatory variables.

In keeping with such a mechanism are studies reporting that the baroreceptor reflexes are suppressed during isometric work (Lind *et al* 1964) and that hypothalamic stimulation (Hilton 1963) and corticohypothalamic impulse discharges to medullary neurons (Follow *et al.* 1965) can supersede the mechanoreceptor reflexes.

Further it has recently been demonstrated (Gebber and Snyder 1970) that the tachycardia associated with the pressor effect evoked by electrical hypothalamic stimulation was the result not only of increased cardiac sympathetic activity (Pitts *et al* 1941) but also of inhibition of baroreceptor induced vagal activation Assuming a similar mechanism in intact man, the rapid changes in heart rate would correspond to the time course of a discontinued vagal stimulation (Warner and Cox 1962)

Thus from the presented arguments and from the absence of a counterregulation via the baroreceptors it might be assumed that the cardiovascular response to muscle activation is the result of a simultaneous somatomotor and autonomic nervous activity and an inhibition of the baroreceptor reflexes.

It might be expected that the cardiovascular adjustments during dynamic muscular exercise from its start up to a steady state level would appear far more complex. From an unpublished investigation made by the author of the cardiovascular effects of rhythmically repeated isometric handgrips at a frequency from 6 to 40 c/min a summation of the cardiovascular responses to single muscle contractions is discerned. Consequently it seems possible through extended studies to evaluate the effect of the described pattern of reaction on the circulatory adjustment to dynamic muscular exercise.

In Chapter VI cardiovascular effects were revealed during intended contractions within restricted paralyzed muscle groups. An accompanying facilitation of spinal reflexes was demonstrated in Chapter VIII. Thus the conditions may exist for a central nervous excitatory drive evoking the observed cardiovascular effects and the facilitation of somatomotor activity as proposed by Landau (1953). The influence on somatomotor and autonomic function might be exerted both via a non-hypothalamic (Landau 1953, Rossi and Brodal 1956) and a hypothalamic route.

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In keeping with such a mechanism are studies reporting that the baroreceptor reflexes are suppressed during isometric work (Lind *et al* 1964) and that hypothalamic stimulation (Hilton 1963) and corticohypothalamic impulse discharges to medullary neurons (Folkow *et al* 1965) can supersede the mechanoreceptor reflexes.

Further, it has recently been demonstrated (Gebber and Snyder 1970) that the tachycardia associated with the pressor effect evoked by electrical hypothalamic stimulation was the result not only of increased cardiac sympathetic activity (Pitts *et al* 1941) but also of inhibition of baroreceptor-induced vagal activation. Assuming a similar mechanism in intact man, the rapid changes in heart rate would correspond to the time course of a discontinued vagal stimulation (Warner and Cox 1962).

Thus from the presented arguments and from the absence of a counter regulation via the baroreceptors, it might be assumed that the cardiovascular response to muscle activation is the result of a simultaneous somatomotor and autonomic nervous activity and an inhibition of the baroreceptor reflexes.

a cardioacceleration might be initiated by a stimulation of beta-adrenergic receptors. The pressure rise could be ascribed both to the heart rate acceleration and to an effect on systemic vascular resistance mediated by alpha-adrenergic receptors.

3 A study of similar experimental design to that previously described was performed in tetraplegic men with a complete transverse spinal syndrome below segments C_6-C_7. Interest was focused on the heart rate response to muscle activation. Evidence suggested that due to the spinal cord lesion at cervical levels the patients were deprived of centrally controlled sympathetic nervous discharges to the heart. The cardiac adjustment elicited in the vasomotor centres was consequently mediated by changes of vagal tone only. The increments of heart rate and aortic pressures evoked by isometric elbow flexion or handgrips exhibited the same rapid onset as in healthy individuals, and the magnitude of the heart rate response did not differ significantly from that of healthy men. Atropine diminished or abolished the heart rate acceleration initiated by muscle contraction. When both atropine and phentolamine were given the heart rate during an isometric contraction was maximally 127 beats/min, while higher values were observed among the healthy individuals.

It was concluded that the heart rate acceleration at the initiation of a muscle contraction was elicited by a mechanism similar to that in healthy men, *viz.* by a withdrawal of vagal tone.

4 Injection of succinylcholine in the brachial artery induced a total neuromuscular blockade in the muscle groups which were activated during handgrips. Intention to perform handgrips in the paralyzed forearm elicited increments in heart rate and aortic pressures which were on an average 64 and 55 per cent, respectively, of appropriate control data. The absence of irrelevant stimuli, such as breathholding with a tendency to Valsalva manoeuvres or activation of muscles proximal to the paralyzed segment, was controlled by inspection, recording of right atrial pressure and EMG records over various muscles of the shoulder.

It was concluded that the increments of heart rate and blood pressure at the initiation of somatomotor activity were at least partly elicited by an autonomic nervous drive of central origin.

5 Similar cardiovascular effects were elicited when one or both hands contracted at the same relative force or when the isometric contraction was performed as elbow flexion. When the same muscle groups contracted at various fractions of maximal force (MVC) there seemed to be a relation between the initial cardiovascular response and the relative force of contraction, so that circulatory data obtained while exerting one

fourth of MVC were lower than those at three fourths of MVC In the phase of restitution of the neuromuscular transmission following blockade, the circulatory effects evoked by small fractions of developed MVC (intended to be higher) were of higher amplitude than contractions performed at higher MVC prior to the blockade.

The results indicated that the initial circulatory effects of isometric contractions were related to the intensity of motor innervation and not to the size of activated muscle groups.

6 Intended muscle contractions during neuromuscular blockade were accompanied by an enhancement of the triceps surae tendon jerk. It was suggested that the cardiovascular response and the reinforcement of spinal reflexes were integral parts of a centrally initiated pattern of reaction.

7 The absence of a counterregulation via the baroreceptor reflexes, indicates a simultaneous inhibition of the baroreceptor function at the initiation of somatomotor nervous activity

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THE HEMODYNAMIC IMPORTANCE OF STRUCTURAL VASCULAR CHANGES IN ESSENTIAL HYPERTENSION

BY

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CONTENTS

INTRODUCTION 5

PREVIOUS STUDIES 6
Cardiac output in hypertensive disease 6
Peripheral resistance in hypertensive disease 6
Blood viscosity 6
Sympathetic nerves 6
Humoral vasoconstrictor agents 7
Electrolytes 7
Increased vascular reactivity or sensitivity to constrictor agents 7
Morphological changes in the arterioles of patients with high blood pressure 9

HEMODYNAMIC CONSEQUENCES OF AN INCREASED WALL/LUMEN RATIO IN THE RESISTANCE VESSELS 14

PRESENT STUDIES 20
Animal experiments 20

I A hemodynamic study on cutaneous A—V shunts in cats 20
Methods 20
Critical aspects of the methods 21
Results and comments 22

II. Adaptive changes in wall/lumen ratio and reactivity in cat blood vessels exposed to a reduced transmural pressure 24
Methods 24
Results and comments 25

Human study 28

III Aspects of the nature of the increased vascular resistance and reactivity to noradrenaline in hypertensive subjects 28
Material 28

Methods 29
Critical aspects of the material and methods 32
Results 34
Discussion 38
Flow resistance at maximal dilatation 38
Basal vascular tone 43
Threshold to noradrenaline 44
Slope of dose-response curve for noradrenaline 44
Conclusions 47
SOME ASPECTS OF THE PATHOGENESIS OF VASCULAR CHANGES IN HYPERTENSION 48
GENERAL SUMMARY AND CONCLUSIONS 50
REFERENCES 53

This survey is based on the following papers which will be referred to in the text by the Roman numerals given below:

I FOLKOW B., and R. SIVERTSSON Aspects of the difference in vascular "reactivity" between cutaneous resistance vessels and A V anastomoses. Angiologica. 1964 *1* 338—345

II FOLKOW B., and R. SIVERTSSON Adaptive changes in "reactivity" and wall/lumen ratio in cat blood vessels exposed to prolonged transmural pressure difference. Life Sci. 1968 *7* 1283—1289

III SIVERTSSON R., and R. OLANDER, Aspects of the nature of the increased vascular resistance and increased reactivity" to noradrenaline in hypertensive subjects. Life Sci. 1968 *7* 1291—1297

INTRODUCTION

This study is primarily concerned with a comparison between patients with hypertension, predominantly of the essential type, and normotensive controls with respect to the pheripheral hemodynamics. Special interest has been devoted to the hemodynamic effects of an increased wall/lumen rano in the resistance vessels. In order to illustrate these effects two series of animal experiments have also been performed.

PREVIOUS STUDIES

Cardiac output in hypertensive disease

Today most authors agree that cardiac output at rest, in patients with established high blood pressure, is within normal limits (Goldring and Chasis 1944 p 39 Pickering 1968 pp 179—180) This means that the blood pressure in most hypertensive patients at rest is elevated because of an increased peripheral resistance rather than an increased cardiac output. This increase in resistance seems to be distributed rather uniformly over the various systemic circuits, possibly being somewhat less pronounced in the skeletal muscle and more intense in the kidneys (Pickering 1968 pp 185—186)

Peripheral resistance in hypertensive disease

Blood viscosity

Pickering (1968 pp 179—180) considers blood viscosity to be within normal limits in essential hypertension. Tibblin *et al.* (1966) on the other hand found about twenty per cent higher values for whole blood viscosity *in vitro* in a small number of hypertensive patients compared with normotensive persons but only slight changes in hematocrit and plasma viscosity

Animal studies indicate, however that viscosity as measured *in vitro* might sometimes be an unreliable indicator of the viscosity *in vivo* (Djojosugito *et al* 1970) These investigators observed fairly large differences in the viscosity *in vitro* of blood from different cats, while the *in vivo* values were similar Furthermore, the viscosity *in vitro* was usually about twice as high as that *in vivo* at corresponding shear rates.

Sympathetic nerves

There is little evidence of an increased vasoconstrictor nerve activity to peripheral vessels of hypertensive subjects during rest (Pickering 1936 Prinzmetal and Wilson 1936, Freis 1960 Pickering 1968 pp 186—187) However this by no means excludes that there may often be an increase during daily life perhaps in the form of intermittent bouts of sympathetic discharge to both heart and blood vessels in hypertensive patients (Hinman, Engel and Bickford 1962, Bevan Honour and Stott 1969)

Humoral vasoconstrictor agents

Increased amounts of circulating substances which may directly or indirectly cause hypertension have been found in some special forms of hypertensive disease *e g* noradrenaline in phaeochromocytoma, corticoids in Cushing's syndrome and Conn's disease, and renin-angiotensin in renal and malignant hypertension In patients with uncomplicated essential (primary) hypertension, however increased amounts of circulating pressor substances have not been convincingly demonstrated.

Electrolytes

Changes in the electrolyte balance of a nature that may lead to an increased smooth muscle tone or increased vascular reactivity have been much discussed, but according to Freis (1960) "cations in the extracellular fluid are normal in uncomplicated essential hypertension" and evidence is lacking for a change in intracellular concentrations" However Pickering (1968, p 191) considers it possible that "a change in the extra/intracellular fluxes of Na and K in the small arteries and arterioles" might be a final common path leading to an increased peripheral resistance in DCA hypertension hypertension due to renal artery stenosis and perhaps also in essential hypertension. An increased amount of water and electrolytes in the vascular wall, "water logging" that reduces the lumen of the vessel, was first proposed by Tobian and Binion (1952) However in their monograph about renal hypertension Page and McCubbin (1968 p 209) summarize that at present there is no firm evidence concerning the importance of water logging" It has been questioned whether "water logging" might simply be another aspect of the morphological changes seen in the vessels of hypertensive subjects (Pickering 1968 p 191)

Increased vascular reactivity or sensitivity to constrictor agents

Terms like increased "sensitivity" or "reactivity" are often used in the studies that will be discussed below As a rule, the authors have used these terms without exact definition, in the general meaning of an increased response to a given stimulus, the definitions used in this study are given below on page 17

There are many papers on this topic, often with contradictory results. Reflex increases of blood pressure, as a result of cold exposure of the hand, were studied by *e g* Hines and Brown (1933) Pickering and Kissin (1936) Alam and Smirk (1938) and Harlan, Osborne and Graybiel (1964) Enhanced responses to cold were found in essential hypertension by Hines and Brown and by Alam and Smirk, but these observations could not be

verified by Pickering and Kissin In their extensive follow up study Harlan, Osborne and Graybiel could not verify the idea proposed by Hines and Brown that the cold pressor test could be used for the diagnosis of prehypertension.

However the explanation of an increased response in such experiments must not necessarily involve an increased vascular reactivity Hines and Brown believed that the cause of the large response in hypertensive patients was an increased reactivity" in the vasomotor centres.

In some studies the change in blood pressure (Clough 1920 Jensen 1930 Gordon and Levitt 1935 Fatherree and Hines 1938 Goldenberg *et al* 1948 Judson *et al.* 1950 a, Judson, Epstein and Wilkins 1950 b Gombos *et al* 1962) or in total peripheral resistance (Goldenberg *et al* 1948) was measured when standard doses of a pressor substance were given intravenously or subcutaneously Most of these studies were performed with adrenaline as the only pressor agent used. The increase in systolic pressure produced by this drug, however is due to an increased cardiac output, and in the doses used it usually reduces overall systemic resistance (Jensen 1930 Goldenberg *et al* 1948) Many of the studies discussed above are therefore of little relevance for the question of vascular reactivity to vasoconstrictors.

Among studies utilizing noradrenaline or some other purely vasoconstrictor drug the one performed by Goldenberg *et al* (1948) indicated increased pressor responses in patients with essential hypertension. However this finding could not be verified by Judson, Epstein and Wilkins (1950 b) nor did Gombos *et al* (1962) observe any increased vascular reactivity in experiments of this type. Furthermore, in studies where blood pressure changes are measured, as well as in those where changes in regional blood flow or flow resistance (Gombos *et al* 1962) are determined during intravenous or subcutaneous drug administration, the results may be considerably modified by secondary reflex adjustments as elicited by *e g* the baroreceptors. The disadvantage of such a type of drug administration to the general circulation can be reduced somewhat by regional or generalized sympathetic nerve blockade, *e g* by means of ganglion blocking drugs (Doyle and Black 1955) application of local anesthetics to regional sympathetic nerves (Bárány and James 1959 Bárány 1963) or for the skin vessels, by indirect heating (Mendlowitz and Naftchi 1958) The studies maid with these techniques have indicated an increased vascular reactivity to noradrenaline in essential hypertension

In many of the studies discussed above changes in blood pressure were given in absolute figures (*e g* Goldenberg *et al* 1948 Doyle and Black

1955) As pointed out by Pickering (1936) and Gombos *et al.* (1962) however it seems more correct to compare relative changes rather than absolute changes.

The most reliable information concerning vascular reactivity is probably obtained if vasoactive substances are given directly into the artery which supplies the tissue or organ studied (Duff 1957 Doyle, Fraser and Marshall 1959 Scroop and Whelan 1968) According to Doyle, Fraser and Marshall (1959) vascular reactivity seems to be unspecifically increased in essential hypertension for many different drugs, *e g* noradrenaline, 5 HT and angiotensin. This is in good agreement with the results reported by Duff (1957) which indicated an increased vascular reactivity for adrenaline. Scroop and Whelan (1968) could, however not confirm these findings

In his review Mendlowitz (1967) summarized "The weight of evidence indicates that there is increased systemic vascular reactivity in essential hypertension" However as discussed above the experimental results reported in the literature, though extensive, are often contradictory and difficult to interpret.

Morphological changes in the arterioles of patients with high blood pressure

The existence of morphological changes in the systemic vascular bed of patients with high blood pressure is well known. In 1868 Johnson reported hypertrophy of the arteriolar walls in the kidney intestine, skeletal muscle, pia mater and skin in Bright's disease. He interpreted the hypertrophy as being a result of "long continued overactivity of the vessels

Moritz and Oldt (1937) in their extensive study gave the following description of arteriolar morphological changes in hypertensive patients. *Intimal hyalinization* is characterised by a subendothelial accumulation of homogeneous acidophilic material. *Medial hypertrophy* implies an increased size (and number) of smooth muscle cells. *Medial degeneration* means an increase of intercellular collagen, which in some cases is associated with atrophy rather than hypertrophy of smooth muscle cells. A fourth type of vascular change, *endothelial hyperplasia (intimal hyperplasia)* was also discussed. All these vascular changes were also seen more or less frequently in healthy persons with normal blood pressure, and in patients with diseases other than high blood pressure. However as a rule, these changes were more severe and frequent in hypertensive patients. Furthermore the occurrence of such arteriolar changes differed from one organ to another *Acute arteriolar lesions* arteriolonecrosis or fibrinoid necrosis, were often superimposed upon the more chronic changes.

In their biopsy study Sommers, Relman and Smithwick (1958) observed smooth muscle swelling and hypertrophy as well as an increased amount of PAS-positive substances in the vascular wall in the early benign cases of hypertension (arteriolar sclerosis, grade I and II) Degenerative changes were found predominantly in the more severe and long standing cases (grade III) "It was inferred" they wrote, "that the sequence of events was spastic arterial contraction intramural vascular edema, muscular overgrowth, replacement of the degenerated muscle by pooled ground substance and ultimately collagenization and hyalinization" Other workers have suggested that the hyaline and fibrinoid deposition in the vascular wall has a hematogenous origin and results from deposition of fibrin and in fact there is considerable evidence to support this opinion (Giese 1966 Page and McCubbin 1968 pp 378—384)

Smooth muscle hypertrophy intimal hyperplasia and hyalinization would tend to increase the wall thickness. Such changes are probably responsible for the increased wall/lumen ratio in the arterioles of hypertensive patients, which has often been reported (*e g* Kernohan, Anderson and Keith 1929 Morlock 1939) The determinations of wall/lumen ratio, however are open to criticism, because it is difficult to decide, if the increased ratio is due to a truly increased wall tissue mass, or simply to a vascular contraction present at the moment of fixation (Andrus 1936) On the other hand, Moritz and Oldt (1937) who also found an increased wall/lumen ratio in the resistance vessels of hypertensive patients, claimed that experimentally induced changes of smooth muscle tone, produced immediately before the animal was killed, did not alter the wall/lumen ratio as observed in a histological section. Changes in the preparation technique were also without effect on this ratio However there are probably situations where differences in *e g* intravascular pressure and in wall distensibility in the arterioles may greatly affect morphological estimations of vascular design.

Short and co-workers claimed (Short and Thomson 1959 Short 1966) that the increased wall/lumen ratio in arterioles from patients with high blood pressure is due to what they called a vascular "contracture rather than to a wall thickening. They proposed that prolonged arteriolar constriction from any cause leads to persistent shortening of the circular muscle They determined the cross-sectional area of the vascular wall in what they considered to be corresponding arterioles in hypertensive and normotensive persons and stated that this was equal. However at the same time the lumen was smaller and the wall/lumen ratio was larger in the vessels of hypertensive patients compared with those in normotensive persons. According to them the wall surface area would better reflect

vascular smooth muscle hypertrophy or hyperplasia than a measure of arteriolar wall/lumen ratio However this almost borders on a semantic problem, and it is a matter of the definition of wall hypertrophy In hollow organs, like the heart and the vessels, the only useful meaning of the term "wall hypertrophy" at least in terms of function of such hollow organs, is an increased wall thickness in relation to the lumen, since the force against which the smooth muscles in the wall act is to a great extent determined by the size of the lumen, according to Laplace s law If then the result of vascular rebuilding in hypertension is that branches of the same generation in the vascular tree are generally decreased in luminal size but have an increased wall/lumen ratio due to an undiminished wall mass, as suggested by Short, one may still define this as wall "hypertrophy' from the functional, hemodynamical point of view The reason is that such a vessel has available for the control of its particular lumen, a relatively greater mass of contractile tissue.

Barrett (1963) studying arteries, found good correlation between the heart weight and the cross-sectional area of the wall in the five largest branches of the superior mesenteric artery in a series of 100 cases of normotensive and hypertensive subjects, where all other causes of cardiac hyper trophy except hypertension were excluded.

Naeye (1967) related the total area of the arteriolar smooth muscle cells to the total area of the nuclei of the endothelial cells in arteriolar cross sections. The size of these nuclei was thought to be a good reference for the estimation of the size of the vessel. He claimed that, in hypertensive patients, arterioles situated proximally to sclerotic obstructions of the vessels, in which the intravascular pressure could be supposed to be higher than normal, showed media hypertrophy and hyperplasia, while those placed distally to such obstructions, where pressure is lower exhibited decreased media muscle mass. He found, like many other authors, that the vascular changes in hypertensive patients varied considerably from one arteriole to another and also within the same arteriole and artery For a more complete discussion of structural vascular changes in hypertension see Giese (1966)

There are surprisingly few physiological studies performed concerning the hemodynamic consequences of these morphological vascular changes. Prinzmetal and Wilson (1936) and Pickering (1936) simultaneously published two rather similar papers, in which the ability for skin and muscle vessels to dilate was compared in normotensive and hypertensive subjects. They found that skin blood flow was the same in the two groups after sympathetic nerve blockade as well as after indirect heating. This means

that flow resistance in the hypertensives was still increased above that in normotensive subjects after elimination of the vasoconstrictor influence, and this increase would therefore have another background than an increased vasomotor activity It was furthermore shown that muscle blood flow was almost equal at rest in normotensive and hypertensive subjects, and increased to about the same level in the two groups after 5—10 minutes of arterial occlusion. This finding was, apparently not only taken as an argument against rigid sclerotic vascular changes, but also against morphological vascular changes of any kind (Prinzmetal and Wilson 1936) These authors suggested that the increased flow resistance in hypertensives, at rest as well during dilatation might be due to some kind of circulating pressor substance that was resistant to ischemia

Stead and Kunkel (1940) made similar observations on hand and foot blood flow after direct and indirect heating and on forearm blood flow after ischemic work. The blood flow in all three tissues reached about the same values in hypertensive and normotensive subjects. These authors also presented evidence indicating that blood flow resistance in the brain could not be lowered to the same level in hypertensive patients as in controls by inducing cerebral anoxia. They discussed both structural factors and humoral vasoconstrictor agents as the cause of the increased resistance in hypertension. Novack *et al* (1953) found that cerebral blood flow could increase in both hypertensive and normotensive persons on 5 % CO_2-breathing, reaching about the same values in the two groups. Flow resistance during vasodilatation was therefore increased in the hypertensive group in proportion to the elevation in blood pressure.

Pickering (1955 p 144) summarized "There would seem little doubt that these organic changes, which encroach on the lumen of small arteries and arterioles, must contribute to the increased peripheral resistance. But the consensus of current opinion is opposed to their being entirely responsible for the increased peripheral resistance, for two reasons which may now be examined. The first reason given is that the increased peripheral resistance in hypertension may be diminished by certain vasodilator agents The second and more formidable reason is that these structural lesions are too slight and too inconstant to constitute the whole abnormality in vascular behaviour which is observed"

Folkow and co-workers (Folkow 1956 Folkow Grimby and Thulesius 1958) have expressed another opinion They demonstrated in principle that for any given degree of smooth muscle shortening the change in flow resistance would always be larger in a vessel with increased wall/lumen ratio than in one with a normal ratio. This might be one, or even the main,

explanation for the increased vascular reactivity to constrictor agents often found in hypertensive patients, and it would eliminate the first of Pickerings two arguments against the hemodynamic importance of morphological vascular changes. They also found an increased flow resistance in forearm vessels at maximal vasodilatation, suggesting that the proposed increase of vascular wall mass had encroached upon the lumen of the resistance vessels also at complete relaxation of the smooth muscles. If these findings reflected a generalized change of the systemic resistance vessels this would eliminated also the second of Pickerings arguments. These findings were fully confirmed in an extensive study of the forearm vessels in hypertensive patients by Conway (1963) The present work is a further analysis of this conception, including its hemodynamic consequences.

HEMODYNAMIC CONSEQUENCES OF AN INCREASED WALL/LUMEN RATIO IN THE RESISTANCE VESSELS

Folkow and co-workers have previously pointed out the hemodynamic effects of an increased wall/lumen ratio in the resistance vessels (Folkow 1956 Folkow Grimby and Thulesius 1958) They conclude on the basis of mathematical deductions that the presence of a wall thickening which practically always implies an increased wall/lumen ratio, in the resistance vessels might affect the flow resistance in two principally different ways

1 By potentially influencing *flow resistance already at maximal vasodilatation* by changing the morphologically set dimensions of the vascular bed

a) *if the wall "grows inwards" flow resistance at maximal dilatation will* increase (Fig 1 H and 2 H_a)

b) if the vascular wall grows in such a way that the outer diameter increases to the same extent as the wall thickness, the result will be an unchanged inner radius and resistance at maximal dilatation (Fig 1 H_b);

c) if finally the wall "grows outwards" to the extent that the outer diameter increases more than the wall thickness, the flow resistance at maximal vasodilatation will be decreased (Fig 1 H_c)

2 By changing the *relation between the degree of vascular smooth muscle contraction and flow resistance* The changed vascular architecture behind an increased wall/lumen ratio in the resistance vessels, with an increased wall mass situated inside the "line of force" of the smooth muscle ring, will lead to exaggerated luminal reductions for given degrees of vascular smooth muscle shortening (Fig. 1 and 2) Now suppose that the smooth muscle cells for any degree of excitation (*e g* sympathetic nerve stimulation or intraarterial noradrenaline infusion) show a perfectly normal per cent decrease in length. In such vessels with thickened walls this would produce a *steeper dose-response relation* between sympathetic nerve stimulation (or intraarterial noradrenaline administration) and flow resistance than in normal vessels. The slope would be increased also if resistance were given in *relative* units, *e g* as a fraction of basal resistance, as in the present study (for definitions see below p. 29) This difference in slope

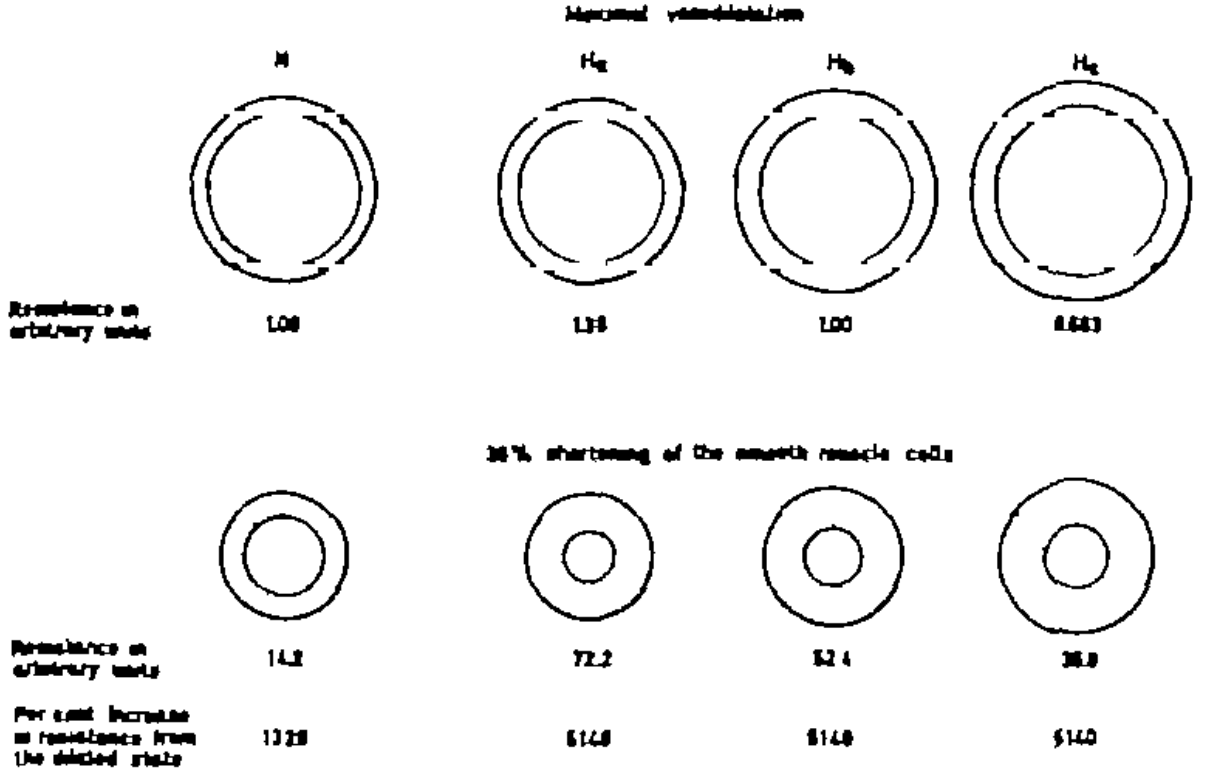


Fig. 1 Illustration of the hemodynamic effects of an increased wall/lumen ratio in the resistance vessels (Folkow 1956, Folkow Grimby and Thulesius 1958).

N represents a normal vessel which at maximal dilatation has a flow resistance of one unit and a wall/lumen ratio of 0.2. All the vessels represented by H have wall/lumen ratios of 0.3 at maximal dilatation, 50 per cent larger than in the normal vessel. H_a has an increased, H_b an unchanged and H_c a decreased resistance at maximal vasodilatation compared with the normal vessel. In the upper series all the vessels are maximally dilated. In the series below the "line of force" in the smooth muscle sheath, represented by the outer circumference, is shortened by 30 per cent. Due to the larger wall mass situated inside this line in the vessels denoted H, the ensuing increase in resistance is much larger in these vessels than in the normal vessel. It should also be observed that the percentual increase in resistance produced by the vascular contraction is the same in all three vessels denoted H because their wall/lumen ratios are equal.

might be one explanation of the potentiated vasoconstrictor response often seen in patients with high blood pressure.

If the intention is to separate the effect of an increased wall/lumen ratio *per se* on the slope of the dose-response curve from that of an increased resistance at maximal dilatation it is important to calculate the slopes from dose-response curves where the resistance is given in *relative* units, either related to the resistance at maximal dilatation or to the basal resistance, if this is proportional to the resistance at maximal dilatation, which is the case in the present study This is important because slopes calculated from curves, where resistance is given in *absolute* values, will depend on the level of the resistance at maximal dilatation (Fig 3) If the resistance at maximal

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b) if the vascular wall grows in such a way that the outer diameter increases to the same extent as the wall thickness, the result will be an unchanged inner radius and resistance at maximal dilatation (Fig 1 H_b)

c) if finally the wall "grows outwards" to the extent that the outer diameter increases more than the wall thickness, the flow resistance at maximal vasodilatation will be decreased (Fig 1 H_c)

2 By changing the *relation between the degree of vascular smooth muscle contraction and flow resistance* The changed vascular architecture behind an increased wall/lumen ratio in the resistance vessels, with an increased wall mass situated inside the "line of force of the smooth muscle ring, will lead to exaggerated luminal reductions for given degrees of vascular smooth muscle shortening (Fig 1 and 2) Now suppose that the smooth muscle cells for any degree of excitation (*e g* sympathetic nerve stimulation or intraarterial noradrenaline infusion) show a perfectly normal per cent decrease in length. In such vessels with thickened walls this would produce a *steeper dose-response relation* between sympathetic nerve stimulation (or intraarterial noradrenaline administration) and flow resistance than in normal vessels. The slope would be increased also if resistance were given in *relative* units, *e g* as a fraction of basal resistance, as in the present study (for definitions see below p 29) This difference in slope

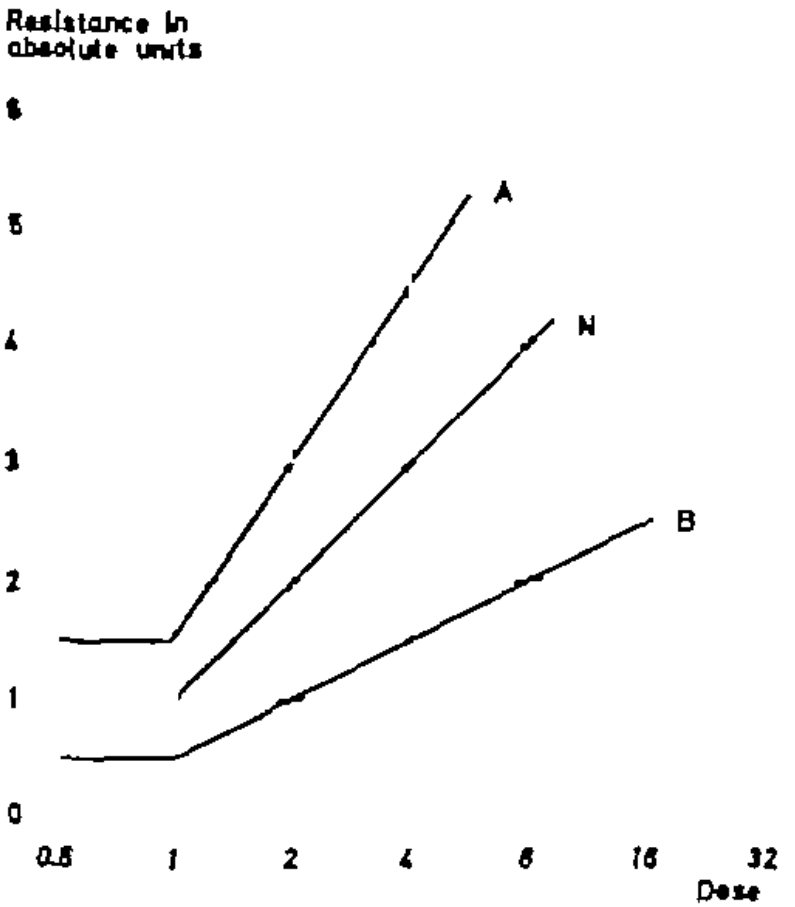


Fig. 3 Hypothetical dose-response curves showing the effect of an increased resistance at maximal dilatation on the slope of the curves when resistance is given in *absolute* values. In all cases the wall/lumen ratio is thought to be the same.
N: Normal resistance at maximal dilatation.
A: 50 per cent increased resistance at maximal dilatation.
B: 50 per cent decreased resistance at maximal dilatation.

wall/lumen ratio and one due to an *increased smooth muscle sensitivity* to noradrenaline. Usually these two different factors are not distinguished in previous studies. The terminology used in the literature to describe these and similar aspects often differs from one author to the other.

By *increased sensitivity* it will here be meant that a normal response is obtained within the entire dose spectrum for any dose which constitutes the same fraction (say one tenth) of that normally needed for this response (see e g Trendelenburg 1963). In other words, if a dose-response curve is plotted with the logarithm for the dose on the abscissa and the response on the ordinate an increased sensitivity will according to this definition, move the curve to the left in a *parallel* fashion, implying a *decreased threshold with unchanged steepness* as is schematically illustrated in Fig. 4 A. A wellknown example of a sensitization in this meaning is that seen after

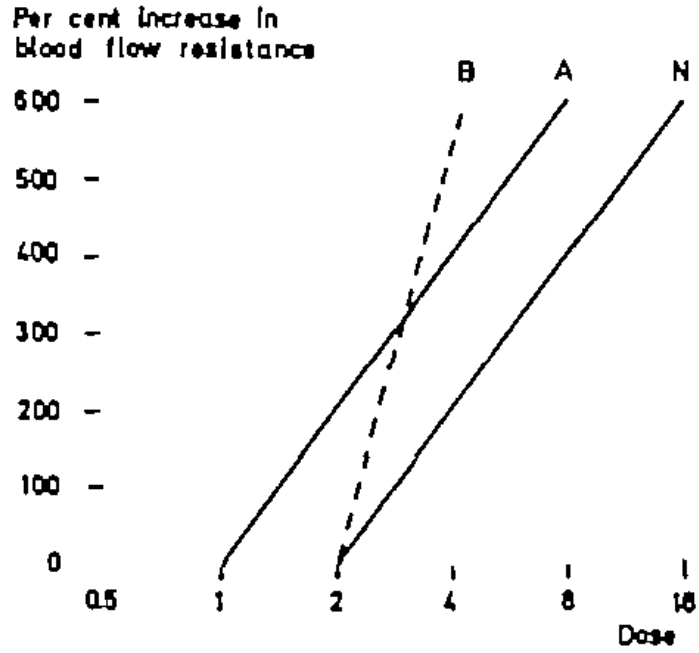


Fig. 4 Schematic illustration of the effect of an increased sensitivity of the vascular smooth muscle cells for noradrenaline (A) and an increased wall/lumen ratio in the resistance vessels (B) on the dose-response curve. N refers to the normal situation.

denervation (Cannon and Rosenblueth 1949) During competitive interaction the dose-response curve is moved to the right in a parallel fashion.

An *increased wall/lumen ratio* in the resistance vessels, on the other hand, will give an *unchanged threshold* but a *steeper dose-response curve* (see Fig 4 B) An increased ability of the smooth muscle sheath to develop contraction force as a consequence of smooth muscle hypertrophy (or hyperplasia) may affect the shape of the dose-response curve. Smooth muscle hypertrophy or hyperplasia is almost inevitably combined with an increased wall/lumen ratio in the resistance vessels, since the media normally makes out the greater part of the wall in these vessels. Such changes appear to be common in hypertension, and the increased contractile strength, which is a consequence of the increased muscle mass as related to the lumen will here be included in a somewhat widened concept of an increased wall/lumen ratio

By *increased vascular reactivity* it is here meant that an exaggerated luminal change is obtained when a standard dose of a vasoconstrictor drug is given. This expression thus includes a lowered threshold as well as an increased slope.

It should be observed that there are other theoretically possible explanations of a raised flow resistance at maximal vasodilatation than an increased wall/lumen ratio of the resistance vessels. These vessels may simply be

symmetrically decreased in size both in lumen and in wall thickness they may be *elongated* there may be *fewer vessels per tissue volume* or the *viscosity* of the blood may be increased. None of these factors would however lead to an increased steepness of the dose-response curve, if the response is measured in relative units.

A steeper slope of the dose-response curve relating flow resistance to vasoconstriction stimulus need not necessarily imply an increased wall/lumen ratio of the resistance vessels. It could also be achieved by other types of changes, but none of these alternatives could explain an increased resistance to flow at maximal vascular relaxation. Thus, a steeper slope of the dose-response curve might be due to an *increased degree of shortening of the smooth muscle cells* for the same stimulation intensity without any change of vascular structures. One example, the only one known to the author where a given drug increased the slope of the dose-response curve of the smooth muscle cell itself (membrana nictitans) to both sympathetic nerve stimulation and intravenous adrenaline administration, is discussed by Cannon and Rosenblueth (1949 p 15) After thyroxine administration the response was increased by about 50 per cent at every stimulation intensity An increased slope could also be explained by a *decreased vascular reactivity to vasodilator metabolites* which accumulate during vasoconstriction. Furthermore, where several effectors are involved, a dose-response curve may at least to some extent, be looked upon as a *cumulative frequency-distribution curve* which provides another possibility to explain a difference in slope between two curves. Suppose that the individual vessel in a vascular bed reacts in an all-or none fashion, changing from maximal relaxation to complete closure, whenever the threshold for vasoconstriction is surpassed. In such a hypothetical situation a shift in threshold within a population of parallel-coupled resistance vessels might change the slope of the curve comprised of the response of the entire vessel population of the tissue. If the difference between the highest and lowest threshold were large a flat curve would be obtained, if it were small a steep curve would ensue. It is, however very unlikely that resistance vessels exposed to physiological stimuli would react in such an all-or none fashion (see *e g* Van Citters 1966) but it is probable that the slope of the dose-response curve to some extent is due to differences in reactivity between the individual vessels in a vascular bed

PRESENT STUDIES

Animal experiments

In order to test the hypothesis discussed above, that an increased wall/lumen ratio in the resistance vessels will increase the slope of the dose-response curve without changing the threshold, the two following series of animal experiments were performed

I. A hemodynamic study on cutaneous A V shunts in cats

Two cutaneous vascular beds with different proportions of thermo-regulatory arterio-venous shunts and of "nutritional resistance vessels were compared. Cutaneous A—V shunts normally display a higher wall/lumen ratio than the regular resistance vessels (Clara 1956 p 18) and possibly but not necessarily their smooth muscles are more sensitive to noradrenaline As both sets of vessels are located in the skin of the cat s paw the local chemical environment of their smooth muscle cells may be expected to be fairly similar

Methods

In 19 anesthetized cats the large saphenous vein was cannulated at the ankle and the total venous effluent from the paw directed to this vein by means of a moderately tight rubber string around the ankle, allowing for a recording of the blood flow in the paw (for details see Paper I) It is known from many animal species that the cutaneous A—V shunts are mainly located in the pads and toes (Clara 1956 pp 25—28) By studying the paw blood flow with the pads and toes included and excluded, respectively it was possible to compare the responses of two parallel-coupled vascular circuits which differ in wall/lumen ratio From these data the blood flow in the pads and toes could be calculated separately and thus the response of their vessels, dominated by the A—V anastomoses evaluated. Blood flow from the cat s hind paw was thus recorded during direct stimulation of the lumbar sympathetic nerve trunk with stepwise increases of frequency and also during intraarterial and intravenous infusion of noradrenaline (Nor-exadrin® Astra) with stepwise increases of dosage. Intraarterial blood pressure was simultaneously recorded with a mercury

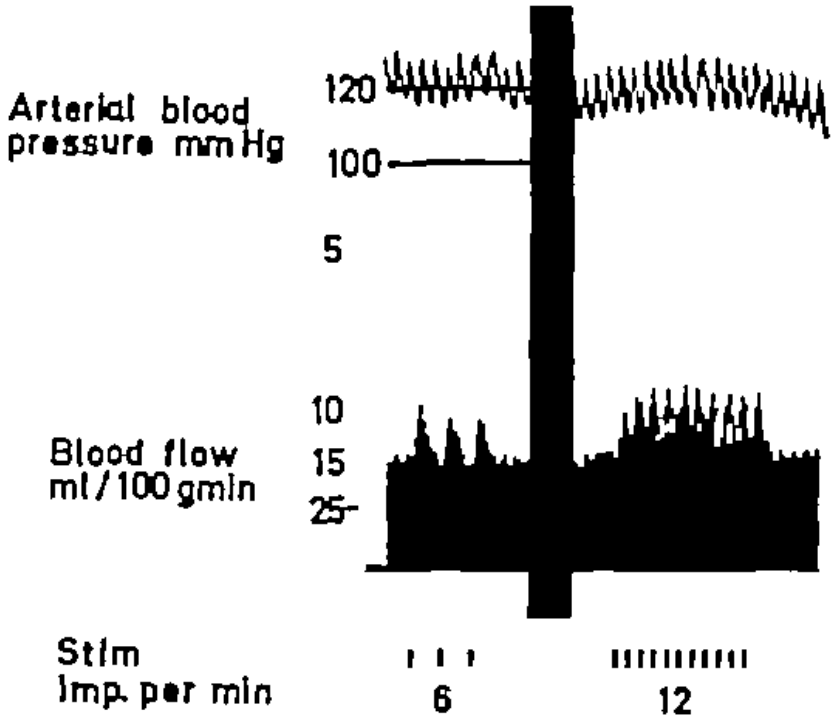


Fig. 5 Blood flow from the cat's hind paw during low frequency vasomotor nerve stimulation.

manometer From these data dose-response curves for sympathetic nerve stimulation and for noradrenaline infusion could be deduced for the paw blood flow resistance, with pads and toes excluded ("skin ') with pads and toes included ("skin + pad") and for pads and toes alone ("pad")

Critical aspects of the methods

With respect to nerve stimulation no distinct threshold in the dose-response correlation could be obtained, simply because even a single impulse produced a clearcut response (Fig 5) Therefore the sensitivity of the acutely denervated vascular smooth muscle had to be judged from the nor adrenaline infusion experiments.

In some of the infusion experiments noradrenaline was given into the femoral artery When a drug is given in this way "close intraarterially there is a risk for incomplete mixture between the drug and the blood stream, causing an uneven distribution of the drug to the tissues. In order to determine whether this factor affected the results significantly some experiments were performed where noradrenaline was administered intra venously which ensures a complete mixture between drug and blood in the heart and lungs. There was little difference in the results, whether nor adrenaline was infused intraarterially or intravenously

For intraarterial infusions a solution of noradrenaline (0.5μg/ml) dis-

solved in 0 9 per cent saline was administered into the femoral artery By changing the speed of infusion different amounts of noradrenaline could be administered per time unit. The highest infusion rates used in the experimentes were often between 0.2 and 0.5 ml per min, but sometimes 1 ml per minute was given This relatively high infusion rate no doubt changed the hematocrit and blood viscosity in the limb vessels to some extent, especially as blood flow to the limb was maximally reduced when this infusion was given. However the changes in hematocrit and blood viscosity which depend on the amount of saline infused per min in relation to the entire blood supply to the hind limb of the cat, must be supposed to be largely the same whether the pads and toes were included or not. Thus, this factor will not significantly affect the comparison of the two vascular circuits studied.

The infusion and stimulation experiments were as a rule first performed with the pads and toes included and then repeated with these parts excluded. It could therefore be argued that the decrease in slope of the dose-response curve, occurring after ligation of the pads and toes, might be due to the fact that one vascular bed was examined isolated after such a long time that a deterioration of the vascular responses might have occurred. However as the pads and toes were excluded only by rubber strings, which left their vessels and nerves relatively undamaged, they could again be included into the perfusion system after a period of exclusion This was done in some experiments and brought about a considerable increase in slope of the dose-response curve. The slopes, however did not quite reach the initial values, which may be due to slight injuries to the vessels, caused by the procedure for exclusion. In any case, this control indicates that the difference between paw vessels with pads and toes included and with these organs excluded could not be entirely explained by the fact that the skin" vessels were studied later in the day than the former but reflected a true difference in the relationship between dosage and resistance response of the two types of vessels.

Results and comments

In the experiments where noradrenaline was infused intravenously or intraarterially there was no difference in threshold when pads and toes were included or excluded. However once the dose surpassed the threshold the vascular bed which included pads and toes usually exhibited more pronounced constrictor responses than the other one. This means that the vascular bed where the vessels displayed the highest mean wall/lumen ratio also showed the steepest dose-response curve (see Fig. 1 in Paper I)

In the experiments where the sympathetic vasoconstrictor fibres were stimulated the vascular bed that was rich in A—V shunts also exhibited the steeper dose-response curve (Fig. 2 in Paper I). However as discussed above (p. 21) it is not possible to evaluate the sensitivity to sympathetic nerve stimulation in this way as no distinct threshold could be recorded.

From these results it is evident that there is no significant difference in sensitivity to noradrenaline between "skin" resistance vessels and the A—V shunts of the pads. However once the threshold is surpassed the "pad" vessels constrict more powerfully than the "skin" vessels. This increased reactivity characterized by a steeper dose-response curve but by an unchanged noradrenaline threshold, is what would be expected from vessels with a higher wall/lumen ratio other things being equal.

In addition, the relatively high reactivity of the A—V shunts to sympathetic stimulation may to some extent be due to a more dense adrenergic nerve supply to these vessels (Celander and Folkow 1953). Such a factor alone, however cannot explain all the findings: there was also, for example, a distinct difference in the slope of the dose-response curve to noradrenaline infusion. Neither does it fully explain the results of the sympathetic nerve stimulation experiments. If the only difference were an increased number of nerve endings in the smooth muscle cells one would expect a tendency of the frequency response curve to move in a parallel fashion to the left without any appreciable change in its slope.

The possibility of an increased strength of smooth muscle contraction, despite an unchanged morphology (p. 19) cannot be *a priori* excluded as an explanation of the steeper dose-response curve in the "skin+pad" vascular bed. Were this the case one might then expect more powerful responses also at noradrenaline concentrations close to the threshold (p. 19). The second alternative explanation discussed above (p. 19) that the "nutritional" skin vessels might be more reactive to vasodilator metabolites accumulated during vasoconstriction, seems unlikely because the cutaneous vessels in general react very little to such factors, and the flow of the skin in relation to its metabolism is high. As "skin+pad" consists of more different types of vessels than "skin" with "pads" excluded, it is likely that the former vascular bed is more heterogeneous with respect to the reactivity of its individual vessels. This would give a less steep curve in "skin+pad" than in "skin" instead of the steeper one observed. Therefore the finding of a steeper curve in the bed rich in A—V shunts can hardly be explained as the effect of a changed frequency distribution curve — the third alternative discussed on p. 19.

II Adaptive changes in wall/lumen ratio and reactivity in cat blood vessels exposed to a reduced transmural pressure

The presence of a high wall/lumen ratio in the resistance vessels of patients with hypertension is usually considered to reflect an adaptation to an increased load on the vessels produced by the high blood pressure. If this is true, the reverse would also occur *i e* prolonged hypotension would lead to some reduction in wall/lumen ratio. In this series of animal experiments a unilateral arterial occlusion was produced in one hind limb of the cats to test whether after some weeks the consequent hypotension caused an adaptive decrease in arterial and arteriolar wall/lumen ratio of that leg. After this time the reactions of the two legs were compared in acute experiments.

Methods

Under ether anesthesia the superficial and deep femoral arteries and the inferior epigastric artery were ligated unilaterally in the cat. This initially decreased the mean arterial blood pressure in the leg to about one third of the normal but the blood supply still proved to be sufficient at rest. Due to widening and growth of collaterals the arterial pressure distal to the occlusion successively rose to about two thirds of the normal value.

After 3—5 weeks the two hind limbs were compared in an acute experiment, in which an isolated calf muscle preparation was arranged on each side (for details see Paper II) so that the only intact communications to the calf besides the femur were the popliteal artery and vein and the sciatic nerve. After heparinisation a normal arterial blood pressure was reestablished on the occluded side ("test limb") by a tube connecting the common iliac artery with the popliteal portion of the femoral artery. In series with this tube a drop recorder operating an ordinate writer was inserted, allowing a continuous measurement of the arterial inflow to the calf. An identical arrangement was made in the "normal limb" so that both had identical arterial supply routes. Arterial blood pressure was continuously measured.

In some experiments, when intraarterial infusions of drugs were given to the limbs, the arterial blood had first to pass a common drop chamber before entering the two femoral arteries. This established a "mixing chamber" where the infused substance was thoroughly mixed with the arterial inflow to the two limbs. In these experiments venous outflow from the two calves was recorded by the drop chamber technique instead of the arterial inflow.

The lumbar sympathetic trunks as well as the intact sciatic nerves could be directly stimulated on both sides so that separate and graded excitations of the vasoconstrictor fibres and the somatomotor fibres to the calf muscles, respectively could be performed. In one series of 13 experiments the relation between increasing rates of constrictor fibre stimulation and flow resistance was investigated. In another series of 12 experiments dose-response curves were determined, relating stepwise increases in rate of noradrenaline infusion (2.5 and 10 μg/ml) to the ensuing resistance responses.

In order to allow the neurohumoral constrictor influences to affect the resistance vessels over the "middle physiological" range of smooth muscle tone, the motor fibres to the calf muscles were continuously and selectively stimulated at an impulse frequency of 1 per sec. Under these conditions the resistance vessels were moderately dilated to start with. As a rule the "basal flow", *i. e.* the flow level during the moderate exercise hyperemia from which vasoconstriction was induced, was about equal on the two sides. Experiments were accepted only when this was the case.

For statistical evaluation paired differences in response between the test limbs and the normal limbs were calculated and tested by Student's t test and Wilcoxon's test (Documenta Geigy 1962 p. 191) at each frequency and dose level. Calculation of thresholds and slopes according to the technique used in Paper III (se the present text p. 31) was used in the infusion experiments.

Results and comments

In the experiments where the vasoconstrictor nerves were stimulated in a graded fashion the slope of the dose-response curve was smaller in the test limb than in the normal limb (Fig. 1 in Paper II and Fig. 6). The response was significantly decreased ($P<0.05$) in the test limb for stimulation frequencies of 4 impulses per sec or above. Here, as well as in the experiments described on page 21 where responses to constrictor fibre stimulation were studied, no distinct threshold could be noted.

The results of the experiments where noradrenaline was given intra arterially are shown in Fig. 7. There was no difference in threshold between the two legs. However once a constrictor response was elicited the slope of the dose-response curve was lower in the previously hypotensive limb (test limb) than in the normal limb. The difference in slope was significant ($P<0.05$).

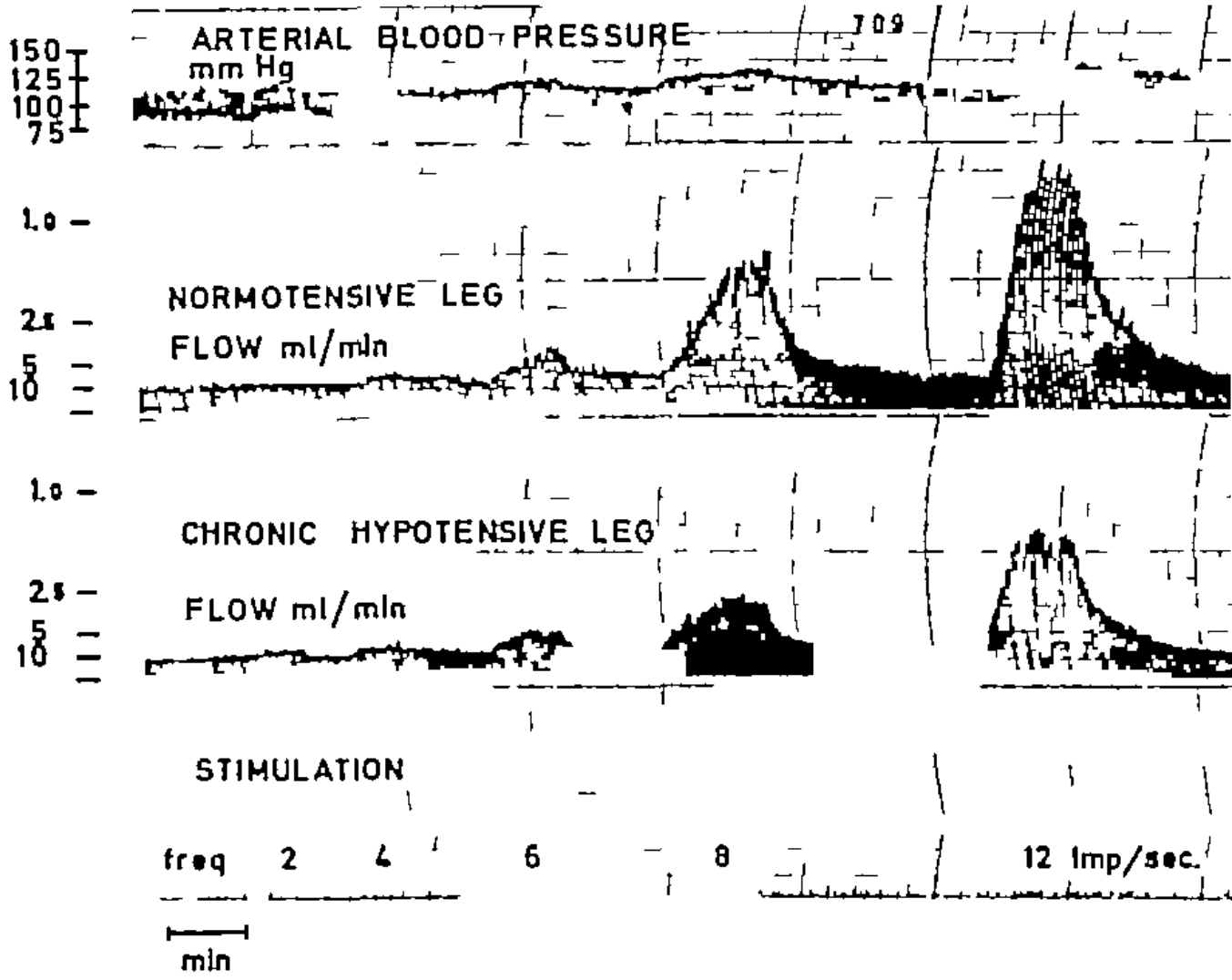


Fig 6. Registration of calf blood flow during graded vasomotor nerve stimulation to the legs. One leg (lower curve) had been exposed to moderate arterial hypotension, by means of arterial occlusions, for about 4 weeks. During the acute experiment the two legs had identical perfusion pressures, since the arterial communications had been established in the hypotensive leg.

The findings of a decreased slope of the dose-response curve of the test limb and of identical thresholds to noradrenaline in the two limbs are what would be expected if the wall/lumen ratio of the resistance vessels in the test limb had been reduced, in this case as a result of some weeks of arterial hypotension with consequent relaxation of the regional vessels.

From a theoretical point of view there are alternative explanations (p. 19) It seems unlikely however that the decreased slope of the dose-response curve in the test limb should be due to a reduced ability of morphologically unchanged vascular smooth muscles to develop contraction force. It was thus observed that the difference in slope between the test

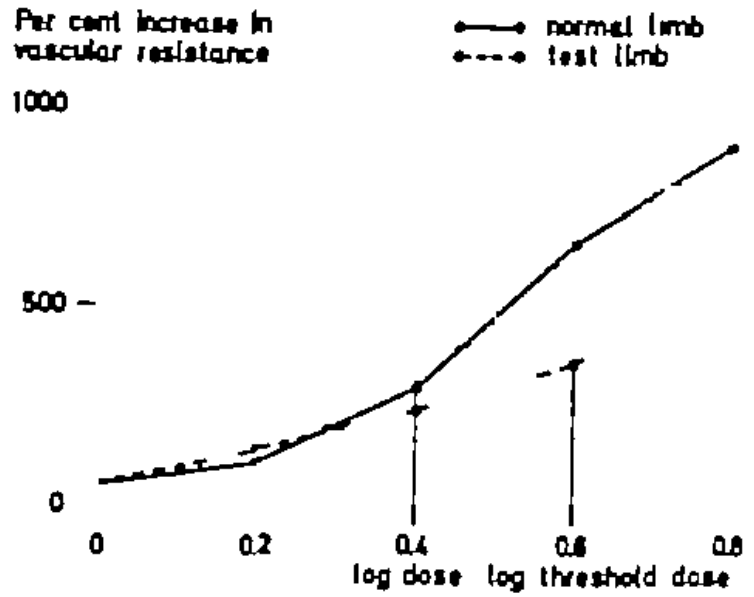


Fig. 7 Relationship (mean curves) between graded intraarterial noradrenaline infusion and percentage changes of blood flow resistance in calf muscle of cats, where the arterial blood pressure in the test limb had been kept reduced by about 40 per cent for 3—5 weeks. In order to illustrate differences in slope individual differences in sensitivity (threshold) are compensated by giving log dose minus log threshold dose on the abscissa. Note the steeper curve in the normal limb.

limb and the normal limb was about the same whether the perfusion pressure was kept high or low This indicates that there was no "overload of any weakened vascular smooth muscle cells that might lead to a difference in slope. Moreover had a reduced contractility of the smooth muscles occurred, causing them to shorten to different extents to identical noradrenaline doses or stimulation frequencies, then one might expect this difference to be largely proportional to the response to all doses or stimulation frequencies analogous with the observations reported by Cannon and Rosenblueth (1949) (see p 19) On the contrary there was a clear tendency for the difference to become more marked as the constriction became larger (Fig. 7) which suggests the influence of a difference in wall thickness. It does not seem reasonable to explain the difference in slope simply as an effect of a changed frequency distribution curve or as a changed vascular reactivity to vasodilating agents (p 19)

In five cats exposed to unilateral arterial hypotension for about five weeks corresponding sections of the femoral arteries from the two sides were compared histologically*) In order to distend the vessels to the same inner lumen the arteries from three of the cats were cannulated with

*) For these examinations dr Johan Silve-Söderberg, at the Department of Pathology in Göteborg, gave valuable advice as well as important technical assistance.

catheters of the same size and fixed in this position Special care was taken to cut the vessels at right angles to their longitudinal axis. The vessels were then photographed and the wall thickness measured (on equidistant points along the inner circumference) In two cats the surface area of the sections was calculated on photographs from undistended vessels. In all five experiments the measurements showed a decreased arterial wall mass in the vessel exposed to the reduced blood pressure compared with the normal vessel. The measurements were, however performed on comparatively large arteries and can therefore only be taken as a circumstantial evidence in favour of structural changes in the arterioles.

Human study

III. Aspects of the nature of the increased vascular resistance and reactivity to noradrenaline in hypertensive subjects

Since Paper III was published in 1968 further experimental results have accumulated and permitted additional calculations on the material These new results and calculations are therefore included in the presentation given below

It was the aim of this work to study the hemodynamic situation in a peripheral vascular bed of hypertensive patients from certain aspects, and to compare it with the corresponding situation in matched controls.

Material

Twenty seven patients, referred to the medical clinics of Sahlgren s hospital in Göteborg for examination and treatment of their hypertension have been studied. The clinical examinations included disease and family history physical examination X ray study of the heart, ophthalmoscopic examination of the eyes, ECG test for proteinuria, urine sediment, urine culture pitressin test of the renal ability of urine concentration, urography determination of vanillomandelic acid in the urine and determination of serum creatinine and serum electrolytes. Almost all patients were examined with renal angiopraphy Uric acid and renin were sometimes determined in blood as well as aldosterone in urine.

In sixteen patients twelve men and four women, no obvious cause of the high blood pressure could be found The results from these patients with essential benign hypertension will be presented together Three of them earlier received small doses of hypotensive drugs (betanidine, reserpine hydralazine or saluretics) but all these drugs were withdrawn at least 24 hours before the experiment was done

In four cases, three men and one woman, the high blood pressure was probably of renal origin and the results from them will be commented upon separately two of them had malignant hypertension and were on treatment with hypotensive drugs (reserpine+hydralazine and betanidine respectively) also during the test. Seven patients were excluded from the material for reasons given in Table I

The hypertensive patients were compared with healthy blood donors, matched for sex, age, height and weight. Mean values for these variables in the two groups are given in Table II. The examination of the controls included anamnesis, physical examination of heart and lungs, palpation of radial and brachial pulses, ECG ophthalmoscopic examination of the eyes (by ophthalmologist) determination of electrolytes in serum, protein and glucose in urine, and urine excretion of electrolytes in 24 hours. Four controls were excluded (Table III)

Methods

Hand blood flow was determined by venous occlusion plethysmography and blood pressure measured intraarterially through a catheter in the ipsilateral brachial artery (for details see Paper III) During the whole experiment the patients were exposed to intense indirect heating in order to abolish vasoconstrictor activity to the skin vessels (Pickering 1936 Prinzmetal and Wilson 1936) The heating procedure was used because vasomotor tone to the skin vessels is mainly determined by the thermobalance and therefore of little interest in this connection Furthermore flow resistance in the hand with the patient resting at room temperature is very unstable. The plethysmograph temperature was kept at 33 C. Flow resistance in the hand, measured during indirect heating and at 33 C plethysmographic temperature and expressed as PRU_{100} was called "*basal*" *resistance*

At the end of the experiment *flow resistance at "maximal" dilatation* of the hand vessels was determined by first increasing the plethysmograph temperature to 43° C, followed by 5 min of arterial occlusion with hand muscle exercise to exhaustion. Blood flow was then determined. The highest flow value was taken as maximal blood flow and from this flow value, and the corresponding mean arterial pressure, flow resistance was calculated in PRU_{100}. The quotient between basal resistance and resistance *at maximal vasodilatation is called "basal tone" as it is regarded as reflect*ing the extent of smooth muscle contraction in the hand vessels during basal conditions (*i e* when virtually all vasoconstrictor influence was abolished)

Table I Hypertensive patients excluded from the material

Initials	Reason for exclusion
A.E.	Incomplete clinical examination
V.B.	Suspected primary hyperaldosteronism
G.A.	Suspected primary hyperaldosteronism
L.E.	Too high temperature in the plethysmographs
M.L.	Diabetes and malignant hypertension
E.A.	Unstable basal flow
K.P.	High and oscillating blood pressure, probably due to insufficient baroreceptor function

Table II Anthropometric data

	Mean values		Maximal difference between a hypertensive patient and his control
	Hypertensives	Controls	
Age (years)	43	44	+ 6, −11
Height (cm)	170	172	+ 6, −9
Weight (kg)	75	75	+ 7 −8

Table III Control subjects excluded from the material

Initials	Reason for exclusion
L.O.	High blood pressure
U.A.	Brachial artery thrombosis
R.E.	Unstable basal flow
B.O.	Chronic nephritis

Noradrenaline (0.5 μg per ml in 0.9 per cent saline) was given in stepwise increasing doses through the arterial catheter mentioned above. From the results obtained in this way dose-response curves were constructed with μg noradrenaline per min and 100 ml tissue volume (hand + forearm, since the infusion was given in the brachial artery) given in a loga

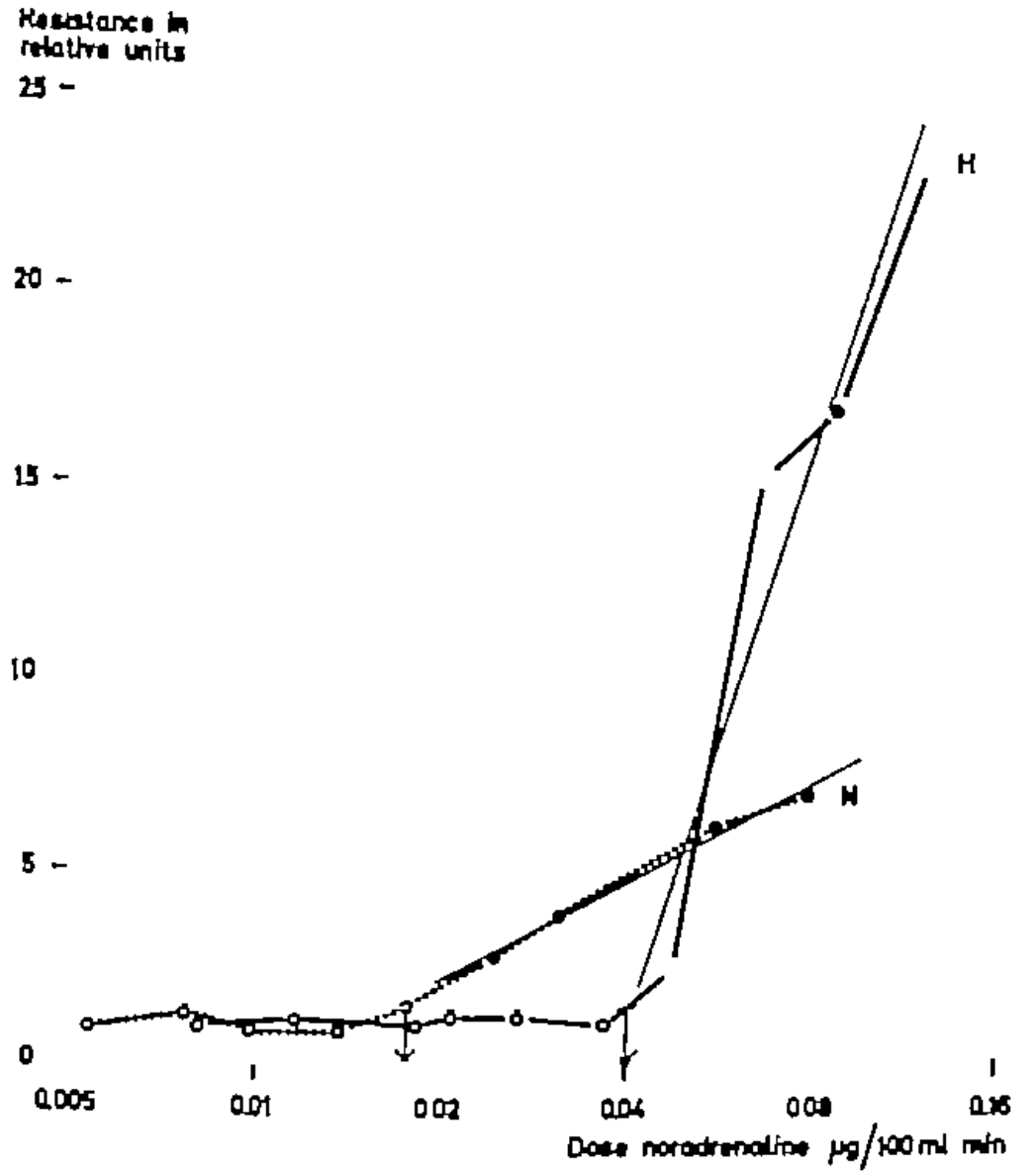


Fig. 8 Dose-response curves from one patient with essential hypertension (H) and his matched normotensive control (N). The regression lines, which characterise the slopes of the curves, are calculated according to the least squares method from the values superior to the threshold (solide circles). The threshold doses are indicated by arrows.

rithmic scale on the abscissa and the actual flow resistance in the hand divided by the basal resistance on the ordinate, since the basal resistance proved to be proportional to the resistance at maximal vasodilatation (p 15) From these curves the *threshold dose* and the *steepness* of the individual dose-response curve were determined in the following way (Fig. 8) The threshold was defined as the dose which produced a fifty per cent increase in resistance compared with the basal resistance. This dose was interpolated from the dose-response curve. The slope was calculated in two different ways. In Paper III the slope of the dose-response curve was given

as the increase in resistance from the threshold up to the resistance at a nor adrenaline dose twice as large as the threshold dose. In the calculations of the slope, presented in Table IV a straight line was adapted to the values above the threshold according to the least squares method (Fig 8) The highest resistance values, corresponding to rather small flow values, were as a rule excluded, as the methodological error in the flow determination here was comparatively large. Attempts were also made to describe the curve with other functions than the straight line, but none of these functions were substantially superior to the straight line.

In all experiments blood flow was simultaneously determined in the opposite hand in order to check that no transient activations of the vasoconstrictor fibres were disturbing the results, *e g* when noradrenaline was given Recordings, where these control measurements in the other hand revealed the presence of intermittently enhanced sympathetic activity were excluded from the subsequent calculations (Fig. 9 see below)

The hypertensive patients and their controls were compared in pairs. Student s t test and Wilcoxon s test (Documenta Geigy 1962 p 191) were used P values less than 0 05 were considered significant*)

Critical aspects of the material and methods

All the controls were blood donors There is, however no reason why they should differ in their blood pressure level and hemodynamic situation from the general population.

Eight essential hypertensives and five controls were smokers. They were not allowed to smoke before or during the test, but three hypertensives (no controls) had done so in the morning before the test. However as the test began at least two hours later there was probably no nicotine effect left at that time. It is unlikely that these differences between the two groups can affect the main conclusions of the study

The crucial question whether it is possible in a physiological study in man to reach a truly maximal vasodilatation of the hand vessels, is discussed later (p 38) as well as the concept of basal tone, as defined above

In the hypertensive subjects, as well as in the normotensive controls, basal hand blood flow was usually comparatively stable. It was, however intermittently reduced in both hands for brief periods (Fig 9) These periods of symmetric flow reduction were probably due to transient bursts of vasoconstrictor fibre activity reaching both vascular beds simultaneously and to an equal extent. The degree of stability of basal blood flow

*) The statistical evaluation of the results was supervised by fil. kand. Bo Eriksson at the Statistical Department of the University of Goteborg.

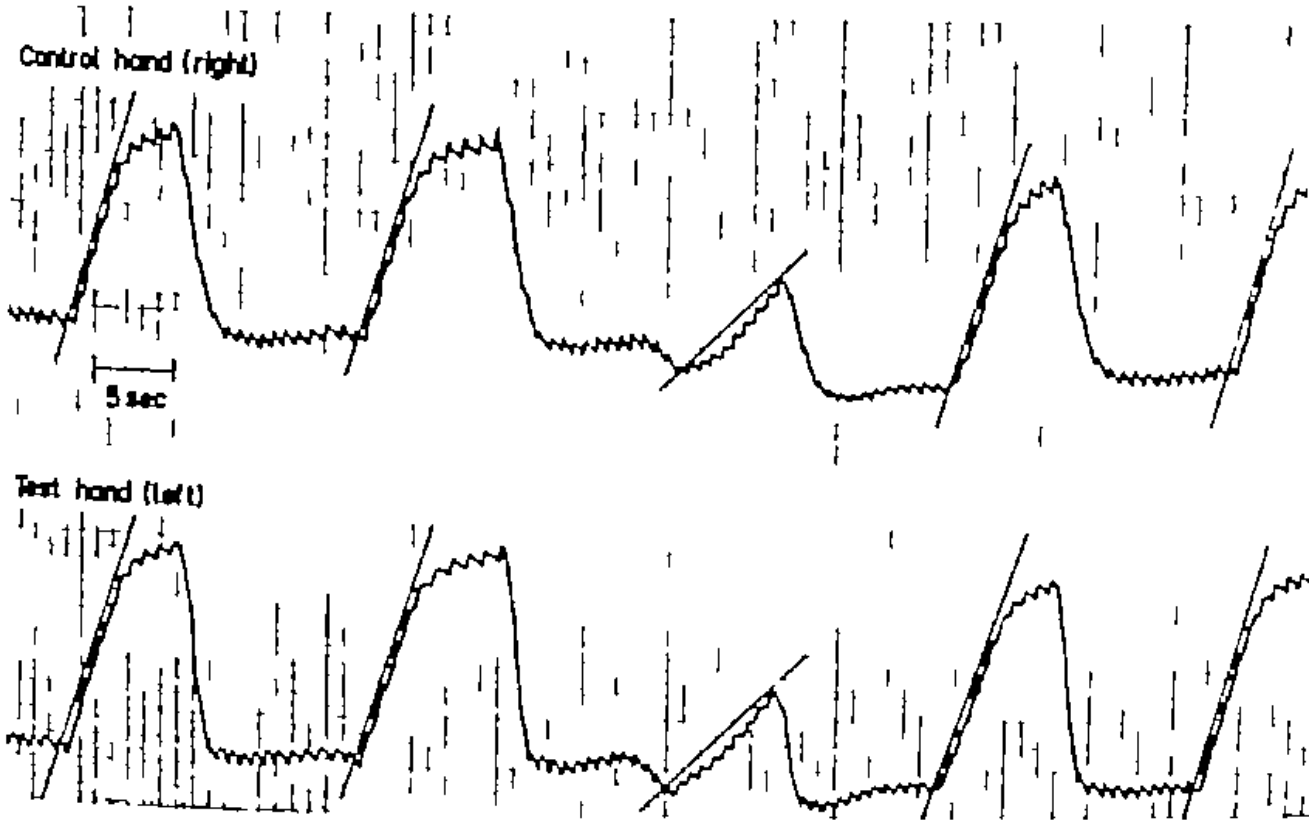


Fig. 9 Plethysmographic recordings of hand blood flow in an indirectly heated control person. Observe the episode of symmetric vasoconstriction in the two hands, probably due to bursts of vasoconstrictor activity to the hands.

varied from one patient to another However only one hypertensive patient and one control had to be excluded from the study because unstable basal blood flow (Table I and III) In order to further eliminate the effect of vasoconstrictor fibre activity such flow determinations were excluded from the calculations, where the control hand indicated the presence of sympathetic activity These exclusions were of the same frequency and changed the flow values to the same extent in the two groups.

If dose-response curves for vasoconstrictor or vasodilator drugs are to be compared in corresponding vascular beds from two subjects along the lines described in this study it is important that the vascular smooth muscles in the basal state display about the same degree of contraction (Duff *et al* 1953) For one and the same type of vessel an increase in resistance from five to ten units is probably neither equivalent to an increase from ten to fifteen nor to an increase from ten to twenty since these changes occur at different levels on the dose-response curves. As will be discussed later there was, however no significant difference in basal tone between hyper

tensives and controls in the present study and therefore the degree of muscle contraction in the basal state can be assumed to be about equal.

Another factor of importance for the interpretation of the dose-response curve is the magnitude of the hand and forearm blood flow during basal conditions. The blood concentration of the drug depends of course not only on the amount of drug injected per time unit but also upon the blood flow into which it is admixed. This is probably the reason for some correlation ($r=0.6$) in the control group between the basal hand blood flow and the threshold of the dose-response curve. Average basal hand blood flow however was equal in the hypertensive and the control groups. Furthermore, when the threshold doses were related to the individual basal hand blood flow values, the slight difference in mean threshold doses between hypertensives and normotensives decreased. The noradrenaline was, however diluted in the total flow passing the brachial artery that is, hand and forearm blood flow together Forearm blood flow was not recorded in the present experiments. The values reported in the literature, for resting forearm blood flow in hypertensives and healthy controls, differ somewhat from one author to another but are as a rule slightly higher in the hypertensive than in the normotensive groups. Therefore, when total blood flow in hand and forearm was calculated from the present flow values for hand and from average figures for forearm blood flow found in the literature, this calculated total flow was somewhat higher in the hypertensives than in the controls, but the difference was only between 0 and 13 per cent. It is therefore probable that the blood flow in which the infused noradrenaline was diluted during basal conditions, was very nearly the same in the two groups.

Results

In Table IV it is evident that there is a large difference between the two groups in flow resistance at maximal vasodilatation a difference that was statistically highly significant. The difference between basal resistance in the two groups was also significant and of the same order of magnitude. This means that the basal tone, reflecting the degree of smooth muscle shortening from maximal dilatation to basal state, was equal in the two groups. The threshold dose showed a slight tendency to be smaller in the hypertensive group than in the control group but the difference was not significant.

In view of the finding, discussed above, that the threshold dose in the control group was correlated to the basal blood flow the individual threshold doses were divided by the corresponding basal hand blood flow

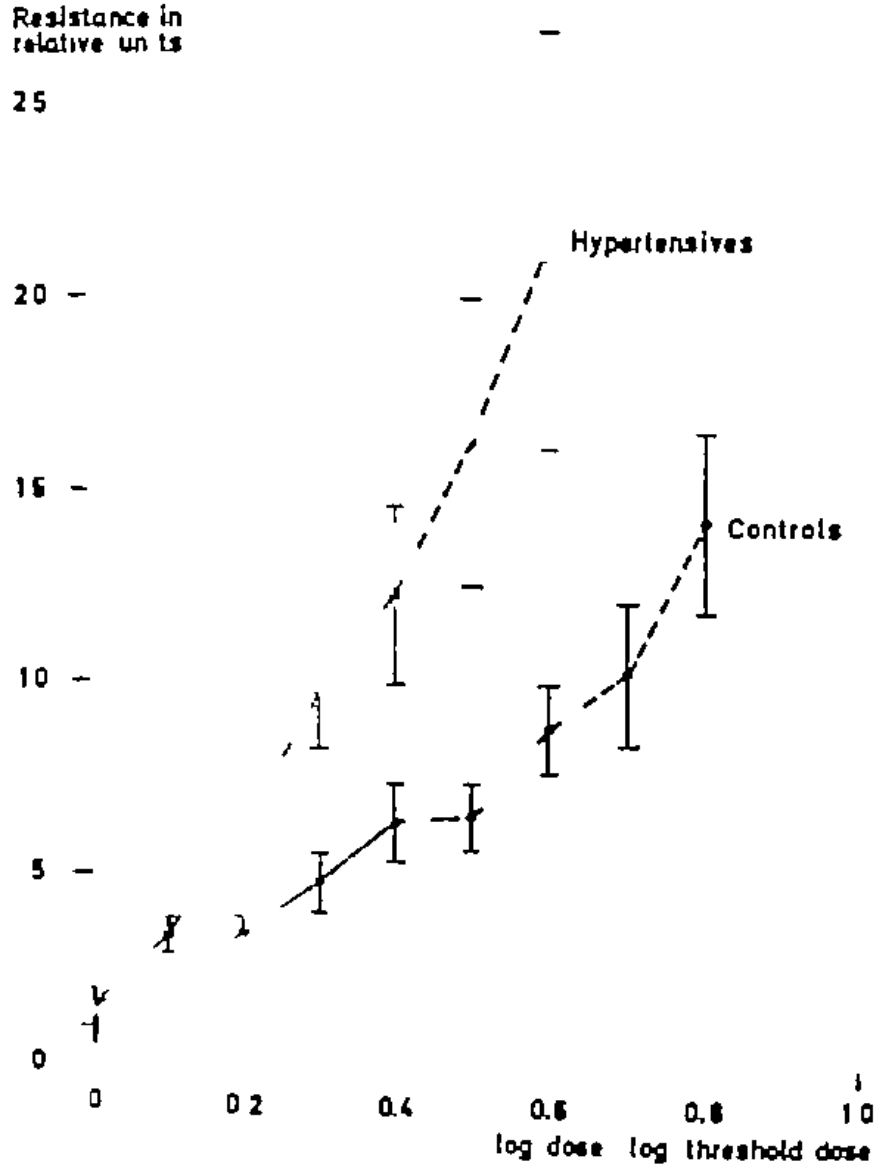
values. The figure obtained in this way can be regarded as a closer estimation of the lowest blood concentration of noradrenaline, which in the individual case produced a barely noticeable vasoconstriction in the hand. When the threshold doses were calculated in this way the difference between hypertensives and controls was reduced from thirteen to seven per cent.

The slopes of the dose-response curves of the hypertensive group and of the control group overlapped to some extent. Some hypertensives had comparatively flat curves, some controls rather steep ones. However when the mean values for the slopes of the lines adapted to the individual dose-response curves were calculated, the value for the hypertensives was much higher than that for the controls, the difference being significant both with Student's t test and with Wilcoxon s test. Figure 10 illustrates the mean dose-response curves for hypertensives and controls, compensated for the differences in individual threshold values. Among the other differences, shown in Table IV those which were significant with Student's t-test, were also significant with Wilcoxon s test, which, unlike the t test, does not call for a normal distribution of data.

In order to test the effect of the duration of the high blood pressure and the effect of different degrees of severity of the hypertensive disease on flow resistance at maximal vasodilatation the patients were regrouped for

Table IV Hand blood flow resistance and its changes during i. a. infusion of noradrenaline in patients with benign, essential and practically untreated hypertension as compared with matched normotensive controls. For definitions and details see text.

	Normotensives n = 16	Hypertensives n = 16	Significance	
			t-test	Wilcoxon test
Mean arterial blood pressure (mm Hg)	100 % (83)	146 % (121)	$P < 0.001$	$P < 0.01$
Resistance at maximal dilatation (PRU∞)	100 % (1.6)	169 % (2.7)	$P < 0.01$	$P < 0.01$
Basal resistance (PRU∞)	100 % (3.7)	170 % (6.3)	$P < 0.05$	$P < 0.05$
Basal tone	100 % (2.3)	100 % (2.3)	$P > 0.10$	$P > 0.10$
Noradrenaline threshold dose (μg/100 ml min)	100 % (0.035)	87 % (0.030)	$P > 0.10$	$P > 0.10$
Steepness of noradrenaline dose response curve	100 % (19)	210 % (40)	$P < 0.01$	$P < 0.05$



1 10 Dose sponse curves where the individual differences in threshold value have been eliminated in order to illustrate the difference in slope between hypertensives and controls. On the abscissa is given the logarithm for the dose minus the logarithm for the threshold dose, on the ordinate the resistance as a multiple of basal resistance. Means ± SEM are given for 16 patients with benign essential hypertension and 16 matched controls (the solid lines). The dashed lines represent reduced numbers of persons, between 9 and 15. For details see text.

comparison in two different ways. Firstly they were divided into three subgroups in group 1 the high blood pressure had been known for less than 2 months in group 2 for more than 2 months but less than 2 years, and in group 3 for more than 2 years. Secondly the patients were grouped in stages according to the recommendations of a WHO expert committee (1962) Table V gives the blood flow resistance in the hand at maximal dilatation for the hypertensive groups compared with their matched controls.

Table I Blood flow resistance in the hand at maximal vasodilatation in patients with benign essential hypertension as compared with matched controls. The patients were grouped in three groups according to the known duration of the high blood pressure. They were also grouped in WHO stages.

Known duration	n	Normotensives	Hypertensives
< 2 months	7	100 % (1.77)	125 % (2.20)
2 months – 2 years	2	100 % (1.30)	203 % (2.64)
> 2 years	7	100 (1.59)	195 % (3.11)
WHO stage			
I	7	100 % (1.54)	178 % (2.74)
II	7	100 % (1.80)	138 % (2.49)
III	2	100 % (1.36)	213 % (2.90)

Table II Hand blood flow resistance and its changes upon i.a. infusion of noradrenaline in patients with renal hypertension as compared with matched normotensive controls. Two patients had malignant hypertension and were treated with hypotensive drugs. For definitions and details see text.

	Normotensives n = 4	Hypertensives n = 4
Mean arterial blood pressure (mm Hg)	100 % (82)	138 % (113)
Resistance at maximal dilatation (PRU_{max})	100 % (1.8)	150 % (2.7)
Basal resistance (PRU_{ba})	100 % (3.8)	161 % (6.1)
Basal rate	100 % (2.1)	110 % (2.3)
Noradrenaline threshold dose (μg/100 ml min)	100 % (0.032)	128 % (0.041)
Steepness of noradrenaline dose-response curve	100 % (4.4)	150 % (6.6)

The subgroups are too small to permit valid statistical analyses, and the durations are, of course, not entirely safeguarded in this way but there is a tendency towards increasing flow resistance at maximal dilatation with increasing known duration of the high blood pressure. It is also interesting to note the comparatively high flow resistance in the group with the mildest disease and even in the group with a known duration of less than 2 months.

The results from four cases with renal hypertension and their matched controls are given in Table VI Two of these patients had malignant hypertension and were treated with hypotensive drugs.

This material is too divergent and too small to permit safe conclusions, but there is a tendency here too towards an increased basal resistance as well as resistance at maximal dilatation in the hypertensive group It should be observed that in Table VI the slope is calculated in a somewhat different way from that used in Table IV (*i. e* in the same way as was done in Paper III see the present text p 31) and therefore the absolute figures for steepness are not directly comparable in the two tables.

Discussion

Flow resistance at maximal dilatation

The findings in this study dealing with the hand vessels, strongly indicate the presence of structural changes in the resistance vessels as the cause of the higher resistance at maximal dilatation in patients with benign essential hypertension. Studies by Folkow Grimby and Thulesius (1958) and by Conway (1963) indicate that the same is true also for the forearm vessels. Data given by Pickering (1936) Prinzmetal and Wilson (1936) and Stead and Kunkel (1940) on forearm blood flow can also be taken to support this opinion though the interpretation given by these authors at that time was different. Results concerning the cerebral blood flow (Stead and Kunkel 1940 and Novack *et al* 1953) point in the same direction (see Table VII)

A possible objection to this opinion would be that in the present study the vascular muscles might after all not have been completely relaxed at maximal dilatation and, if so the remaining tone might be different in the two groups. Such a tone could then hardly be explained by an increased vasomotor nerve activity as all the experiments were performed during indirect heating which is known to produce an almost complete inhibition of the activity in these nerves in both normotensive and hypertensive subjects (Pickering 1936 and Prinzmetal and Wilson 1936)

As for the idea of a circulating vasoconstricting substance as the cause of a residual tone it must be stressed that such a substance would then have to be quite unique in its ability to withstand the measures in the experimental procedure for establishing maximal dilatation. Thus, model experiments in two control subjects (Fig 11) showed that this procedure completely eliminated the effects of noradrenaline, when given intraarterially even in amounts two to three times as large as those which during basal conditions increased resistance more than 200 per cent.

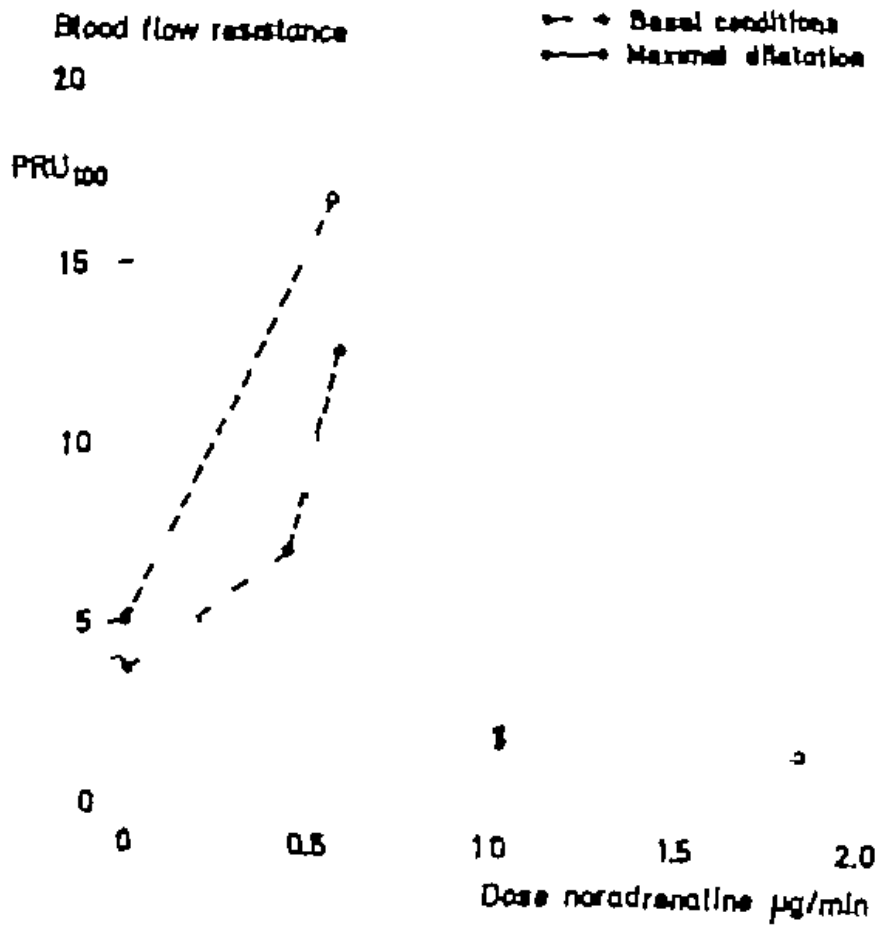


Fig 11 Effect of noradrenaline, given intraarterially on hand blood flow resistance during basal conditions and maximal dilatation in two healthy controls.

Likewise model experiments with angiotensin exclude this substance as a possible cause of the increased resistance at maximal dilatation since its effect is abolished by indirect heating alone. Fig. 12 shows one of two similar experiments, in which angiotensin was given intravenously to a healthy subject in amounts which increased systemic mean blood pressure about 30 per cent. When the subject was in "normal" thermoequilibrium at room temperature hand blood flow resistance increased about 100 per cent when angiotensin was given but when the same, and even the double, amount was given with the subject indirectely heated, hand blood flow resistance remained unchanged despite a 30 per cent increase in mean arterial pressure. The effect of angiotensin during ordinary thermal equilibrium is in agreement with results of Brod *et al* (1969) who observed that angiotensin given intravenously to resting patients at room temperature reduced skin blood flow despite a rise in blood pressure.

One explanation of the present findings on the indirectly heated patients may be that a great part of the angiotensin effect on skin vessels is medi-

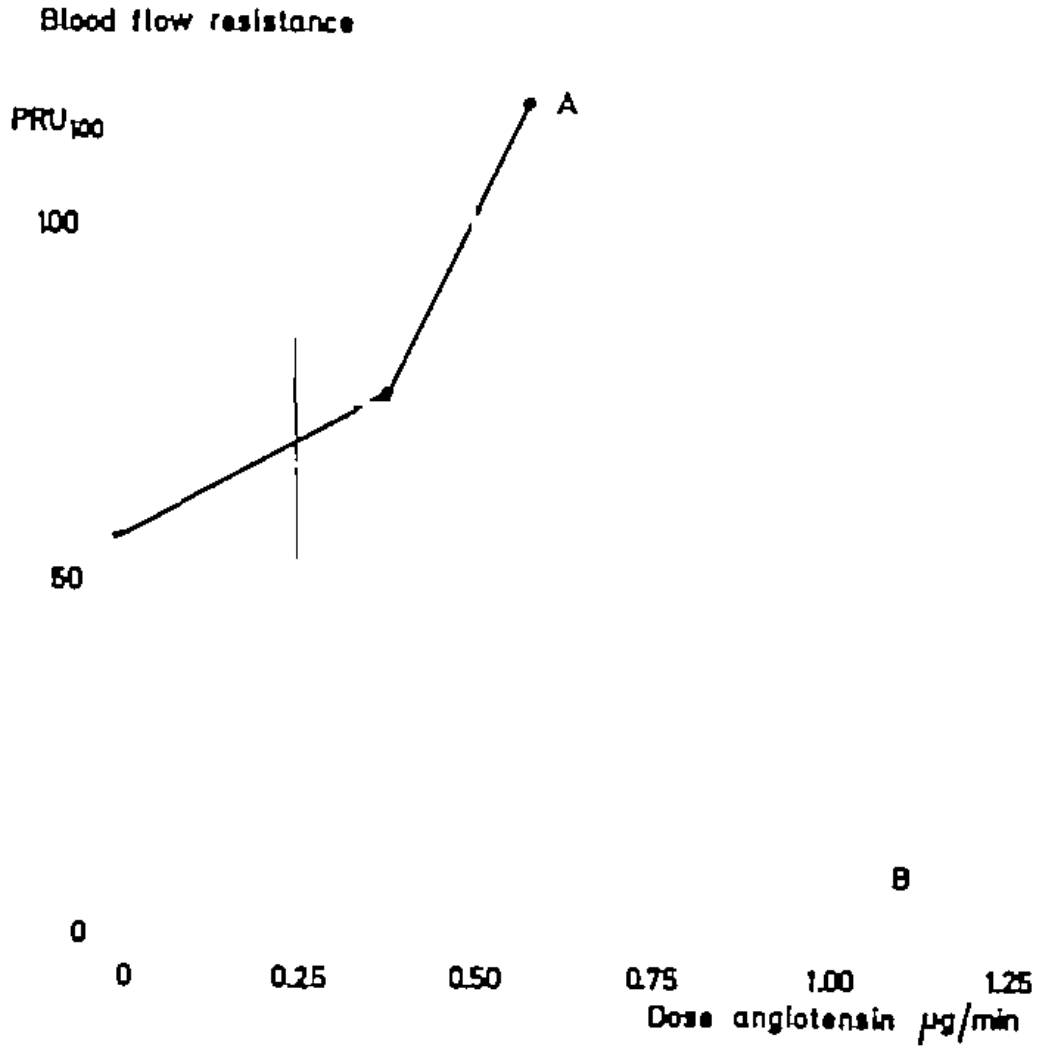


Fig. 12. Effect on hand blood flow resistance of angiotensin given intravenously to healthy person, resting at room temperature (A) and indirectly heated (B).

ated by the vasoconstrictor fibres, the activity of which is inhibited durir indirect heating. Johnsson, Henning and Åblad (1965) could block major part of the angiotensin effect on the hand blood vessels by pher benzamine and guanethidine. Therefore a neurogenic increase of tone, as caused by the possible presence of increased amounts of sin, could not be determined in the present experiments, since the genic influence was eliminated by the indirect heating. However follows that such a factor cannot be the cause of the increased f stance at maximal vasodilatation in the hand found in the pres ments.

Even if the extensive accumulation of vasodilator influen produce maximal dilatation were unable to fully block the circulating vasoconstrictor substance, this substance would be

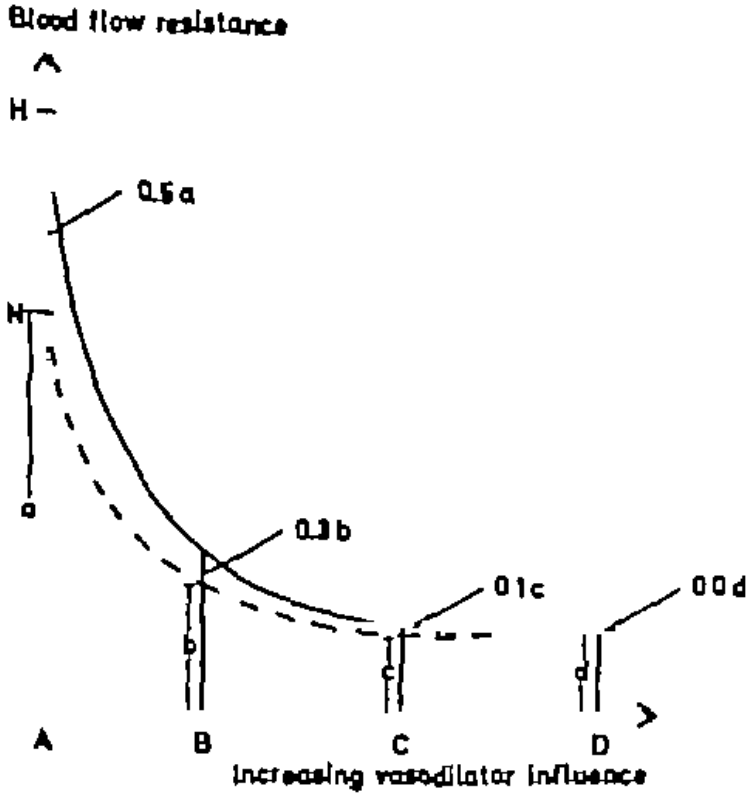


Fig. 13 Hypothetical curves illustrating the effect of increasing vasodilator influence on normal vessel (N) and on another vessel (H) which is exposed to constant vasoconstrictor activity e.g. a circulating pressor substance. At A (basal condition) resistance is supposed to be 50 per cent higher in H than in N, but at maximal dilatation D resistance is equal in the two beds. During submaximal dilatation the percentual increase in resistance produced by the vasoconstrictor acid Iry in H must be somewhere between 0 and 50 per cent. This means that even if the procedure for maximal dilatation produces only submaximal vasodilatation, basal tone, calculated as defined in the text, will still be higher in the vascular bed exposed to vasoconstrictor influence than in the other one, see the table below

	Basal tone	
	N	H
D (maximal dilatation)	a/d	1.5a/d
C (submaximal dilatation)	a/c	1.5a/1.1
B (" ")	a/b	1.5a/1.3b

exert greater vasoconstrictor effects during basal conditions than during dilatation (Fig 13) thus producing a high "basal tone" Basal tone was, however found to be equal in the two groups.

Reports in the literature support the opinion that the procedure used in the present study to relax the vascular smooth muscles is very effective Krog *et al.* (1960) used a similar method to dilate the hand vessels maximally They had the same high plethysmograph temperature and increased the length of the ischemic period in a stepwise fashion until blood flow fell

not increase any further This means that the maximal dilator response had been reached by way of the naturally occuring vasodilator metabolites which are known to be very effective. The authors found maximal blood flows of the same magnitude in healthy persons as was found in the present study *i e* about 60 ml per minute and 100 ml tissue. It is not possible to reach the same high flow levels by pharmacological means. The highest flow values reported by Duff *et al* (1953) with intraarterial administration of high doses of acetylcholine and histamine was 15—30 and 20—40 ml per min and 100 ml tissue, respectively High doses of adenosine triphosphate increased hand blood flow maximally to between 25 and 55 ml per min and 100 ml tissue (Duff Patterson and Shepherd 1954)

An increased blood viscosity in hypertensive patients is a theoretically possible explanation of the increased flow resistance during basal conditions as well as during maximal vasodilatation in this group of patients. However most authorities seem to agree that blood viscosity is largely normal in well-established hypertension (Pickering 1968 pp 179—180) Tibblin *et al* (1966) observed in a small number of hypertensive subjects a 20—25 per cent increase of blood viscosity *in vitro* which seems somewhat surprising, since the difference in both plasma viscosity and hematocrit were only minor Recent comparisons of blood viscosity *in vivo* and *in vitro* indicate, however that *in vitro* values are much higher and also exhibit much larger spread than *in vivo* values (Djojosugito *et al* 1970) For such reasons it is difficult at present to evaluate the true relevance for the *in vivo* situation of a small or moderate difference in blood viscosity as measured *in vitro* Whatever the case, an increase of blood viscosity would have to be in the order of 60 per cent to explain the whole increase in resistance at maximal dilatation and would not explain the increased vascular reactivity observed (see below)

As pointed out on page 18 many different types of structural change in the vascular bed may explain an increased resistance at maximal vasodilatation. However an increased wall/lumen ratio is the only single change which can also explain the increased slope of the dose-response curve

The fact that the blood vessels could be dilated also in hypertensive patients (Prinzmetal and Wilson 1936, Goldring *et al* 1941) may be an argument against a quantitatively important sclerosis of the resistance vessels, leading to a rigidity of their walls, but it is no argument against other types of morphological changes of the vessels. In fact, an increased wall/lumen ratio due to vascular smooth muscle hypertrophy and/or an increase of other normal wall tissue elements inside the vascular smooth muscle sheath, will increase vascular reactivity rather than decrease it (Folkow

1956 Folkow Grimby and Thulesius 1958 Paper I and Paper II) The claim that histological changes cannot be seen in all vascular regions and not in all patients with hypertension (Pickering 1936 Moritz and Oldt 1937 Duff 1957) is likewise no valid argument, because most methods for demonstration of a true wall/lumen change are rough and inadequate to estimate minor changes. It should be stressed that even small shifts of the inner radius of the vessel have large effect on flow resistance, as this is inversely proportional to the fourth power of the radius. Furthermore most morphological studies of the vessels in hypertensive vascular disease have dealt with qualitative changes in the vascular wall instead of quantitative changes in the normal constituents of the vascular wall, which are much more difficult to evaluate. The absence of an increased vascular reactivity in Raynaud's disease is, of course, no argument against structural changes of major hemodynamic importance in hypertensive patients, as Mendlowitz *et al* have claimed (1962) since the nature of the vascular changes present Raynaud's disease may differ fundamentally from that in hypertension.

Basal vascular tone

The finding of a largely normal basal tone in patients with benign essential hypertension indicates that significantly increased amounts of direct acting circulating constrictor agents, or other factors causing vascular smooth muscle excitation, cannot be present during the prevailing experimental conditions. They would then have elicited an increased basal tone, even if the dilatation were not truly maximal (p. 40 Fig. 13) This does, of course, not exclude the possibility that in other situations such factors may be involved, being then superimposed on the structurally based increase of flow resistance. Thus, in hypertensive patients during daily life, there might occur at least intermittently an increased vasomotor fibre activity which in this study was largely inhibited by the indirect heating (p. 29)

As Gaskell and Diosy (1959) pointed out, the increase in critical closing and critical opening pressures in the fingers of hypertensive patients after sympathetic nerve blockade (Yamada and Burton 1954 Gaskell and Kruman 1958 Gaskell and Diosy 1959) does not necessarily imply an increased activity level of the individual smooth muscle cells. It may as well be due to some kind of wall thickening, *e g* edema, or smooth muscle hypertrophy in the arteriolar wall.

The concept of an increased smooth muscle tone, as caused *e g* by circulating pressor substances, has been widely accepted as the main hemodynamic factor behind the increased blood pressure in essential hyperten-

sion although no one has definitely proved the existence of such an increased tone. Such a proof would call for a determination of flow resistance both at maximal dilatation and at rest relating these to each other as proposed by Folkow in 1956 Quite contrary to the widely accepted but unproved concept of an increased smooth muscle tone in hypertension many data given in the literature, when recalculated from this point of view indicate that vascular tone at rest is normal in hypertension and that in the forearm it may even be lower in hypertensive patients than in normal subjects (Table VII) One would expect the quotient between the flow resistance at rest and during maximal dilatation to be higher in the hypertensives than in the controls in case smooth muscle tone had really been increased, even if the vasodilatation were submaximal (Fig 13)

Threshold to noradrenaline

The threshold dose of noradrenaline was not significantly different in the two groups, even though it tended to be slightly smaller in the hypertensive group However because the threshold dose was defined as the dose which gave a significant vasoconstriction (50 % increase of resistance) the tendency to a lower threshold in the hypertensive group might simply be due to the steeper dose-response curve in this group Further after compensation for the individual differences in basal flow the slight difference in mean threshold values for the two groups was almost abolished. Thus, there was no evidence of any increased sensitivity of the vascular smooth muscles to noradrenaline in patients with essential hypertension.

The normal noradrenaline threshold in the hypertensive group is hardly compatible with the hypothesis that the fundamental fault in essential hypertension should be a more or less blocked noradrenaline pump reuptake in the nerve endings. Such a block would probably decrease the noradrenaline threshold (e g Svedmyr 1968)

Slope of dose response curve for noradrenaline

The increased slope of the dose-response curve in the hypertensive group is in good agreement with what one would expect to find if the wall/lumen ratio in the resistance vessels were increased, whether this were due to smooth muscle hypertrophy or to hypertrophy of other wall elements situated inside the smooth muscle sheath A possible "water logging" of the arteriolar walls would have the same effect (Tobian and Binion 1952) but there is little evidence of this type of change in essential hypertension. However there may be other explanations also as discussed on page 19 but as mentioned earlier these explanations are for various reasons unlikely As

Table VII Data taken from the literature and recalculated according the principles adopted in the present study Resistance during — often *submaximal) — vasodilatation (C) and at rest (B) is regularly higher in the hypertensive than in the normotensive groups. Vascular tone t rest* (B/C), which is calculated in an analogous manner to basal tone in the present study is usually of the same order in the two groups. (E = essential hypertension; R = enal hypertension; A = primary hyperaldosteronism; Mal. = malignant hypertension, Mixed = mixed group).

Reference	Organ studied	Procedure for dilatation	Normotensives					Hypertensives				
			n	Mean blood pressure at rest A	Resistance: Rest B	Resistance: Vasodilatation C	Vascular "tone" at rest B/C	Type of hypertension	n	Mean blood pressure at rest A	Resistance: Rest B	Resistance: Vasodilatation C	Vascular "tone" at rest B/C
Pickering 1936	forearm	ischemia	3	96	22.8	2.6	8.8	E	4	150	40.5	3.6	11
								R	3	131	31.5	3.6	8.8
								Mal.	1	180	41 9	5.8	7.2
Prinzmetal and Wilson 1936	forearm	local heat + ischemia	14	91	67.8	3.3	21	E	14	159	125	6.4	20
								R	4	149	110	5.9	19
								Mal.	5	176	143	5.5	26
Novack *et al* 1953	brain	5 % CO_2 breathing	12	83	1.8	1.2	1.5	Mixed	6	127	2.6	1 7	1.5
Conway 1963	forearm	ischemic work	50	83	31.3	2.0	16	E (mild)	58	1.1	32.7	2.5	13.1
								E (sever)	44	137	47 4	3.7	12.8
								R	21	120	36.3	2.9	12.5
								A	8	138	39.0	2.7	14 4

to a decreased reactivity to vasodilator metabolites in hypertensive patients, there are experimental results speaking against this possibility (Abramson and Fierst 1942) The vessels of hypertensive patients dilate as readily to e g brief periods of ischemia or exercise as do the vessels of normotensive subjects.

Most previous investigators have used only a single or a few doses of constrictor drugs in testing vascular sensitivity and reactivity Usually they have not differentiated between a true increase of sensitivity i e a decreased threshold as combined with a parallel shift of the dose-response curve to the left, and an increased slope of the curve. It is then impossible to say whether the vascular smooth muscles display any increased sensitivity it can only be stated that the vessels are more reactive, but this can as mentioned, be due to very different mechanisms.

In studies by Duff (1957) Doyle, Fraser and Marshall (1959) Scroop and Whelan (1968) more complete dose-response curves were given. In Duff's experiments the hypertensives generally showed steeper dose-response curves than the normotensives, but this was apparently not the case in the experiments performed by Doyle. In both studies there was a tendency towards a decreased threshold in the hypertensive group Scroop and Whelan found no difference at all between hypertensives and controls.

Part of the explanation of the discrepancy between these and the present results may be technical. It seems, for instance, preferable to analyse separately the thresholds and slopes of the individual dose-response curves and then use these values in the calculations, instead of calculating mean curves for the hypertensive and control groups as did the authors mentioned above According to the experience gained in the present study, [illegible] differences in threshold within the groups are rather large. [illegible] where som subjects showed quite marked responses, others had [illegible] to react. In such conditions it would not be appropriate [illegible] "mean" curves according to the principles applied [illegible] Furthermore, Duff's study has been criticized because [illegible] did not correspond in age and sex to the hypertensives [illegible] performed their study on patients on current [illegible] pressure and this may of course seriously [illegible] material was furthermore small, and it also [illegible] sex to the hypertensives.

Another question, which has not been co[illegible] the effect of an increased basal smooth [illegible] curve. It is difficult to predict how such an [illegible] curve and if no attempt has been made to [illegible]

two groups is equal, as was done in the present experiments, it is difficult to evaluate the significance of differences in response. The similarity in basal tone found in the present study in the two groups of subjects indicates that the dose-response curves were performed from about equal initial states of vascular smooth muscle activity. This fact greatly facilitates the interpretation of the results.

Conclusions.

The present finding of an increased resistance at maximal dilatation combined with a normal basal tone, in agreement with previous observations by Folkow, Grimby and Thulesius (1958) and by Conway (1963) strongly suggests the presence of hemodynamically important morphological changes in the resistance vessels of patients with essential hypertension. The observation of an increased slope of the dose-response curve combined with an essentially unchanged threshold to noradrenaline also lends support to this idea. When taken together these findings indicate *the presence of a wall thickening in the resistance vessels of such a nature that it encroaches upon the lumen even at maximal dilatation and causes an increased wall/lumen ratio*.

Such a structural change, which may be a simple expression of the normal tendency of tissues to respond with adaptive hypertrophy to increase load (see Paper II), will freely allow for a completely normal, perhaps even enhanced, range of vasodilatation — vasoconstriction but from a "raised baseline". If generalized the change may in fact account for the major part of the rise of resistance in the resting equilibrium of well-established essential hypertension. None of the alternative factors discussed above can alone explain *all* the experimental findings. Moreover several of them are highly hypothetical or even unlikely, while the presence of "hypertrophic" changes in arterial-arteriolar walls can hardly be doubted. It is striking however that the important hemodynamic consequences of such changes have been largely overlooked, until attention was drawn to the fact that both the resistance at maximal dilatation and the entire range of vascular adjustments will be "reset to a higher level" by such changes and that hypertensive subjects indeed display a raised flow resistance also at maximal dilatation (Folkow 1956, Folkow, Grimby and Thulesius 1958, Conway 1963).

SOME ASPECTS OF THE PATHOGENESIS OF VASCULAR CHANGES IN HYPERTENSION

In essential hypertension many causative factors have been discussed, but up to now no single cause of the elevated blood pressure has been definitely demonstrated It is generally agreed upon that this, by far the most common type of high blood pressure, has a distinct genetic element (see Pickering 1968 p 236) Even if the genetic element is one factor it is likely that several factors are involved to establish this disease, and the pressure level *no doubt is the resultant of a great number of influences* (Page and McCubbin 1965) Two factors, which may be of particular importance for the pathogenesis of essential hypertension, will be discussed below

The cardiovascular system in healthy persons as well as in hypertensive patients, is normally exposed to a sympathetic nervous tone, which varies from one moment to another For many reasons, particularly because of the impact of psycho-emotional factors, initiated at cortico-hypothalamic levels, large variations in blood pressure also occur in subjects with normal blood pressure as a normal effect of the "stress of daily life" (Hinman Engel and Bickford 1962, Pickering 1968 p 53 Bevan, Honour and Stott 1969)

It is possible that the vascular changes in essential hypertension are caused by an intermittently increased nervous drive on the cardiovascular system, as a result of a more or less stressful environment It is also possible that there exists a hereditary tendency of the highest autonomic centres to respond in an especially marked manner in terms of efferent discharge, to *per se* ordinary stress situations of daily life (Hines and Brown 1933) If the response to stressful situations are mimicked in rats by weak but often repeated electric hypothalamic stimulations in the defence area, this will gradually lead to hypertension which remains during periods when no stimulations are performed (Folkow and Rubinstein 1966) Furthermore, if the living conditions for mice are so arranged as to lead to repeated stressful situations, this produces high blood pressure, which persists for at least a month after the stimulus is eliminated (Henry Meehan and Stephens 1967) There is no contradiction between such a view concerning the role of the autonomic nervous system and the observations by *e g* Prinzmetal and

Wilson (1936) and Pickering (1936) and others, who found that the el v tion in peripheral resistance in resting subjects persisted after sympath nervous blockade and therefore concluded that the high flow resistance this situation could not be explained by an increased vasomotor activit Naturally such a measurement does not take into account the possibility o an intermittently increased vasomotor activity during daily life. Such factor may well constitute the trigger mechanism for the gradual development of other changes, particularly structural adaptations of the resistance vessels in the direction of an increased wall/lumen ratio

Another possibility has been stressed by Folkow (Folkow 1956 1961 Folkow Grimby and Thulesius 1958) Suppose there is a genetically bound disposition in patients with essential high blood pressure to develop vascular smooth muscle hypertrophy and other adaptive structural changes in the resistance vessels more readily than "normal" persons. Such a disposition might be one of the expressions of an increased "mesenchymal endowment" (Backer 1956) which in other respects seems to reveal itself in subjects with essential hypertension as a 'mesomorphic' body build (Robinson and Brucer 1940) If so adaptive vascular changes might develop as a response to vasomotor and other stimuli on the cardiovascular system, which are *per se* fully normal in both magnitude and duration. Furthermore, in hemodynamic studies in rats with genetically based high blood pressure (Folkow *et al.* 1970 a, b) the same type of changes were found to be generalized to the entire systemic vascular bed as those that have been observed in the present study on the hand of patients with essential hypertension. Thus, these rats with "primary" hypertension display an increased resistance at maximal vasodilatation, a normal threshold to noradrenaline, an increased slope of the dose-response curve and an increased maximal contractile strength of the resistance vessels. All these findings are in complete accord with the hemodynamic consequences of an increased wall/lumen ratio in the resistance vessels and such a change seems to be the only single factor which can explain them If this hypothesis were correct, *i. e.* if the increased tendency to exhibit morphological vascular changes would constitute the genetic element, then such changes should be looked upon not just as a consequence of the rise in blood pressure but as the *primary* factor in the pathogenesis of essential hypertension.

GENERAL SUMMARY AND CONCLUSIONS

The study has been concerned with the hemodynamic importance of structural vascular changes in essential hypertension particularly the hemodynamic effects of an increased wall/lumen ratio in the resistance vessels.

In Paper I two kinds of vessels in the cat's hind paw were compared cutaneous A—V shunts which have thick walls and a high wall/lumen ratio and "nutritional" resistance vessels, with relatively thinner walls. Blood flow resistance was measured during stepwise increases in dosage of noradrenaline, infused intraarterially or intravenously and stepwise increasing frequencies of vasomotor nerve stimulation. When dose-response curves were calculated there was found to be no difference in threshold indicating an equal noradrenaline sensitivity of the smooth muscles in the two types of vessels, but once the threshold was surpassed the vascular bed rich in thermoregulatory A—V shunts exhibited a much steeper curve than the bed mainly consisting of "nutritional" resistance vessels. An increased slope of the dose-response curve must be expected from a theoretical point of view in vessels like A—V shunts as a consequence of the high wall/lumen ratio

In Paper II the rebuilding of a vascular bed due to a changed intravascular pressure was studied. The arterial inflow to one hind limb the test limb of the cat was reduced some 30—40 per cent by arterial ligatures. After 3—5 weeks the test limb was compared with the "normal limb at the same perfusion pressure and otherwise identical conditions. As in Paper I blood flow resistance was measured during graded vasomotor nerve stimulation and graded intraarterial noradrenaline infusion and the thresholds were found to be the same in the two legs, indicating an identical smooth muscle sensitivity to noradrenaline However for doses exceeding the treshold, the response was significantly smaller in the test limb implying a decreased slope of the dose-response curve in the leg which had been exposed to hypotension These findings suggest that a hemodynamically important structural change with a decreased wall/lumen ratio in the resistance vessels takes place already within 3—5 weeks in a vascular bed exposed to a moderate hypotension These structural vascular changes, which go in the opposite direction to those seen in hypertension, may be considered as an inactivity atrophy consequent to the compensatory de-

crease in smooth muscle tone in the resistance vessels [illegible] d perfusion pressure.

In Paper III sixteen patients with benign essential [illegible] re compared with sixteen matched controls (sex, age, hei[illegible] d flow resistance in the hand was determined in vari[illegible] plethysmography and intraarterial blood pressure mea[illegible] *mal vasodilatation resistance* was increased in the hyp[illegible] om pared with the controls in the same proportion as the [illegible] that is about 50 per cent. This finding, which strongly [illegible] tural changes in the vascular bed, implies that, for identical [illegible] nooth muscle shortening, flow resistance will always be about [illegible] gher in the hypertensive group. Resistance during indirect hea[illegible] d *basal resistance* was also about 50 per cent higher in the hype[illegible] than in the controls. This means that *basal tone* which was defin[illegible] mal resistance divided by the resistance at maximal dilatation [illegible] which was thought to reflect the degree of smooth muscle shortening [illegible] maximal vasodilatation to the basal state, was the same in the two group[illegible]

This finding is contrary to the common opinion that blood flow resistance in essential hypertension is increased because of an increased smooth muscle activity. However as basal resistance was recorded during indirect heating, which implies almost complete vasomotor blockade, basal tone has no bearing on the question of an increased vasomotor tone to the vessels, produced e. g. by angiotensin.

Blood flow resistance was also measured during graded infusions of noradrenaline intraarterially and dose response curves were plotted. The *smooth muscle sensitivity* in the resistance vessels, as expressed by the *threshold* was equal in the two groups, but the *slope of the dose response curve* was considerably increased in the hypertensive patients, and this fact may explain the increased vascular reactivity observed in patients with high blood pressure.

The increased slope of the dose response curve and the increased resistance at maximal dilatation, in combination with the normal basal tone and normal noradrenaline threshold, strongly indicate hemodynamically important structural changes of a nature which increases vascular wall thickness and partly encroaches on the lumen at maximal dilatation. If other vascular beds are structurally changed in the same degree as the hand vessels, these morphological changes may almost completely explain the increase in peripheral resistance observed in cases of established essential hypertension.

Such changes can not be considered as late complications to the high

blood pressure, as they are also seen in patients with high blood pressure known for less than two months and in patients with mild degrees of high blood pressure, belonging to WHO stage I The animal studies discussed in Paper II also indicate that adaptive structural changes in the vessels take place very quickly

It is probable that any situation where vascular smooth muscle activity is increased, with or without blood pressure elevation, can cause adaptive structural changes in the vessels. In such a case a primary blood pressure elevation may become permanent and potentiated due to structural changes. An alternative hypothesis concerning the pathogenesis of the structural vascular changes in essential hypertension is that the basic fault in this disease would be a hereditary increased tendency to smooth muscle hyper trophy as one expression of a mesomorphic" body build (Folkow 1956)

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ACTA PHYSIOLOGICA SCANDINAVICA

Supplementum 344

FUNCTION OF THE LEFT ATRIAL RECEPTORS

BY

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Helsinki, April 1970

Martti Hakumäki

CONTENTS

	Page
INTRODUCTION	7
SURVEY OF LITERATURE	8
EXPERIMENTAL PROCEDURES	10
Material and experimental conditions	10
Left atrial and aortic pressure and ascending aorta flow measurements	11
Calculation of the pressure impulses of the v-wave of the left atrial pressure curve and the left ventricular relative stroke volume	13
Nerve preparation and recording of the action potentials	14
Determination of left atrial vagal afferent B- and A type impulses and sympathetic efferent postganglionic impulses	15
CALCULATION OF THE RESULTS OF NEURAL ACTIVITY	16
Calculation of the difference in the sums of B- and A type impulses during *sympathetic silence*	16
Calculation of the ratio of B- and A type impulses	16
STATISTICAL METHODS	17
RESULTS	19
Left atrial B- and A-type impulses under conditions of normal and changed left atrial pressure	19
The pressure impulse and the number of left atrial B-type impulses in single and multifibre nerve preparations	19
A-type impulses under conditions of raised and lowered left atrial pressure	22
Effect of B- and A-type left atrial impulses upon the sympathetic postganglionic activity of the heart under conditions of normal and changed left atrial pressure	24
B-type impulses and the activity of sympathetic cardiac fibres	24
A-type impulses and the activity of sympathetic cardiac fibres	26
Ratio of B- and A type impulses and the number of sympathetic impulses	27
Left atrial B- and A-type impulses, and the left ventricular relative stroke volume	29
DISCUSSION	31
Experimental conditions	31
Left atrial pressure and pressure impulse	32
B-type impulses and the sympathetic activity of the heart	33
A-type impulses and the sympathetic activity of the heart	33
Theoretical background of calculation of the ratio of B- and A-type impulses nd the difference in the sums of B- and A-type impulses	34
Ratio of B- and A-type impulses	35

Page

Difference in the sums of B- and A-type impulses 36

Left atrial B- and A-type impulses and the left ventricular relative stroke volume 37

Role of baroreceptor activity 37

Bainbridge reflex 38

SUMMARY 39

REFERENCES 41

APPENDIX 45

INTRODUCTION

In study of the left atrial receptors of the heart, note has been taken of the correlation between the atrial pressure and the vagal afferent nerve impulses. It has been demonstrated that a linear correlation exists between the atrial volume and the average frequency of discharge of B-type left atrial impulses (PAINTAL 1953 a)

The relationship between the mean femoral artery pressure and the average frequency of impulses in the splanchnic nerve has been analysed quantitatively (MILLAR and BISCOE 1965) A positive inverse correlation was found between these two variables.

The dependence of the sympathetic efferentation upon direct or indirect baroreceptor stimulation has been shown by several workers (ADRIAN 1933 BRONK et al. 1936, GREEN 1959 DOWING and SIEGEL 1963) A positive inverse correlation has been found between the average frequency of impulses of the sympathetic nerves and the carotid sinus pressure (SPICKLER, KEZDI and GELLER 1965)

In the present work an attempt has been made to examine the v-wave of the left atrial pressure curve as a function of time and the corresponding number of B-type impulses (preliminary results HAKUMÄKI et al. 1968) Although the ventricular filling is only partially and to a varying extent regulated by atrial filling and contractility the sum of B- and A type impulses was determined and correlated with the relative stroke volume of the left ventricle

The neural regulation of the heart from cycle to cycle was studied by simultaneous recordings of left atrial vagal afferent and third sympathetic postganglionic efferent nerve impulses under conditions of raised (preliminary results HAKUMÄKI 1968, 1969) and lowered left atrial pressure.

SURVEY OF LITERATURE

In a study of the vagal afferent impulses from the neck of a cat, ADRIAN (1933) found that a slender nerve could easily be separated from the trunk of the vagus and that this had impulses associated with the rhythm of the heart. The impulses were increased by raising, and diminished by lowering the blood pressure The cardiac vagal afferent nerves have been studied by WHITTENRIDGE (1948) and JARISCH and ZOTTERMAN (1948), who demonstrated that these impulses had their origin in the atria and large veins. According to JARISCH and ZOTTERMAN two types of impulses existed one appeared during atrial filling, and the other during atrial contraction PAINTAL (1953 a) named these impulses B- and A types, and showed that the average frequency of discharge of the B-type impulses bears a linear correlation to the volume of haemodynamically isolated atrium. PAINTAL (1953 b) also found that the number of right atrial B-type impulses in a cardiac cycle is linearly correlated with the venous pressure when the pressure is raised with pituitrine. In his monograph, PAINTAL (1963 a, p 114) states that a positive correlation exists between the number of left atrial B-type impulses per cardiac cycle and the difference in peak pressure and threshold pressure of the atrial pressure curve

Atrial A-type impulses have been studied by DICKINSON (1950) who found a linear correlation between the "frequency of discharge per second" and the atrial pressure. KOLATAT KRAMER and MÜHL (1957) found a positive correlation between the number of A type impulses and the peak pressure during atrial contraction

The cardiac minute output and the number of B-type impulses per minute have been investigated by LANGREHR and KRAMER (1960) who did not find any distinct correlation. However they demonstrated a distinct positive correlation between the number of B-type impulses per minute and the thoracic blood volume According to LANGREHR (1960) a positive correlation exists between the stroke volume per duration of systole and the relative frequency of B-type impulses.

The sympathetic efferentation has been studied by ADRIAN, BRONK and

PHILIPS (1932) They found in cats and rabbits that the activity of cervical and abdominal sympathetic nerves underwent a change with the frequency of the heart beats or of the respiration. This finding was supported by the work of BRONK et al. (1936) who found both "tonic" and "grouped" activity in cardiac sympathetic efferent nerves. Asphyxia increased the tonic activity while hyperventilation and a rise in blood pressure reduced it. The grouped activity was similar in the right and left cardiac sympathetic nerves, and was diminished by stimulation of the aortic nerve. GREEN (1959) has also demonstrated that stimulation of the baroreceptors depressed the efferent sympathetic activity and induced bradycardia. The activity of the left inferior sympathetic cardiac nerve has been investigated in cats by DOWNING and SIEGEL (1963), who reported that the spontaneous activity in the nerve varies with the rhythm of the heart and of the respiration. Bilateral vagotomy and carotid sinus denervation were found to increase the influence of the respiration upon the sympathetic discharge. They also suggested that systemic hypoxia has little effect upon the cardiac sympathetic centre. Sympathetic cervical and splanchnic efferent activity has been studied by MILLAR and BISCOE (1965) with anaesthetized rabbits. They indicated four types of activity (1) Spontaneous activity with respiratory rhythm, inhibited partially or completely with a rise in the arterial pressure induced by intravenous adrenaline, (2) an irregular spontaneous activity without relation to respiratory or arterial blood pressure with the possibility of increasing the activity of the nerve by adrenaline injection (3) fairly regular spontaneous activity increased on a rise in arterial pressure, (4) brief high-frequency bursts of action potentials not affected by the arterial pressure. The authors also demonstrated a positive inverse correlation between the average frequency of the impulses in the splanchnic nerve and the mean femoral arterial pressure when hypotension was induced with haemorrhage.

EXPERIMENTAL PROCEDURES

Material and experimental conditions

The experimental animals were 21 adult cats, eight females and 13 males weighing between 1 9—4 8 kg. Action potentials were recorded after preparation of the nerves from the left vagus an third cardiac sympathetic nerves. Simultaneous recording of the blood pressure from the left atrium was made in all the experimental animals and the flow and pressure of the ascending aorta in four of them The combinations of different recordings are listed in Table 1 contained in the Appendix.

The cats were anaesthetized with chloralose urethane mixture (chloralose 0 09 g and urethane 0 125 g in 7 ml Ringer solution per kg body weight intramuscularly) The level of the anaesthesia was evaluated by examination of the reflexes to touch and sound When necessary and additional 3—5 ml of the mixture was administered intravenously The bladder was cannulated to prevent shivering (JEWETT 1964). The reduction in blood volume was roughly estimated, according to the excreted amounts of fluids in urine, respiration bleeding and evaporation during the surgical and preparation phase It was compensated with Ringer solution infusion into the femoral vein Wet compresses were used on and around the wounds to minimize evaporation With the animal supine the trachea was cannulated towards the lung and closed towards the larynx. Artificial respiration was given with an E & M Instrument Co Inc Physiograph at a frequency of 13—20 per minute with a positive pressure of 8—12 cm H_2O The respired gas was room air

The thorax was opened on the left side between the fourth and fifth intercostal space In those cases in which the nervous action potentials were recorded inside the thoracic cage the first four ribs were removed and the upper lobe of the left lung was resected.

The pressure measurement and injection cannulas were inserted in the left atrium through the apex of the auricular appendage so that the injection cannula was above the opening of the pressure measurement cannula In

this way an attempt was made to preclude any direct influence of the injections upon the pressure measurement. In the flow determination experiments, the ribs were intact, and the thorax was closed partially after implantation of the flow transducer (see p. 10) around the ascending aorta and the cannulas to the left atrium. In these cases, the action potentials were recorded from the left cervical vagus nerve. The thorax was half filled with body warm saline to obtain fluid between the outer wall of the ascending aorta and the flow transducer.

The acid base balance was determined in four animals by the customary Astrup technique. The results indicated a tendency towards respiratory alkalosis and metabolic acidosis. The pH values ranged from 7.20 to 7.27 and the readings for P_{CO_2} from 17.0 to 31.0 mm Hg.

The durations of the different phases of the experiments were as follows: induction of anaesthesia ½—1 hour, surgical procedures ½—1 hour, mineral oil pool formation and nerve preparation ½—1 hour. The actual recording phase lasted 1—2 hours, in accordance with the condition of the experimental animal.

The atrial B- and A type receptors (PAINTAL 1953 a) were stimulated by an injection of Ringer solution (8—15 ml with 6—15 sec) into the femoral vein or blood (3—8 ml within ½—8 sec) into the left atrium. The blood for successive injections into the left atrium was taken direct from the left atrium of the experimental animals. The reaction of the receptors was also tested by a reduction in the atrial pressure, with blood suction (5—8 ml, within 3—8 sec) from the left atrium or (15—30 ml within 10—30 sec) from the femoral vein.

The temperature of the experimental animal was tested, the rectal and mineral oil pool temperatures being taken with an electric (Ellab Copenhagen) thermometer. The temperature was regulated with a heating lamp, and held between 37—38° C.

Left atrial and aortic pressure, and ascending aorta flow measurements

The left atrial pressure was recorded through a cannula inserted into the left atrium through the apex of the auricular appendage. The aortic pressure was recorded by a needle inserted through the right carotid artery into the aorta. The cannula and needle were connected by polyethylene tubings led to two Sanborn 267 BC transducers, and on to the Sanborn 350—1100 cm carrier preamplifiers. (Fig. 1)

The aortic pressure was recorded in flow measurements only. In some experiments, the results were also stored in a Pi-6200 tape recorder and subsequently filmed. The flow was recorded with a Nortec ultrasonic Doppler

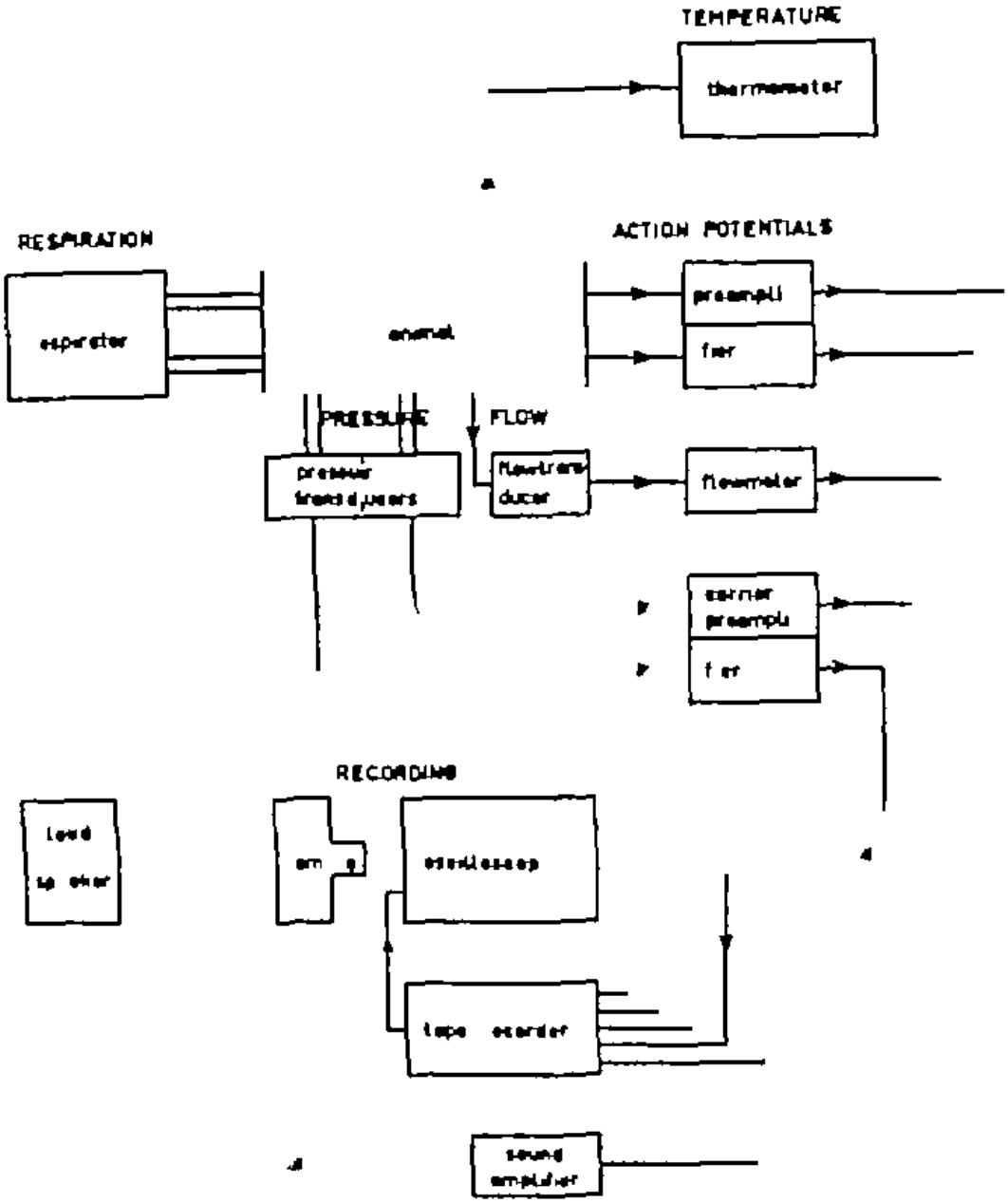


Fig 1 Recording system

shift transducer from the ascending part of the aorta The signal generator and the amplifiers for the flow transducers were made by the Technical Univeruty Applied Electronics Laboratory Bio-electronics Research Group. Otaniemi, Finland.

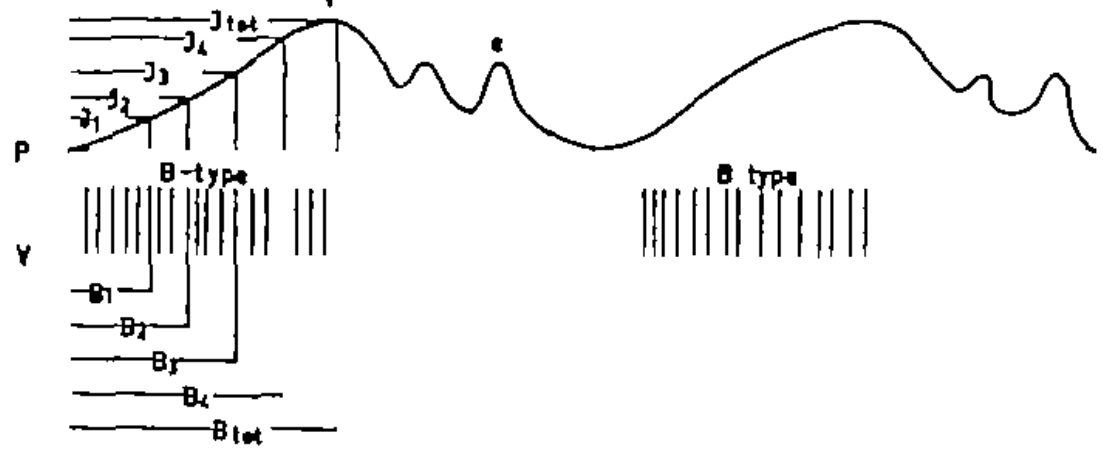


Fig. 2. P — left atrial pressure curve (with threshold pressure level — horizontal line), v a and c are the waves of the pressure curve. V — vagal afferent left atrial (B-type) impulses. I_1, I_2 I_{tot} are the pressure impulses (— the area between the threshold pressure and v-wave) and B_1 B_2 B_{tot} are the correspondi g numbers of B-type impulses.

Calculation of the pressure impulses of the v-wave of the left atrial pressure curve and the left ventricular relative stroke volume

The pressure impulse (I) of the v-wave of the left atrial pressure curve can be expressed by an equation

$$I = \int P_{v\text{-}diff} \times dt,$$

in which $P_{v\text{-}diff}$ is the pressure difference between the threshold pressure (Fig. 2 horizontal line from P) and some occasional point of the v wave, and t is the time interval between these two points.

On calculation of the pressure impulse during one atrial cycle the v-wave was divided into arbitary parts, and the corresponding areas between the horizontal line and v-wave measured (I_1 I_2 I_{tot}) In calculating the pressure impulses in different cycles, the I_{tot} was measured from different v-waves. I_{tot} was determined from the point at which the B-type impulses normally ceased at the top of the v wave.

The relative stroke volume of the left ventricle was calculated from the following equation

$$V = \int v \times dt,$$

in which V = relative stroke volume (shadowed area of Fig. 3) v = velocity of the blood stream, and t is the time interval from the 0-level (horizontal

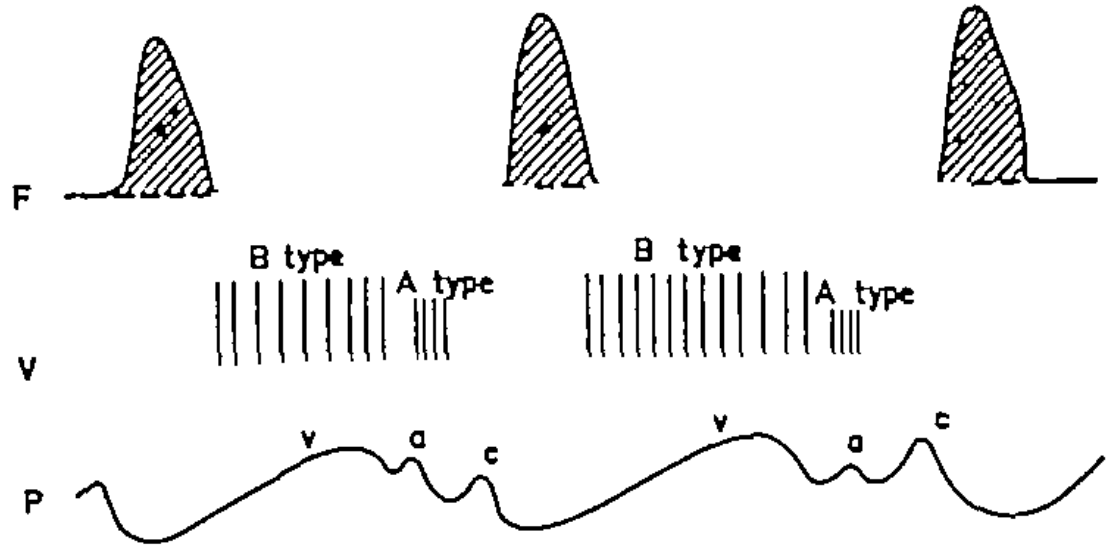


Fig. 3 F = blood velocity curve and 0-level. V = vagal afferent B- and A type impulses, P = left atrial pressure curve shadowed area = relative stroke volume.

line of F in Fig. 3) of the blood stream velocity curve, just before the ventricular contraction to the point at which the velocity curve is again at the 0-level after the contraction

The pressure impulse and the relative stroke volume were determined by a planimeter

Nerve preparation and recording of the action potentials

With the animal in a Faraday cage, preparation of the nerves was effected with a Zeiss binocular operation microscope. In the thorax, the mineral oil pool was formed of pleura parietale, and on the neck of muscles and fascias. The movement resulting from respiration and pulsation was minimized by means of bent metal plates inserted into the thoracic cage. The compression of vessels and nerves was avoided. Generally the aim was that of getting a calculable multifibre nerve preparation of the vagus, but some experiments also resulted in single fibre recordings. The action potentials were recorded monopolarly by platinum electrodes from thin vagal cardiac branches after the central connection of the fibre had been cut. Multifibre postganglionic efferent sympathetic activity was recorded from the third sympathetic cardiac nerve without cutting. The nerve impulses were led to Tektronix 122 preamplifiers, and on to a Tektronix 565 oscilloscope with six traces (Fig. 1) The action potentials were filmed from the screen with a Physiologie Milano (Arduine) camera the speed of the film was 15.2 cm per sec. Some of the results were stored on a magnetic tape and subsequently filmed

Determination of left atrial vagal afferent B- and A type impulses and sympathetic efferent postganglionic impulses

Preparation of the vagal nerves was selective, so that the recording was made only from the left atrial fibres The localization of the receptors in this phase of the experiment was determined by means of PAINTAL s (1955) identification system and with intra-atrial injections Finally localization was checked at the end of each experiment by the point of maximal impulsation to the touch of a needle All the receptors recorded were in the left atrium

The sympathetic postganglionic impulses were counted from separate sympathetic bursts, and in all the experiments under conditions of normal and raised left atrial pressure. The bursts were easily distinguishable from each other (Fig. 9 a, d, e) Some few impulses between the two bursts were counted to the nearest burst (Fig. 9 a the few impulses marked with a black wedge between the third and fourth sympathetic burst were counted to the third one) Under hypovolaemic experimental conditions in which the sympathetic activity was quite continuous the line between two bursts was determined as the point at which the activity was least (Fig. 9 b and c the vertical lines between the bursts)

All the vagal and sympathetic impulses were counted with the aid of a magnifying glass The maximal difference between the results counted independently by two persons from the films of multifibre preparation was 4 ± 1 %. No difference was observable between the results in single fibre counting.

CALCULATION OF THE RESULTS OF NEURAL ACTIVITY

Calculation of the difference in the sums of B- and A type impulses during sympathetic silence

The difference in the sums of B- and A type impulses was calculated as follows all the B-type impulses of each cycle were summed up from the artificial rise in the left atrial pressure to the cycle before the sympathetic activity recommenced (Fig. 9 d and e from point A to point B) From this sum was substracted the sum of A type impulses, counted in the same way *The control values were counted by substraction of the A type impulses* per heart cycle from the B-type impulses in the same cycle (Fig. 9 d, from A_0 to A = a heart cycle)

The time of sympathetic silence was measured from the point at which the sympathetic activity ceased to the point at which it recommenced (Fig. 9 d and e from point C to point D)

Calculation of the ratio of B- and A type impulses

The ratio of the numbers of B- and A type impulses was calculated by the following equation

$$\frac{B}{A} = \frac{B_0 + B_{R1} + B_{R2} + B_{R3}}{A_0 + A_{R1} + A_{R2} + A_{R3}}$$

in which B_0 and A_0 are the numbers of impulses counted from the film (Fig. 4) 200—400 msec before the comparable sympathetic burst (n_s).
B_{R1} B_{R2}, B_{R3}, and A_{R1} A_{R2}, A_{R3} are the values calculated from the numbers of impulses (B_1 B_2, B_3 and A_1 A_2, A_3) by the following equation

$N_R = N \cdot e^{-\frac{}{k}}$ in which N_R = B_{R1} B_{R2}, B_{R3} or A_{R1} A_{R2}, A_{R3} N = B_1 B_2, B_3 or A_1 A_2, A_3 and e is the Neper's number (e = 2.71) k is a coefficient (= regression coefficient from sympathetic silence time, differ

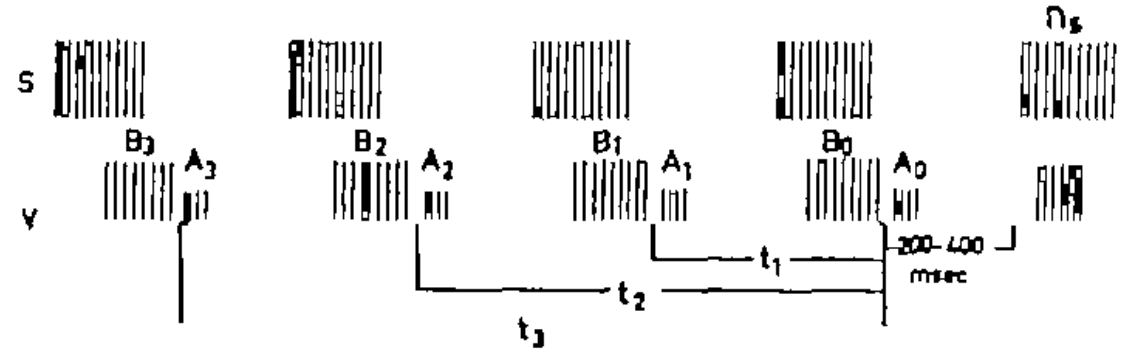


Fig 4 S = sympathetic impulses, n_s = number of sympathetic impulses of a sympathetic burst, V = vagal B- and A-type impulses, B_0 B_3 and A_0 A_3 number of B- and A-type impulses per heart cycle. t t_3 = time differences.

ence in the sums of B- and A type impulse correlation, Table 5 of the Appendix, b), t = the time difference from the corresponding cycle (B_1A_1, B_2A_2, B_3A_3) to the B_0A_0. t was determined from the P wave of the EKG which occurred as a mild artefact in each recording of action potentials (see Discussion p 34)

STATISTICAL METHODS

The mean $\bar{x}$ of observations x_1 x_2 x_n was calculated by the following formula

$$\bar{x} = \frac{\sum_{i=1} x_i}{n}$$

and variance s^2 from

$$s^2 = \frac{\sum_{i=1} (x_i - \bar{x})^2}{n - 1}$$

and the standard deviation is then s_x.

The covariance of observations x_1 x_2, x_n and y_1 y_2, y was arrived at from

$$s_{xy} = \frac{\sum_{i=1} (x_i - \bar{x})(y - \bar{y})}{n - 1}$$

The coefficient of linear correlation was calculated by the following formula

(1) $$r = \frac{s_{xy}}{\sqrt{s^2_x \; s^2_y}}$$

and the regression coefficient from

$$b = \frac{s_{xy}}{s^2_x}$$

and the point at which the regression line meets the y axis $a = \bar{y} - b\bar{x}$

The z transformation of the correlation coefficient was determined from

(2) $$z = \tfrac{1}{2}\ln \frac{1 + r}{1 - r}$$

and the mean of the transformed correlation coefficients $z_1 \; z_2, \quad z_k$ from

$$\bar{z} = \frac{\sum_{i=1}^{k} (n_i - 3) z_i}{\sum_{i=1}^{k} (n_i - 3)}$$

in which z_i were calculated by means of formulae (1) and (2) from observations $x_1 \; x_2, \quad x_{n_i}$ and $y_1 \; y_2, \quad y \quad i = 1 \; 2, \quad k.$

The variance of $\bar{z}$ is now

$$V(\bar{z}) = \frac{1}{\sum_{i=1}^{k} (n - 3)}$$

$$\text{The variable } u = \frac{\bar{z}_1 - \bar{z}_2}{\sqrt{V(\bar{z}_1) + V(\bar{z}_2)}}$$

is now approximately normally distributed It can employed for comparison of the similarity of the means of transformed correlation coefficients of two groups.

To test the significance of the difference between two means, the t test was calculated from the following formula

$$t = \frac{\bar{x}_1 - \bar{x}_2}{\sqrt{\frac{n_1 s^2_{x_1} + n_2 s^2_{x_2}}{n_1 + n_2 - 2} \left(\frac{1}{n_1} + \frac{1}{n_2}\right)}}$$

(cf HALD 1960)

RESULTS

Left atrial B- and A type impulses, under conditions of normal and changed left atrial pressure.

The pressure impulse and the number of left atrial B-type impulses in single and multifibre nerve preparations

The number of left atrial B-type impulses, and the corresponding pressure impulses, were studied in six single and 13 multifibre nerve preparations in 13 experimental animals.

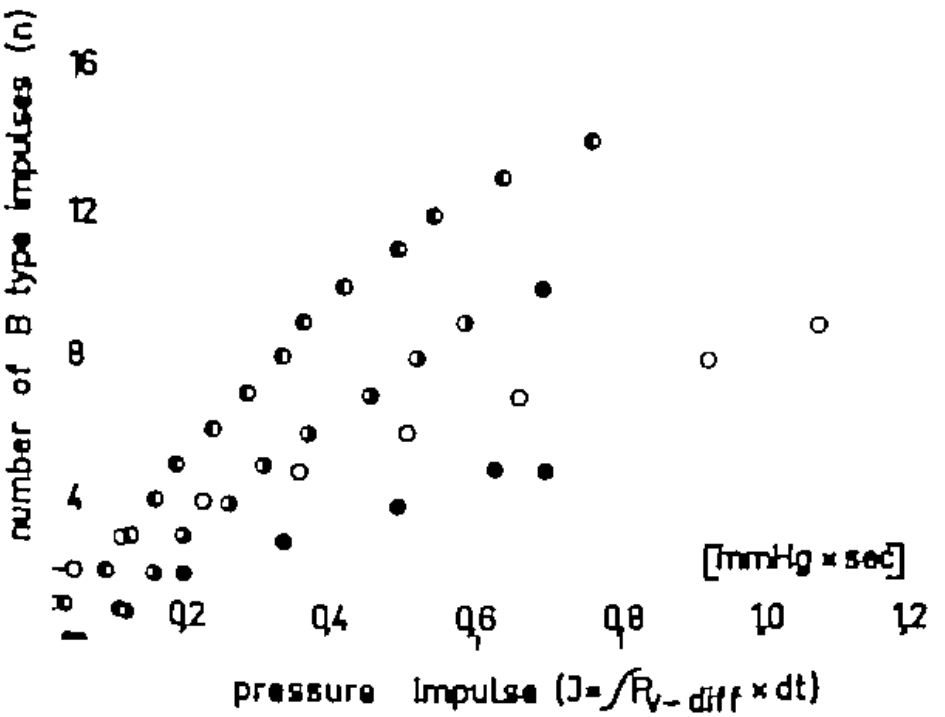


Fig 5 Number of the vagal afferent left trial B-type impulses (n) as a function of the pressure impulse (I) of the v-wave of the left atrial pressure curve, during one and the same heart cycle in single fibre nerve preparations. The figure relates to four normal cycles of four experimental animals.

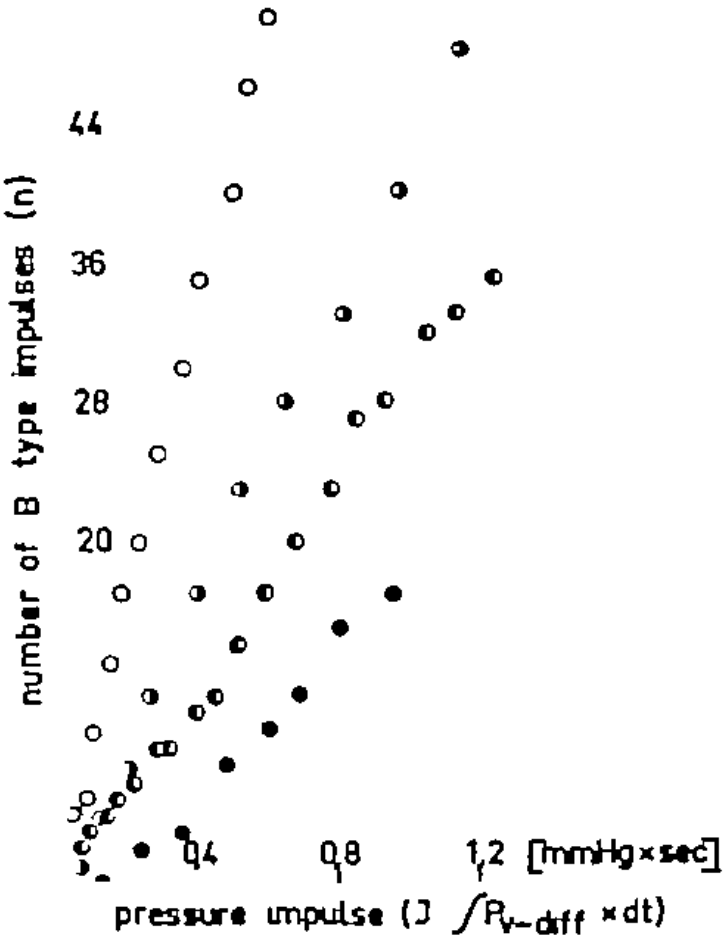


Fig. 6 Number of the vagal afferent left atrial B-type impulses (n) as a function of the pressure impulse (I) of the v-wave of the left atrial pressure curve during one and the same heart cycle in multifibre nerve preparations. The figure relates to four normal cycles of four experimental animals.

In single fibre preparations, the number of B-type impulses, as a function of the pressure impulse of the v wave during one and the same heart cycle, has a positive correlation in normal cycles (Fig. 5)

The arithmetical mean of the correlation coefficients, calculated from randomly chosen 12 heart cycles of six experimental animals, was 0.986 The other statistical values are listed in Table 2 of the Appendix.

In multifibre nerve preparations, it seems that the number of B-type impulses as a function of the pressure impulse of the v wave during one and the same normal heart cycle has a linear correlation (Fig. 6)

The mean of the correlation coefficients, calculated from 12 randomly chosen heart cycles in six different experimental animals, was 0 995 The other statistical values are listed in Table 3 of the Appendix. It is observable that a linear relationship exists between these two variables in multifibre nerve preparations

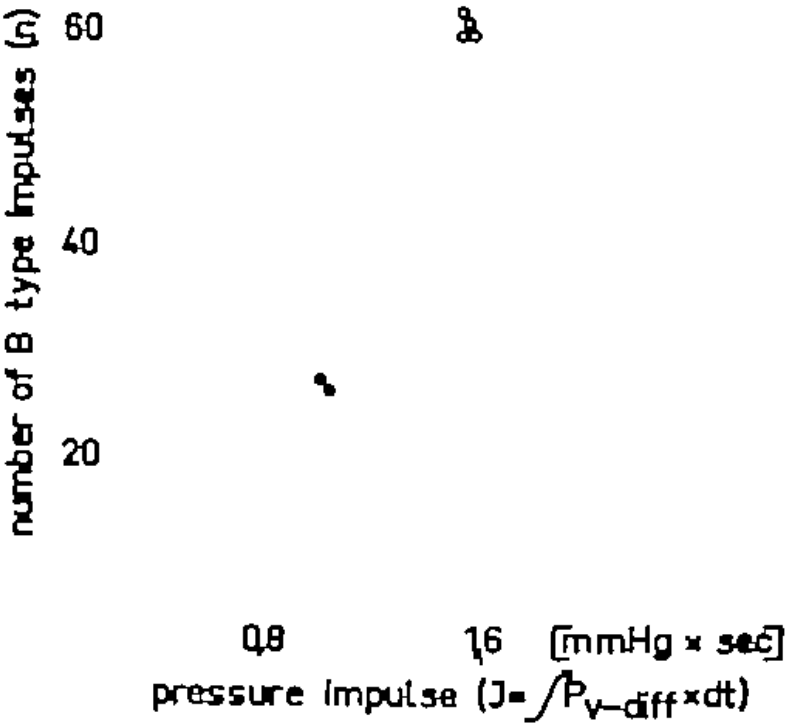


Fig 7 Number of vagal afferent left atrial B-type impulses per heart cycle (n) as a function of the pressure impulse (I) of the v-wave of the left atrial pressure curve, in different cycles, when the atrial pressure was raised by an injection (open circles). The closed circles illustrate the variation during artificial respiration in normal cycles. (Table 4 exp. animal No 16).

The difference between the correlation coefficients of multifibre pressure impulse and single fibre, pressure impulse was tested by z transformation. The u-value was + 3 432 and $P < 0.001$ (P = risk) This result implies that the correlation coefficients of multifibre, pressure impulse differ significantly from single fibre, pressure impulse correlation coefficients.

In different successive cycles, when the left atrial pressure was elevated by an injection (3—10 ml during 2—10 sec), the number of B-type impulses displayed a positive correlation with the pressure impulse in single and multi fibre nerve preparations (Fig. 7 and 8)

The correlation coefficient of 19 different cases of a rise in the left atrial pressure in 13 experimental animals varied from 0 701 to 0.970 and the number of tested cycles from 6 to 50 The mean of the correlation coefficients was 0.889 The other statistical values are listed in Table 4 of the Appendix. The correlation between these two variables may be linear No obvious difference was apparent in the relationship between the single and multi-fibre nerve preparations.

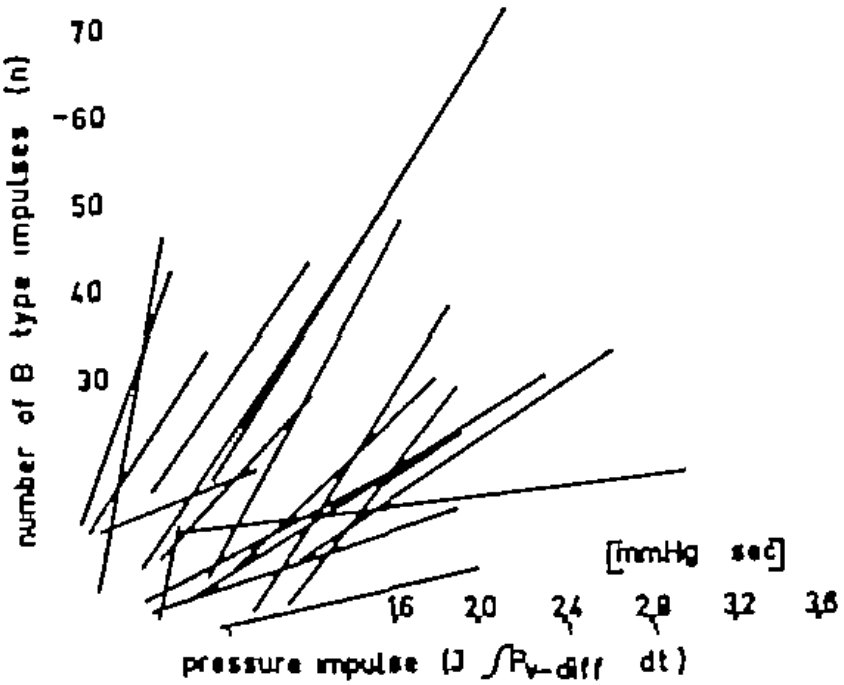


Fig 8 Number of vagal afferent left atrial B-type impulses per heart cycle (n) as a function of the pressure impulse (I) of the v-wave of the left atrial pressure curve, in different cycles, when the atrial pressure was raised by an injection The figure relates to 19 rises in 13 experimental animals. The lines are illustrative and drawn in accordance with statistical alues. (Table 4 of the Appendix).

l type impulses under conditions of raised and lowered left atrial pressure

The increase in left atrial A type impulses was induced by intra atrial and femoral injections The effect of the injections upon the number of A type impulses was calculated in 37 rises of 10 different experimental animals. The mean of the control values was 9 3 impulses per cycle, and the mean of the raised values 12.5 impulses per cycle The difference between the two values is significant ($t = 4\,57$ $f = 223$ $P < 0\,001$ t = t value of Student t test, f = degrees of freedom and P = risk)

The suction of blood from the left atrium increased the number of A type impulses The control and the raised values of A type impulses were tested in six different experimental animals, and in six different suction cases The mean of the control values was 8 5 imp per cycle and that of the raised values 12.2 imp per cycle The difference between these two numbers is significant ($t = 5\,07$ $f = 111$ $P < 0\,001$)

These results imply that the raising and lowering of the left atrial pressure increases the number of A type impulses.

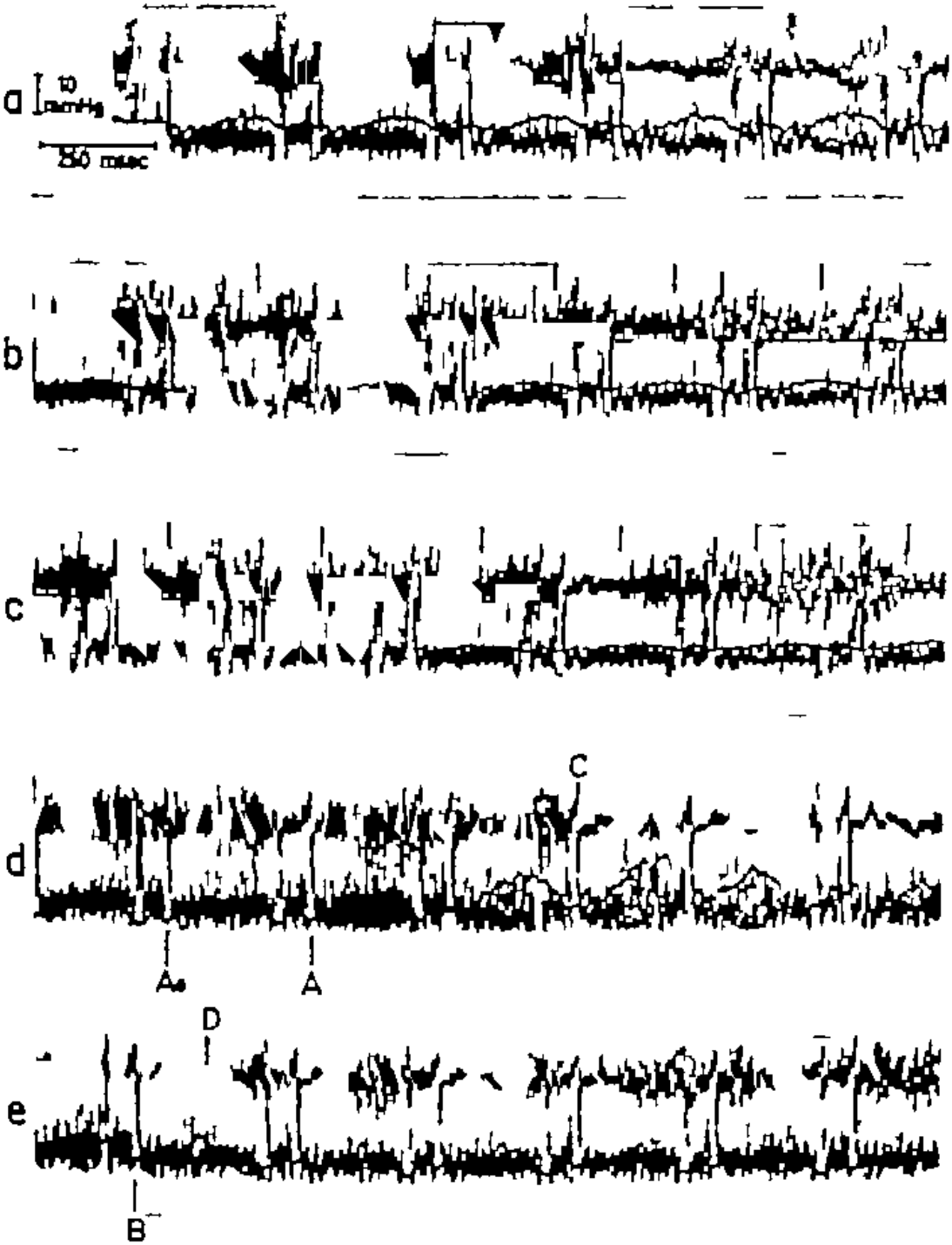


Fig 9 Influence of lowering or raising the left atrial pressure upon the left atrial vagal afferent impulses (B-type and A-type) and upon the sympathetic postganglionic efferent cardiac impulses. The upper impulses are sympathetic, and the lower impulses vagal. The lower oscillating curve, below the upper straight, is the left atrial pressure curve. (The alphapeths A_e, A, B, C, D include to the text of p. 16). a) control b) reduction in blood volume by 15 ml, c) after reduction by 30 ml, d-e) influence of the left atrial pressure rise upon the vagal (B- and A-type) impulses and sympathetic impulses (with 5 ml of blood/1 sec, weight of animal 3.8 kg) estimated total blood volume 212 ml.

Effect of B- and A-type left atrial impulses upon the sympathetic postganglionic activity of the heart under conditions of normal and changed left atrial pressure.

B-type impulses and the activity of sympathetic cardiac fibres

The rise in the left atrial pressure increases the number of B-type impulses, and diminishes the sympathetic activity of the heart (Fig. 9 d-e)

The inhibition of the sympathetic activity was partial or total, depending on the injected volumes per time unit. In particular the blood injections (3—8 ml within ½—5 sec) into the left atrium in most cases induced total sympathetic silence (Fig. 9 d-e) The diminution in sympathetic impulses followed 200—400 msec (mean 308 msec) after the increase in B-type impulses (calculated from the P wave of the EKG which occurred as a mild artefact in the recording of action potentials to the beginning of the next sympathetic burst)

A positive inverse correlation was apparent between the number of

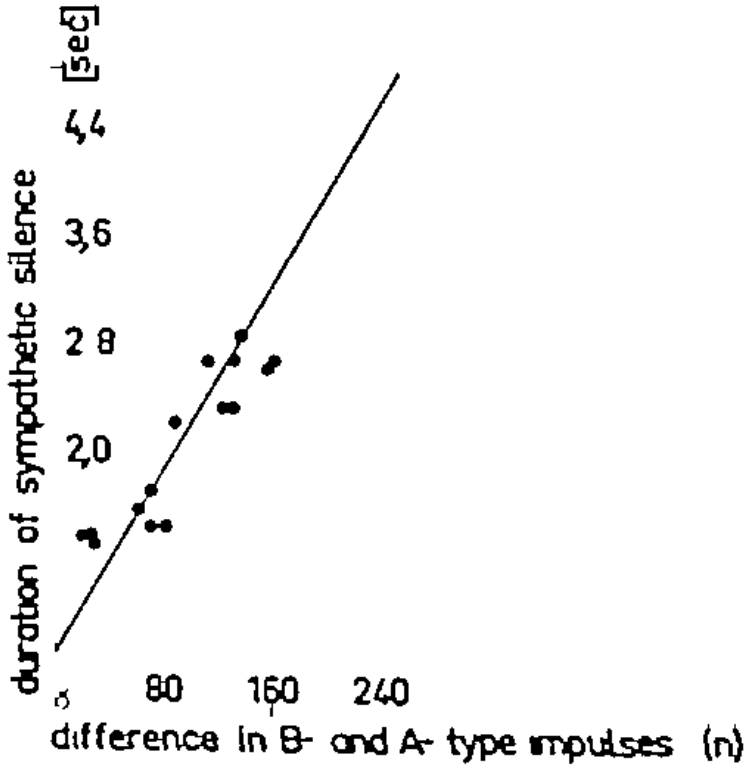


Fig 10. Duration of the silence of the sympathetic postganglionic efferent cardiac activity as a function of the difference in the sums of the left atrial afferent vagal B- and A-type impulses (n) under conditions of normal (open circles) and raised (closed circles) left atrial pressure. The impulses were recorded from multifibre nerve preparations, and the volume injected varied from 3 ml to 8 ml within ½—5 sec. The weight of the animal was 3.8 kg. (Table No 5 p animal No 11)

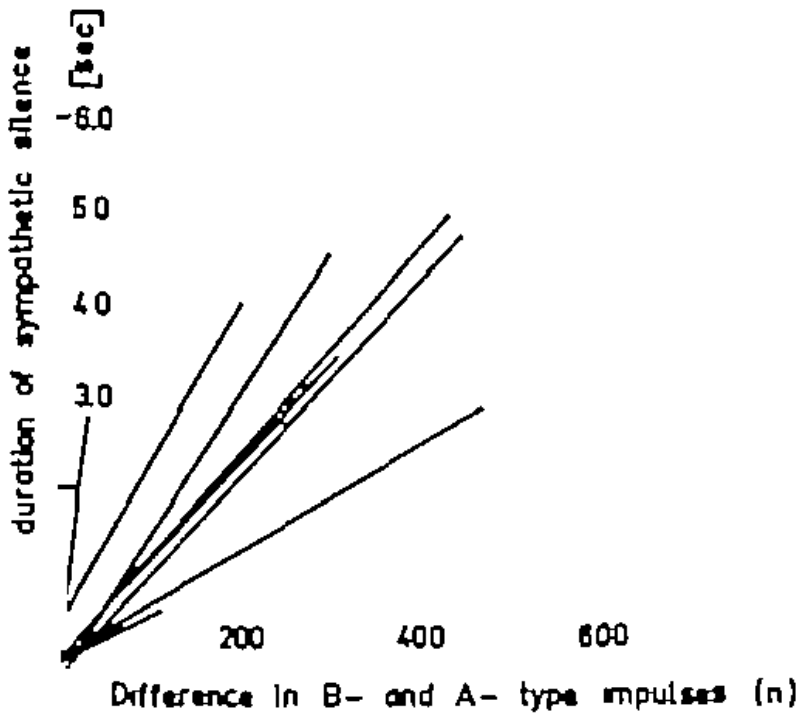


Fig. 11 Duration of the silence of the sympathetic postganglionic efferentation to the heart as a function of the difference in the sums of the left atrial afferent vagal B- and A type impulses (n) under conditions of normal and raised left atrial pressure, of eight multifibre nerve preparations from seven experimental animals. The lines are drawn in accordance with statistical values. (Table 5 of the Appendix).

B-type impulses and the number of sympathetic impulses in all cases of pressure rise.

The mean of the correlation coefficients calculated for 15 rises in seven experimental animals was —0 659

An attempt was made to test the nature of the inhibition in the central nervous system (= CNS) with eight nerve preparations from seven experimental animals forming the difference in the sums of the B- and A-type impulses of each injection These differences were correlated with the corresponding silence time of the sympathetic activity within the same nerve preparation (see p 16)

It seems that a linear correlation exists between these two variables (Fig. 10 and 11).

The correlation coefficient varied from 0.925 to 0.992, and the number of points from 8 to 25. The mean of the correlation coefficients was 0.965 The other statistical values are listed in Table 5 of the Appendix.

In suction experiments, the left atrial pressure was lowered and the number of B-type impulses diminished, with a simultaneous increase in sympathetic outflow to the heart (Fig. 9 a-c). A positive inverse correlation

was observable in all the tests with blood suction, between the number of B-type impulses and the number of sympathetic impulses (Fig. 12) In six suction tests in six cats the mean of the correlation coefficients between the number of B-type impulses and the number of sympathetic impulses was —0 682.

A type impulses and the activity of sympathetic cardiac fibres

In most instances, the rises in the left atrial pressure induced an increase in A type impulses, particularly under normo- and hypovolaemic conditions. The increase was not always demonstrable under hypervolaemic conditions by an injection It was found that the diminution in sympathetic activity was delayed in those cases in which an increase in A type impulses occurred during atrial injection as compared with the experiments in which the increase in A type impulses was unobservable or cases in which the sympathetic silence was induced by a very fast injection within one cardiac cycle.

In three experimental animals, the atrial contractions, which occurred during ventricular premature beats in response to intra-atrial injections, caused an increase in A type impulses. This was followed by one two bursts of sympathetic impulses after a delay of 300—400 msec in the middle of the sympathetic silence

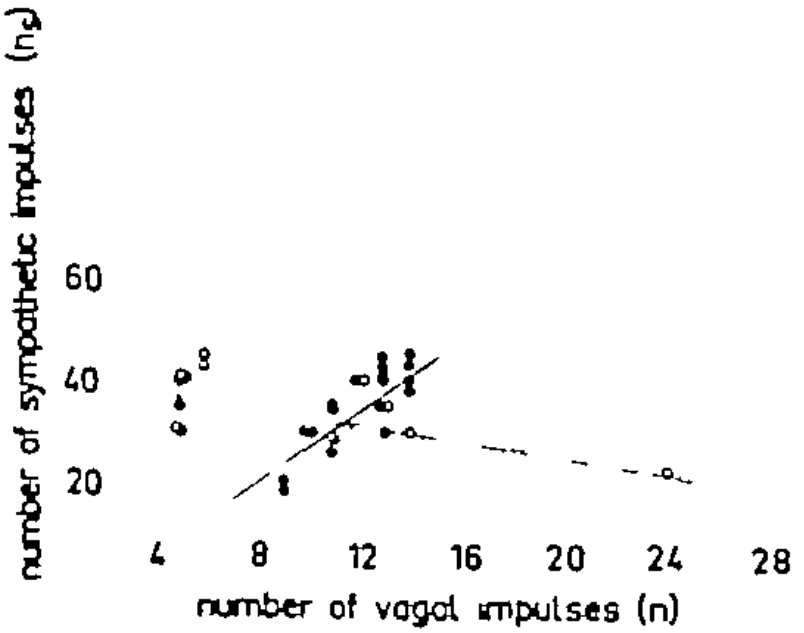


Fig 1 Number of sympathetic efferent postganglionic cardiac impulses (n_s) as a function of the v gal afferent left atrial impulses (n) when the left atrial pressure was lowered by blood suction. The points in respect of one experimental animal are calculated from cycle to cycle during the course of the suction. The lines are drawn in accordance with statistical values. B-type mpulses pen circles A type impulses = closed circles, (Table No 6, exp. animal No 11).

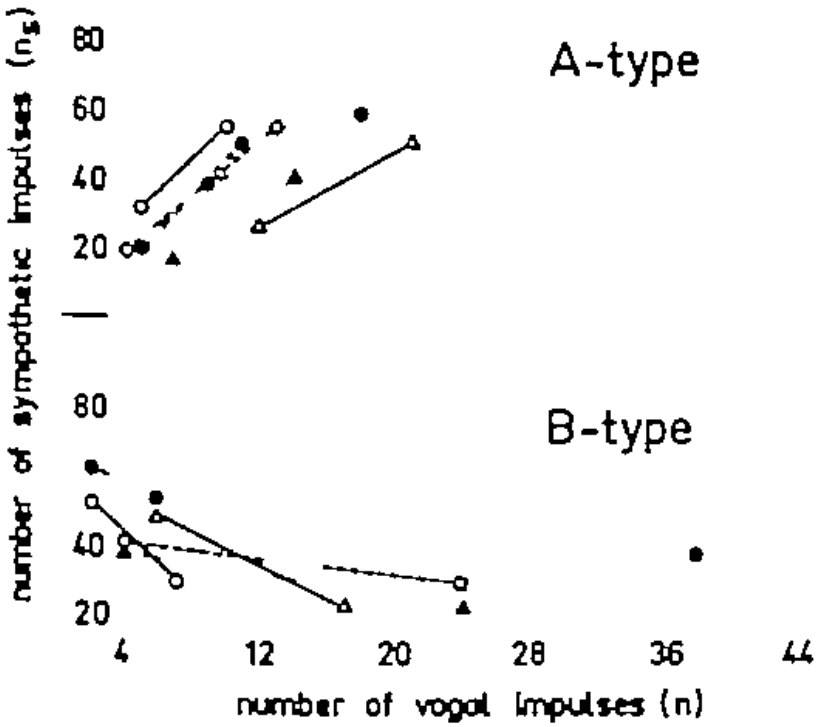


Fig 13 Number of sympathetic efferent postganglionic cardiac impulses (n_s) as a function of the vagal afferent left atrial impulses (n) (B-type, A-type) when the left atrial pressure was lowered by blood suction. The lines are illustrative, and drawn in accordance with the statistical values from six suctions in six experimental animals. (A-type in Table 6 of the Appendix).

In suction tests, a linear positive correlation was apparent between the number of A-type impulses and the number of sympathetic impulses following after a delay of 200—300 msec (Fig. 12 and 13)

The correlation coefficient varied from 0 761 to 0 917 and the number of points, from 12 to 24 was calculated from six suction cases in respect of six different experimental animals. The mean of the correlation coefficients was 0 864 The other statistical values are listed in Table 6 of the Appendix.

Ratio of B- and A type impulses and the number of sympathetic impulses.

The ratio (see p 16) of B- and A-type impulses was calculated and correlated with the number of sympathetic impulses in cases of pressure rise A positive inverse correlation seems to exist between these two variables (Fig. 14 and 15).

The correlation coefficient, calculated from 15 cases of pressure rise in seven experimental animals varied from —0 455 to —0 926 (mean —0.659)

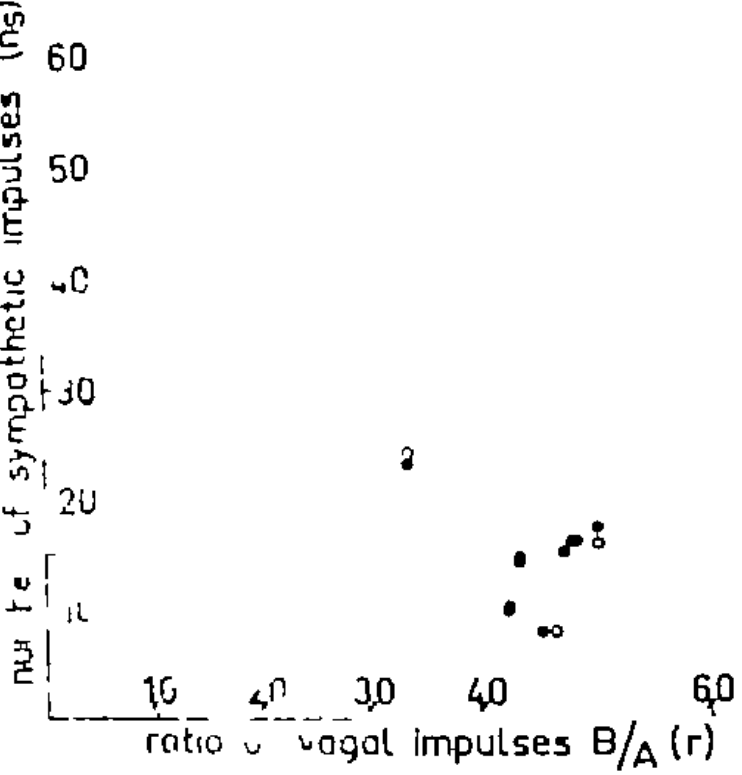


Fig 14 Number of sympathetic postganglionic efferent cardiac impulses (n_s) as a function of the ratio (r) of the numbers of B- and A type afferent cardiac impulses in one experimental animal during two different cases of pressure rise (injection of blood into the left atrium closed circles, 3 ml within 1 sec open circles, 5 ml within 1 sec). (Table No 7 exp. animal No 17)

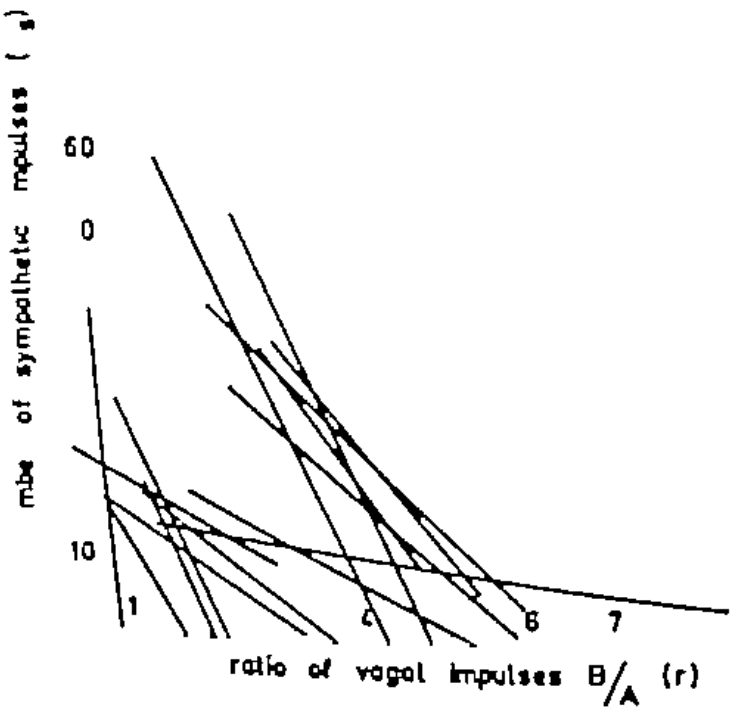


Fig 15 Number of sympathetic postganglionic efferent cardiac impulses (n_s) as a function of the ratio (r) of the numbers of B- and A type vagal afferent cardiac impulses in 15 cases of pressure rise in seven experimental animals. (3—8 ml within ½—5 sec). The lines are drawn in accordance with statistical values and are illustrative. (Table 7 of the Appendix).

and the number of points from 12 to 23 The other statistical values are listed in Table 7 of the Appendix.

In suction tests a positive inverse correlation also existed between the two variables. The correlation coefficient varied from —0 614 to —0 863 (mean —0 733) and the number of points from 12 to 24 calculated from six *suctions in six different experimental animals The statistical values* are listed in Table 8 of the Appendix.

Left atrial B- and A-type impulses, and the left ventricular relative stroke volume.

The sum of the numbers of B- and A type impulses during one heart cycle, and the following relative stroke volume, were correlated from eight tests of pressure rise and four experimental animals (Fig. 16 and 17) The blood volume *of the cats was first reduced with suction of blood (20 ml)* from the femoral vein or from the left atrium. A part of the blood volume, 8—15 ml, was injected into the femoral vein or 4—8 ml into the left atrium. The response in the stroke volume and nerve impulses was followed during the course of the injection

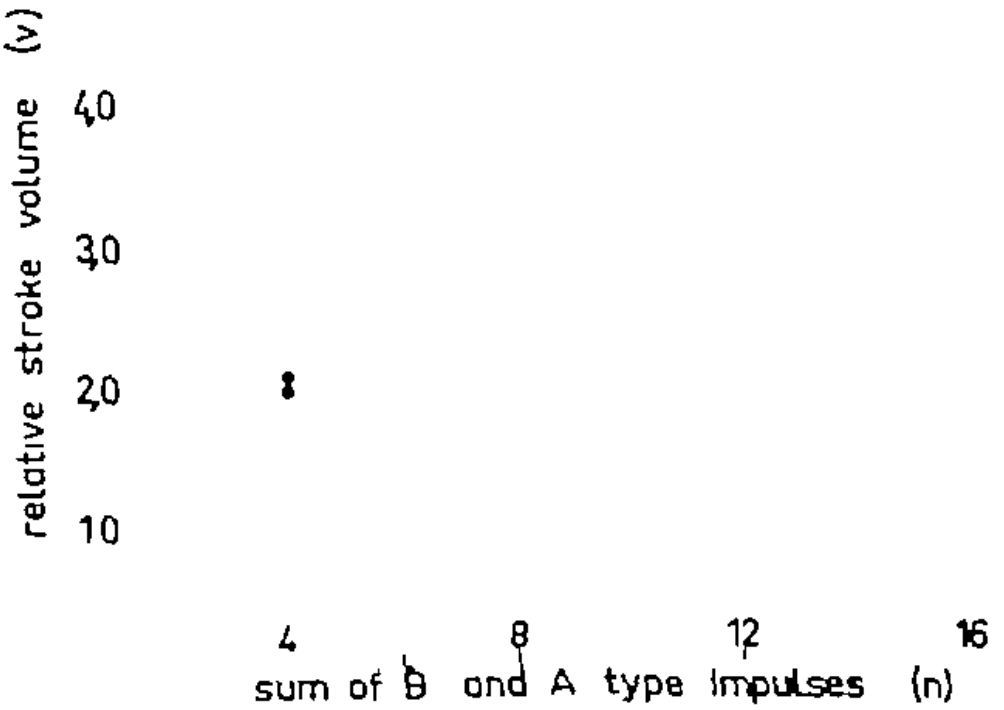


Fig 16 Relative stroke volume (V) as a function of the sum of left atrial vagal B- and A-type impulses ( ) in different successive cycles when relative stroke volume was increased with an intra-atrial injection of blood (4 ml/during 4 seconds). (Table N 9 exp. animal No 12 2).

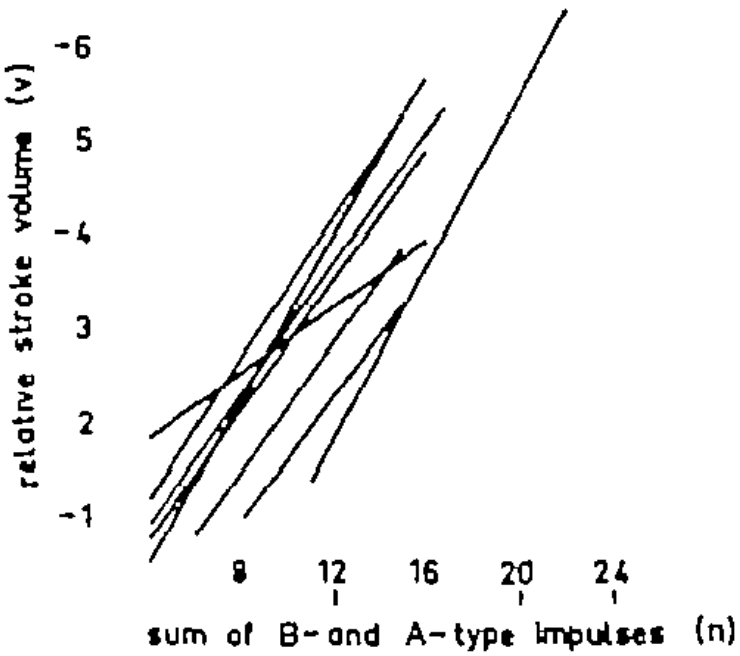


Fig. 17 Relat left ventricular stroke volume (V) as a function of the sum of vagal afferent left atrial B- and A type impulses (n) in different cycles when the relative stroke olume wa nereased with femoral or intra-atrial injections of blood. The lines are drawn accordance w ith statistical values and are illustrative. (Table No 9 of the Appendix).

The correlation coefficient varied from 0 748 to 0 893 and the number of points from 13 to 83 The other statistical values are listed in Table 9 of the Appendix.

DISCUSSION

Experimental conditions

Reference was made to the earlier work of JARISCH and ZOTTERMAN (1948) for use of the chloralose urethane mixture, but the amount of urethane in 7 ml of Ringer solution was reduced from 0.250 to 0 125 g as it was observed that the higher dose caused some cardiac depression which appeared as cardiac arrest (cf GILES, QUIROZ and BURCH 1969)

Since lowering of blood pH both in respiratory and non respiratory acidosis is known to increase the plasma catecholamine concentration (MORRIS and MILLAR 1962 a, b) and a rise of CO_2 in inspired oxygen the sympathetic efferent activity (MILLAR and BISCOE 1965) the acid base balance was followed during four experiments. In general, a tendency toward metabolic acidosis was encountered. An attempt was made to compensate this by hyperventilation. It is suggested here that the acid base balance was not changed when the tests were performed during short periods.

The nerve preparation was made by modification of the techniques of JARISCH and ZOTTERMAN (1948) and PAINTAL (1953 c). In every recording, the nervous connection of the vagus from the heart to the CNS was partially cut. The effect upon the sympathetic activity of this dissection was tested in four experimental animals no difference was noted in the response to the atrial pressure elevation before and after cutting. This indicates that the dissected fibres are responsible for no more than a small part of the total activity coming to the CNS from the left atrium. Moreover in the nerve preparation phases of this work, nerve impulses of right atrial origin were found in the fibres of the left vagus.

The sympathetic postganglionic activity was recorded, as the system of recording was easier than that in preganglionic acitivity Also, samples of the sympathetic activity to the heart were obtainable.

The recorded samples of the nervous activity of the sympathetic nerves are likely to represent the total sympathetic activity to the heart. This is suggested by the findings in the preliminary experiments (not included in

the material) that no distinct difference was apparent in the sympathetic activity of the second and third left sympathetic cardiac nerves, recorded simultaneously In harmony with these findings, BRONK et al (1936) failed to detect any appreciable differences in the activity of the right and the left cardiac sympathetic nerves.

The samples from vagal fibres do not represent the total activity of B- and A-type impulses to the CNS since the share of each type of impulse in the whole recording is occasional However the ratio of B- and A type impulses is comparable during the course of one and the same experiment with the same nerve preparation if the recording and experimental conditions are constant. This is suggested by the mere fact that in pressure rise cases a parallel increase was seen in the numbers of B- and A type impulses.

Left atrial pressure and pressure impulse

PAINTAL (1953 a) observed a linear correlation between the left atrial volume and the average frequency of discharge of B-type impulses in a haemodynamically isolated atrium preparation In a later work PAINTAL *(1963 b) found that the best correlation prevails between the number of* B-type impulses per heart cycle and the amplitude of the v wave. LANGREHR (1960) in turn demonstrated the existence of a positive correlation between the mean left atrial pressure and the number of B-type impulses.

In the present work, the pressure impulse (I) was calculated for study of the pressure as a function of time. A linear correlation seems apparent between the number of B-type impulses per heart cycle and the pressure impulse of the v wave in multifibre nerve preparations. The correlation between the pressure impulse and the number of B-type impulses in a single fibre nerve preparation is probably not linear although the statistical values are significant This result in respect of multifibre nerve preparations is similar to the findings of BERGSTRÖM (1966) and VILJANEN (1967) BERGSTRÖM showed that in cats the time integral of the applied pressure bears, within given limits a linear correlation to the total number of the impulses during the time in question in the sensory nerve fibres of skin. VILJANEN found a linear correlation between the mechanical pressure impulse and the number of EMG impulses of human intercostal muscles in respiration

The finding in the present work is explicable by the hypothesis that multifibre nerve preparations contain many fibres of receptors with different activation thresholds and nerve impulses from the receptors are summed to the same pressure impulse This type of summation has been suggested by BERGSTRÖM (1961) in his study of the touch and stretch receptors of frog s skin

When the pressure impulse reaches a higher level, the relationship deviates, so that a smaller number of impulses corresponds to a given pressure impulse (HAKUMÄKI et al. 1968).

B-type impulses and the sympathetic activity of the heart

In the present study a rise in the left atrial pressure resulted in an increase in B-type impulses and a diminution in sympathetic activity This indicates that the B-type impulses inhibit the sympathetic system. This is in keeping with the results obtained by JARISCH and ZOTTERMAN (1948) and ÖBERG and WHITE (1968), who by means of stimulation of the central end of the cardiac vagal nerves, in most cases induced a bradycardiac response. OKADA, OKAMOTO and NISIDA (1961) showed also that injection into the external jugular vein or compression of the right atrial wall caused inhibition in the interior cardiac sympathetic nerve. The responses were interpreted as reflecting sympathetic inhibition

It is obvious, furthermore, that the B-type impulses regulate the sympathetic silence time. This is supported by the present finding that the increase in B-type impulses during a few cycles induced a sympathetic silence for many cycles after the injection, although the corresponding number of impulses, during silence was not increased (Fig. 9 d-e).

A-type impulses and the sympathetic activity of the heart.

AMANN and SCHAEFER (1943) first recorded A-type impulses from vagal afferent nerves. The correlation between the "frequency of discharge per second" of A type impulses and the pressure of the right atrium was shown to be linear in cats by DICKINSON (1950). KOLATAT KRAMER and MÜHL (1957) have found a positive correlation between the pressure of the a wave and the number of A-type impulses in frogs.

SCHAEFER (1950 1960) has suggested that the atrial receptors both activate and inhibit the sympathetic system, so that a small amount of atrial distension causes cardiac acceleration, and maximal excitation of the cardiac sensory nerves a bradycardiac effect. PEARCE, HENRY and CHAPMAN (1956) have proposed that A type impulses may signify a state of high "tone" and low filling, in the central control of the cardiovascular system

The following findings made here support the hypothesis that A type impulses activate the sympathetic system

1 During intra-atrial injections the dimmution in sympathetic activity was delayed as the A type impulses were increased

2. The atrial contractions, which occurred during ventricular premature

beats in response to intra atrial injections, caused an increase in A type impulses. This was followed by one or two bursts of sympathetic impulses in the middle of the sympathetic silence.

3 In suction tests, a linear positive correlation was found between the number of A type impulses and the number of sympathetic impulses

4 The ratio of B- and A type impulses had a positive inverse correlation with the number of sympathetic impulses.

Some findings in earlier works may be explained by the function of A type impulses JARISCH and ZOTTERMAN (1948) and ÖBERG and WHITE (1968) found that in some experiments stimulation of the central end of the vagal cardiac branches also induced a rise in blood pressure with an associated bradycardia. The finding of HINDERLEIDER et al (1968) that denervation of the dog s heart diminished the constriction capacity of the spleen in hypovolaemic states is very interesting. It is obvious that the *denervation of the heart inhibited the activating influence of A type impulses* upon the sympathetic system.

Theoretical background of calculation of the ratio of B- and A type impulses and the difference in the sums of B- and A type impulses

Calculation of the ratio of B- and A type impulses was based upon the hypothesis that the *influence of the afferent neural activity in the CNS is* "in activated" in accordance with the formula $N_R = N \quad e^{\frac{-t}{k}}$ presented This formula was derived by simplification of the formula for exponential decay applied by GRODINS (1963 p 49) It is patent to all that the afferent activity is "inactivated" according to the formula stated above. This is supported by the response of pupil diameter to light stimulus and the Respiratory Heart Rate reflex, in which the similar exponential decay of the responses is seen after the stimulus (cf CLYNES 1961 1962) Coefficient k decides the slope of the inactivation curve in relation to the time, and might provide information on the capacity of the CNS to inactivate the afferent left atrial activity With the equation above the "residual activity (N_R) was calculated that is, the activity which still affects the CNS after time t It is suggested here that the effect upon the CNS of B- and A type impulses is "inactivated in the same way All the residual activities" were summed up from three successive cycles, and the ratio was derived as indicated earlier $\left(\frac{B}{A} = \frac{B_0 + B_{R1} + B_{R2} + B_{R3}}{A_0 + A_{R1} + A_{R2} + A_{R3}}\right.$ see p 16$\left.\right)$

The regression coefficient of the time of duration of sympathetic silence in relation to the difference in the sums of B- and A type impulses (see p 25 Table 5 of the Appendix) has been used as the value of the k coefficient in the formula $\left(N_R = N \quad e^{\overline{k}}\right)$

The difference in the sums of B- and A type impulses was derived by application of the theory of the opposite functions of B- and A type impulses to the sympathetic activity Thus the difference in the inhibitory and activating units is comparable with the duration of the inactivation of the sympathetic efferentation The regression coefficient was found by mechanical stimulation of receptors feeding to the CNS and thus provides some information of the tonus in the CNS of the animal. The coefficient also indicates the duration of inhibition in the sympathetic outflow per unit of neural inhibition, under those experimental conditions. The regression coefficients are not mutually comparable, as the number of active fibres (B A and sympathetic) in the recording system is occasional.

Ratio of B- and A type impulses

The ratio of B- and A type impulses was also calculated on the assumption that they have an opposite function upon sympathetic cardiac efferentation. The ratio can be correlated with the number of sympathetic impulses. It was further concluded that the sympathetic activity is influenced not only by this proceeding, but also by a few earlier vagal bursts of impulses (B and A, see Fig. 4, p 17) This conclusion was arrived at from the finding made in the present work that intra-atrial injection within a few atrial cycles induced an increase in B-type impulses and in the duration of sympathetic silence during the following cycles, although the numbers of B-type Impulses during silence were near the control values (Fig. 9 d-e) In addition during premature ventricular contractions elicited by the fast intra-atrial injections, there was a massive increase in A-type impulses (associated with simultaneous atrial contraction) followed by one — two successive sympathetic bursts, although the number of B-type impulses was high. In the system of calculation, there was observed the effect exerted by previous cycles upon the sympathetic burst.

It is obvious that changes in vagal afferent cardiac activity influence the next sympathetic burst after 200—400 msec. This delay is supported by Iggo and Vogt (1960), who came to the conclusion that there was a latent period of 200 msec between the stimulus of baroreceptors and the inhibition of sympathetic outflow In the present work, a massive increase in B-type

impulses, induced by an injection resulted in a sympathetic silence after 200 msec.

The ratio of B- and A-type impulses represents the amount of inactivation per unit of activation It appeared that this ratio has a positive inverse correlation with the number of sympathetic impulses. This implies that an increase in the left atrial pressure conditions induced a change in vagal afferentation of the heart which influenced the sympathetic efferentation This efferentation is dependent upon the ratio of B- and A type impulses. The preliminary results have been published by HAKUMÄKI (1968).

For testing of the influence mechanism of the B- and A type impulses upon the sympathetic system, the following combinations were also calculated and correlated with the number of sympathetic impulses (n_s) (see Fig. 4 p 17)

- Number of B-type impulses from the previous cycle (B_0)
- Number of B-type impulses from the previous cycles calculated by the formulae presented (B)
- Number of A type impulses from previous cycle (A_0)
- Number of A type from the previous cycles calculated by the formulae presented (A)
- The ratio of B- and A type impulses from the previous cycle $\left(\frac{B_0}{A_0}\right)$

The coefficients of linear correlation obtained for the above interrelations were not so significant as the correlation coefficient of the ratio of B- and A-type impulses and the number of sympathetic impulses (n_s) The differences between the correlation coefficients were significant This indicates the correctness of the suggestion made in the present work in respect to the influence mechanism of B- and A type impulses upon the sympathetic activity

Difference in the sums of B- and A type impulses

The difference in the sums of B- and A type impulses was calculated for estimation of the amount of inhibition which induces a given sympathetic silence The result near linearity indicates that under these experimental conditions, and within given limits, the CNS inhibits the neural activity mainly in the same way Moreover it indicates that the difference in the inactivating and activating impulses bears a linear correlation to the duration of sympathetic silence The preliminary results have been published by HAKUMÄKI (1969)

Left atrial B- and A type impulses and the left ventricular relative stroke volume.

LANGREHR and KRAMER (1960) did not find a distinct correlation between the cardiac output and the sums of activity of B-type impulses. The correlation between the thoracic blood volume and the atrial B-type impulses was distinct. LANGREHR's finding (1960) of a positive correlation between the stroke volume per duration of systole and the relative frequency of B-type impulses is similar to the results obtained in the present work.

Although the left ventricular filling is regulated only partially and to a varying extent by left atrial filling pressure and left atrial contractility the sum of B- and A type impulses was calculated and correlated with the left ventricular relative stroke volume. The correlation between these two variables seems linear although the result can be considered indirect, as the stroke volume is a result of ventricular contraction and the B- and A type impulses have their origin in the atrial functions.

Role of baroreceptor activity

BRONK et al. (1936), DOWING and SIEGEL (1963) and SPICKLER, KEZDI and GELLER (1965) have shown that both mechanical and electrical stimulation of the baroreceptors or their afferent nerves diminish the sympathetic efferent activity. In preliminary experiments (not included in the present material) the influence of the intra-atrial injection upon baroreceptor activity and the sympathetic system was tested with simultaneous recording of the aortic baroreceptor afferent and cardiac sympathetic postganglionic efferent impulses. No marked increase in baroreceptor activity was apparent during and after the left atrial injections (3—8 ml within ½—5 sec) although sympathetic silence was found to emerge. This finding can be explained by the results of ALLISON and SAGAWA (1967) that the aortic arch reflex receptors operate usually between 100—300 mmHg. DOWING and SIEGEL (1963) found that raising the carotid sinus pressure from 35 to 130 mm Hg reduced the sympathetic activity but little. It might be possible that the intra atrial injections (3—8 ml within ½—5 sec) in the present work do not exert any marked influence upon the baroreceptor activity and, through this, upon the sympathetic outflow. Nevertheless, changes in the sympathetic activity were inducible by stimulation of the left atrial receptors. In this context, it may be mentioned that GUPTA et al. (1966) demonstrated that a progressive increase in blood volume increases the activity of atrial receptors much more than the activity of aortic baroreceptors. Further OKADA, OKAMOTO and NUMDA (1961) have shown that bilateral aortic nerve cutting did not induce any changes in the response of the cardiac sympathetic efferentation to intravenous infusion

However in those tests in which the vagal afferent and sympathetic efferent cardiac activity was followed in different blood volume conditions (Table 7 of the Appendix, marked with x), the influence of baroreceptor activity upon the sympathetic activity is obvious. Premature ventricular beats, induced by intra atrial injections also diminish the baroreceptor activity and affect the sympathetic outflow

It would appear that cardiac sympathetic efferentation is also controlled by the atrial receptors.

Bainbridge reflex

The neural pathways of the BAINBRIDGE reflex (1915) have been studied by LEDSOME and LINDEN (1967) who in their experimental system were able to show that the reflex has an atrial origin

COLERIDGE and LINDEN (1955) found that the cardiac acceleration oc curred more frequently when the control level of heart rate was low before injection When the rate was high, the acceleration was seldom induced and was not very significant. The findings of COLERIDGE and LINDEN are explicable as follows. In the experiments in which the control level of heart rate before the injection, was low the increase in blood volume induced an increase in B- and particularly in A type impulses, so that the ratio of B- and A type impulses diminished and induced an increase in sympa thetic activity and cardiac acceleration. In the experments in which the initial frequency was high, the increase in blood volume mainly induced an in crease in B-type impulses (as the A type impulses were near their maximum number) and the ratio of B- and A type impulses was not changed or was increased This did not introduce any changes in the sympathetic activity or diminish it in cases of bradycardia. The neural regulation of the heart would be expressed by the formula

$$\frac{I}{E} = \frac{\text{number of vagal efferent impulses}}{\text{number of sympathetic efferent impulses}}$$

where I is the number of inhibitory impulses (including the B-type impulses) and E the number of excitatory impulses (including the A type impulses). The regulation system based upon the above formula is supported by JEWETT s (1964) finding of a linear correlation between the vagal afferent impulses and the length of the heart cycle MARGUTH RAULE and SCHAEFER (1951) WEIDINGER, HETZEL and SCHAEFER (1962) and JEWETT (1964) made an interesting observation of the opposite functions of the vagal efferent and sympathetic afferent impulses. The above equation is an attempt to formulate a quantitative presentation of the mechanism of heart regulation on the basis of earlier works and the present findings

SUMMARY

1 A study has been made of the impulsation of the vagal left atrial afferent fibres and of the left third sympathetic cardiac nerve under conditions of normal and changed left atrial pressure in 21 open-chest anaesthetized cats. Simultaneously the left atrial pressure and in some experiments, the flow and pressure of the ascending part of the aorta were determined

2. A comparison was made between

— the pressure impulse of the v wave of the left atrial pressure curve, and the number of left atrial B-type impulses in single and multifibre nerve preparations.

— the difference in the sums of B- and A type impulses and the duration of the sympathetic inhibition.

— the ratio of B- and A type impulses and the number of sympathetic impulses.

— the sum of B- and A type impulses, and the left ventricular relative stroke volume

3 The number of B-type impulses was found to bear a linear correlation with the pressure impulse of the v-wave in a multifibre nerve preparation during normal atrial filling.

4 When the left atrial pressure was raised artificially

— the number of B-type impulses and the corresponding pressure impulse in different cycles were positively correlated

— the difference in the sums of B- and A type impulses had a linear positive correlation with the duration of sympathetic inhibition.

— the ratio of B- and A type impulses presented a positive inverse correlation to the number of sympathetic postganglionic impulses.

5 The number of A-type impulses showed a linear positive inverse correlation with the number of sympathetic impulses upon lowering of the left atrial pressure by blood suction.

6. The sum of B- and A type impulses had a positive correlation with the left ventricular relative stroke volume when the left atrial pressure was elevated from "subnormal" post suction levels.

7 On the basis of the present results and of some findings by others it is suggested that the A type impulses stimulate the sympathetic activity of the heart, while the B-type impulses exert an inhibitory action. The Bainbridge reflex is re-evaluated on the basis of this concept.

8. A hypothesis is presented of the influence and inactivation mechanism of the vagal cardiac afferent impulses in the CNS

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APPENDIX

Number of experiment	Weight of animal (g)	Anæsthetic used	Anæsthetic after cat implanted	Left atria removed	Right atrium removed	Auricles fibrillating	Left ventricle	Right ventricle	Blood	Heparin	Date and no. of injections (sec)	Total number of injections	Rabbit bred	Type of heparin used: Single fibre	Pressure heparin in one cycle	Pressure heparin in different cycles	Left atrial B-type heparin	Left atrial A-type heparin	Difference of B-A and sympathetic blood duration	B/A and auricle of sympathetic heparin	Rabbits urine volume	Serum of B- and A-type heparin
1	1·0								×	×	2—3	6	×	×	×	×	×					
2	1·6		×						×	×	1½—3	6	×	×	×	×	×	×	×	×		
3	1·7			×					×	×	1—2	4	×	×	×		×					
4	4·0			×					×	×	5	1	×	×	×	×	×					
5	1·4			×					×	×	3—4		×	×	×		×	×	×	×	×	×
6	1·6			×					×	×	3—7	2	×	×	×	×	×					
7	1·1			×				×			7	1	×	×	×	×	×					
8	1·6			×				×			9	1	×	×	×	×	×					
9	3·7			×				×			10	1	×	×	×	×	×					
10	1·8			×				×			1½—4	8	×	×	×	×	×	×	×	×	×	×
11	3·8			×				×			1½—5	1	×	×	×	×	×					
12	1·5							×			½—4	5	×	×	×	×	×	×	×	×	×	×
13	3·4			×				×			½—3	12	×	×			×	×	×	×	×	×
14	4·4			×				×			5	1	×	×	×	×	×					
15	3·5			×				×			½—4	12	×	×			×	×	×	×	×	×
16	1·9			×				×			10	1	×	×	×	×	×					
17	1·8			×				×			1½—5	10	×	×			×	×	×	×	×	×
18	4·4			×				×			7—15	2	×	×			×	×				
19	4·8			×				×			8—1	2	×	×			×	×				
20	2·9			×				×			6—8	2	×	×			×	×				
21	3·7			×				×			4—8	1	×	×			×	×				

TABLE 2

Statistical values of the pressure impulse and the number of B-type impulses in single fibre nerve preparations in 12 analysed cycles of six experimental animals.

Number of experimental animal	Mean of pressure impulse	Standard deviation of pressure impulse	Mean of number of B-type impulses	Standard deviation of B-type impulses	Regression coefficient	Constant	Number of points	Correlation coefficient	Statistical significance
	$\bar{I}$	s_I	$\bar{n}$	s_n	b	c_1	N	r	$p<$
1	0.35	0.28	7.50	4.18	13.20	2.71	14	0.883	0.001
	0.41	0.29	7.00	3.89	13.00	1.55	13	0.994	0.001
2	0.43	0.39	5.00	2.74	6.80	2.04	9	0.979	0.001
	0.20	0.16	5.00	2.74	12.80	2.43	7	0.975	0.001
4	0.28	0.24	4.00	2.74	11.40	0.78	9	0.987	0.001
	0.40	0.28	5.00	2.74	9.00	1.07	9	0.998	0.001
5	0.35	0.26	2.86	1.95	7.00	0.24	7	0.986	0.001
	0.31	0.27	2.50	1.87	15.20	0.39	6	0.972	0.01
14	0.33	0.21	5.00	3.32	15.90	−0.25	11	0.994	0.001
	0.37	0.24	4.09	3.18	1.00	−0.66	11	0.992	0.001
16	0.32	0.32	5.00	2.74	8.00	−2.38	9	0.960	0.001
	0.33	0.22	5.00	2.16	9.60	1.86	7	0.998	0.001

TABLE 3

Statistical values of the pressure impulse and the number of B-type impulses in multifibre nerve preparations in 12 analysed cycles of six experimental animals.

Number of experimental animal	Mean of pressure impulse	Standard deviation of pressure impulse	Mean of number of B-type impulses	Standard deviation of B-type impulses	Regression coefficient	Constant	Number of points	Correlation coefficient	Statistical significance
	f	sf	n	sn	b	c_1	N	r	p<
1	0.37	0.21	2.85	16.29	78.80	1.43	13	0.998	0.001
	0.39	0.28	3.31	14.66	5.10	3.28	13	0.985	0.001
3	0.49	0.39	14.55	11.44	29.20	0.09	20	0.996	0.001
	0.33	0.27	14.47	11.30	41.00	0.86	19	0.996	0.001
6	0.41	0.31	10.22	8.32	26.80	−0.77	9	0.995	0.001
	0.43	0.36	12.10	9.75	26.60	−0.70	10	0.989	0.001
7	0.4	0.31	8.90	5.82	18.90	0.25	10	0.996	0.001
	0.36	0.26	9.9	7.97	30.90	−1.40	12	0.995	0.001
8	0.51	0.38	21.20	15.94	41.40	0.06	10	0.998	0.001
	0.39	0.30	23.82	18.20	59.60	−0.36	11	0.995	0.001
9	0.52	0.29	8.25	15.97	40.60	−2.52	8	0.995	0.001
	0.54	0.37	5.87	5.28	14.10	−1.74	8	0.987	0.001

TABLE 4

Statistical values of the pressure impulse and the number of B-type impulses in different cycles, when the left atrial pressure was raised with injections in 19 cases of rise in 13 experimental animals.

Number of experimental animal	Mean of pressure impulse	Standard deviation of pressure impulse	Mean of number of B-type impulses	Standard deviation of B-type impulses	Regression coefficient	Constant	Number of points	Correlation coefficient	Statistical significance
	$\bar{I}$	s_I	$\bar{n}$	s_n	b	c_1	N	r	$p<$
1	0.56	0.20	15.06	2.02	7.8	10.56	15	0.776	0.001
2	0.78	0.42	3.86	3.57	8.1	— 0.43	21	0.946	0.001
4	0.39	0.18	20.44	7.16	35.9	6.55	9	0.880	0.01
5	1.34	0.38	4.67	2.69	6.0	— 3.34	15	0.834	0.001
	0.55	0.35	8.89	3.95	98.6	—45.02	9	0.864	0.01
6	1.21	0.25	16.63	7.46	23.7	—12.22	15	0.792	0.001
	1.86	0.19	22.75	4.40	16.4	— 7.68	20	0.701	0.001
x) 7	1.31	0.39	40.88	16.55	38.2	—10.29	50	0.917	0.001
x) 8	1.35	0.43	16.51	6.60	13.4	— 1.68	29	0.874	0.001
x) 9	1.91	0.36	21.54	6.96	16.35	— 9.74	28	0.845	0.001
10	1.45	0.29	15.70	10.38	31.85	—30.53	10	0.893	0.001
	1.47	0.61	17.11	9.58	15.17	— 5.17	9	0.964	0.001
	1.42	0.32	22.33	13.09	38.6	—32.47	6	0.933	0.01
	0.93	0.33	27.67	12.86	36.1	— 6.07	9	0.919	0.001
11	0.74	0.19	27.30	7.72	35.3	1.14	10	0.890	0.001
12	0.32	0.96	21.67	13.98	145.4	—14.05	12	0.858	0.001
	0.32	0.12	26.82	10.78	67.7	5.39	11	0.749	0.01
14	0.88	0.22	19.67	6.82	97.0	— 4.56		0.8[illegible]	0.01
x) 16	1.16	0.33	25.41	14.70	43.2	— 24.7	4	0.970	0.001

x) Impulses through the femoral vein, (other straight to the left atrium).

TABLE 5

Statistical values of the difference in the sums of B- and A type impulses and the duration of sympathetic silence in eight nerve preparations from seven experimental animals under conditions of normal and raised left atrial pressure.

Number of experimental animal	Mean of difference in B- and A type impulses	Standard deviation of difference in B- and A type impulses	Mean of sympathetic silence (sec.)	Standard deviation of sympathetic silence	Regression coefficient	Constant	Number of points	Correlation coefficient	Statistical significance
	n	sn	t	st	b	c_1	N	r	p<
2	51.26	32.35	0.40	0.24	0.0069	0.04	10	0.925	0.001
10	96.23	117.68	1.10	1.29	0.0108	0.05	13	0.991	0.001
11	84.48	57.26	1.89	1.05	0.0170	0.45	25	0.925	0.001
1	1.88	18.64	1.76	1.46	0.0762	0.09	8	0.975	0.001
13	115.33	126.26	1.32	1.40	0.0110	0.05	9	0.992	0.001
13	200.22	176.66	1.36	1.11	0.0060	0.14	9	0.969	0.001
15	142.35	139.29	1.44	1.48	0.0104	—0.04	17	0.985	0.001
17	115.93	88.54	1.57	1.43	0.0158	—0.27	14	0.981	0.001

TABLE 6

Statistical values of the numbers of A-type impulses and the number of sympathetic postganglionic impulses in six suction cases in respect of six experimental animals.

Number of experimental animal	Mean of A-type impulses	Standard deviation of A-type impulses	Mean of number of sympathetic impulses	Standard deviation of sympathetic impulses	Number of points	Correlation coefficient	Statistical significance
	nA	snA	nS	snS	N	r	p<
2	16.17	3.43	38.89	10.42	18	0.915	0.001
11	11.62	1.93	32.50	8.39	24	0.822	0 001
12	7.91	2.31	34.87	10.84	24	0.917	0.001
13	7.33	1.82	42.17	10.50	12	0.806	0.01
15	12.89	3 18	48 78	9.83	18	0.761	0.001
17	8.00	1 96	35.00	12.06	14	0.878	0.001

TABLE 7

Statistical values of the ratio of the numbers of B- and A-type impulses and the number of sympathetic postganglionic impulses in 15 cases of pressure rise in seven experimental animals. In two experimental animals (marked with x), also in different blood volume conditions. (not in Fig. 15).

Number of experimental animal	Mean of ratio of B- and A-type impulses	Standard deviation of ratio of B- and A-type impulses	Mean of number of sympathetic impulses	Standard deviation of number of sympathetic impulses	Regression coefficient	Constant	Number of points	Correlation coefficient	Statistical significance
	x	s	$\bar{n}$	s_n	b	c_l	N	r	$p<$
	4.83	6.51	15.07	9.71	— 9.69	61.95	15	—0.682	0.01
)	3.67	1.61	34.12	14.29	— 3.70	47.77	69	—0.420	0.001
) 10	1.76	0.92	16.68	11.76	— 7.40	29.91	111	—0.582	0.001
	2.29	0.91	6.87	7.83	— 6.30	21.40	23	—0.733	0.001
11	1.44	0.53	15.00	12.10	—20.70	44.97	16	—0.926	0.001
	1.85	0.83	31.33	7.96	— 8.30	46.83	12	—0.878	0.001
12	0.76	0.18	25.30	18.03	—83.70	89.07	13	—0.816	0.001
	0.96	0.33	14.84	10.31	—13.90	28.23	19	—0.445	0.1
13	2.92	0.90	28.38	22.36	—19.90	86.59	13	—0.797	0.01
	3.36	0.93	32.07	22.75	—20.60	101.57	15	—0.847	0.001
	3.70	0.68	27.07	11.52	—11.90	71.35	15	—0.704	0.01
15	3.87	2.56	11.08	5.19	— 1.50	16.97	12	—0.743	0.01
	1.12	0.96	19.06	8.65	— 5.10	24.90	16	—0.575	0.02
	2.10	0.99	12.12	10.70	— 7.10	27.06	16	—0.659	0.01
	4.17	1.85	7.75	12.29	— 4.80	27.94	16	—0.729	0.01
17	3.79	0.8.	23.37	11.74	—12.30	70.11	18	—0.870	0.001
	4.37	0.92	14.06	9.45	— 8.40	51.12	18	—0.826	0.001

TABLE 8

Statistical values of the ratio of the numbers of B- and A-type impulses and the number of sympathetic postganglionic impulses in six suction cases in respect of six experimental animals.

Number of experimental animal	Mean of ratio of B- and A-type impulses	Standard deviation of ratio of B- and A type impulses	Mean of number of sympathetic impulses	Standard deviation of stympathetic impulses	Number of points	Correlation coefficient	Statistical significance
	$\bar{n}BA$	$snBA$	$\bar{n}S$	snS	N	r	p<
2	0.70	0.33	38.89	10 42	18	—0.863	0.001
11	0.94	0.56	32.50	8.39	24	—0.620	0.01
12	0.24	0.23	34.87	10.84	24	—0.614	0.01
13	0.65	0.31	42.17	10.50	12	—0 734	0.01
15	1 44	0.98	48.78	9 83	18	—0 767	0.001
17	0.62	0.28	35.00	12.06	14	—0.800	0 001

ACTA PHYSIOLOGICA SCANDINAVICA

STUDIES ON THE EFFECT OF PROSTAGLANDINS E_1 and E_2 ON THE SYMPATHETIC NEUROMUSCULAR TRANSMISSION IN SOME ANIMAL TISSUES

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PER HEDQVIST

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Apart from certain hitherto unpublished results, the present thesis is based on the following papers

1 Inhibition by prostaglandin E of noradrenaline release and of effector response to nerve stimulation in the cat spleen. Life Sci. 1969 8 389—395 (Together with J Brundin)

2 Control by prostaglandin E_2 of sympathetic neurotransmission in the spleen Life Sci. 1970 *9* 269—278

3 Inhibitory action of prostaglandins E_1 and E_2 on the neuromuscular transmission in the guinea pig vas deferens. Acta physiol. scand. 1969 *77* 510—512. (Together with U S v Euler)

4 Inhibition by prostaglandin E_2 of sympathetic neurotransmission in the rabbit heart. Acta physiol. scand. 1970. (Preprint) (Together with L. Stjärne and Å. Wennmalm)

5 Antagonism between prostaglandin E_2 and phenoxybenzamine on noradrenaline release from the cat spleen. Acta physiol. scand. 1969 *76* 383—384

6 Antagonism by calcium of the inhibitory action of prostaglandin E_2 on sympathetic neurotransmission in the cat spleen. Acta physiol. scand. 1970. (Preprint)

These papers will be referred to below by their arabic numerals.

Abbreviations used

NA = noradrenaline PG = prostaglandin TA = tyramine PBA = phenoxybenzamine DMI = desmethylimipramine.

CONTENTS

I INTRODUCTION 5

II METHODS 9

III RESULTS AND DISCUSSION 12

A Evidence for PGE_1 and PGE_2 acting as inhibitors of sympathetic neuromuscular transmission

1 Effect of PGE_1 in the cat spleen 12
2 Effect of PGE_2 in the cat spleen 13
3. *Interaction between* PGE_2 *and* phenoxybenzamine in the cat spleen 14
4 Effect of PGE_1 and PGE_2 in the guinea pig vas deferens 14
5 Effect of PGE_2 in the rabbit heart 15
6. Concluding remarks 16

B Analysis of the prejunctional action of PGE and PGE in the sympathetic *neuromuscular system.*

1 Nerve conduction 18
2. Stimulus secretion coupling 18
3 " storage particles 20
4 " synthesis 21
5. Release of transmitter into the extracellular space 21
6. Effect of PGE_1 and PGE_2 on the mechanisms of inactivation of transmitter released.
a) Enzymatic degradation 23
b) Physical binding 23
7 Physiological aspects on pharmacological interference with the effect of PGE_1 and PGE_2.
a) Interaction between PGE_2 and phenoxybenzamine 24
b) Interaction between PGE_1 and atropine 25

C Possible role of PGE_1 and PGE_2 in sympathetic neuromuscular transmission 26

IV SUMMARY AND CONCLUSIONS 28

V REFERENCES 31

I INTRODUCTION

In 1930 Kurzrok and Lieb reported that addition of human seminal fluid to isolated uterine strips alters their contractile state in a complex way causing either contraction or relaxation. This appears to be the first observation of a biological effect that can be accounted for by prostaglandins, although it was for some time believed that acetylcholine was the active principle (Kurzrok and Lieb 1930 Cockrill, Miller and Kurzrok 1935)

Shortly after this Euler and Goldblatt independently described the vasodepressor and smooth muscle stimulating properties of human semen (Euler 1934 Goldblatt 1933 1935) Similar effects were obtained with seminal fluid from other species and with extracts of the vesicular glands of rams (Euler 1934) By preparing different extracts of seminal fluid and prostate and vesicular glands Euler found the active principle to be present in a fraction containing lipid soluble acids. With reference to the tissue source this novel kind of biologically active substance was named prostaglandin (Euler 1935 1936) From further studies it was concluded that the active principle is a lipid soluble fatty acid probably containing a double bond and a hydroxy group (Euler 1939)

More recent chemical work, mainly by Bergström and coworkers, made it apparent that prostaglandin is not a single substance but rather a family of biologically active lipids. In 1957 the first prostaglandin (PGF) was obtained in pure crystalline form (Bergström and Sjövall 1957) Shortly afterwards a more lipid soluble factor was isolated and termed PGE_1 (Bergström and Sjövall 1960) The two PG s proved to be unsaturated hydroxy acids with the empirical formulas C_{20} H_{34} O and C_{20} H_{32} O respectively (Bergström *et al* 1962 a) Further studies led to the isolation of several *related PG s and to determination of their chemical structures* (Bergström *et al.* 1962, b, *c*, 1963 a, b, Samuelsson 1963 1964 a, Hamberg and Samuelsson 1965 1966 Nugteren *et al* 1966) which are shown in Fig 1

The fundamental work of Euler and Goldblatt in the prostaglandin field made it natural that the initial work to isolate PG s was performed with extracts of seminal fluid and of accessory genital glands. However studies with sensitive methods have revealed that PG s are not confined to the

Fig. 1 Structural formula of prostaglandins

genital sphere but have a broad distribution in mammalian tissues. Thus PG s have been shown to be present in or released from a variety of tissues, such as lung (Bergström *et al* 1962 d) brain Samuelsson 1964 b, kidney (Lee *et al.* 1965) adrenals (Ramwell *et al* 1966) and spleen (Davies, Horton and Withrington 1967) The high activity and the wide spectrum of biological actions of PG s has subsequently led to the proposal of several hypotheses concerning their physiological function. These aspects have recently beeen reviewed (Bergström 1967 Bergström, Carlson and Weeks 1968 Horton 1969) and the discussion will therefore be limited to observations considered to be of interest in connection with the present study

The observed vasodepressor and smooth muscle stimulant properties of crude prostaglandin have in many respects been confirmed using pure PGE_1 and PGE_2. Thus the two compounds lower the arterial blood pressure in many animal species and in man (Bergström *et al* 1959 a, b, Holmes, Horton and Main 1963 Bergström and Euler 1963 Berti *et al* 1964) The blood pressure increasing effect of NA is also counteracted by PGE_1 and PGE_2 (Holmes, Horton and Main 1963 Bergström Carlson and Orö 1964) The reduction of the blood pressure is probably mostly due to vasodilation, since the two compounds have been shown to decrease flow resistence and to increase blood flow in tissues (Horton and Main 1963 Nakano and McCurdy 1967 Weiner and Kaley 1969) Peripheral resistance vessel strips in the dog show a biphasic dose—response curve with relaxation after small and contraction after high doses of the two compounds (Strong and Bohr 1967) On the other hand coronary aortic and carotid strips, and nasal vessels *in vivo* respond only by contraction on addition of PGE_1 or PGE_2 (Strong and Bohr 1967 Khairallah, Page and Türker 1967 Jackson and Stovall 1968)

The effect of PGE and PGE_2 on non—vascular smooth muscle is more complex in that they produce either contraction or relaxation, and potentiation or inhibition of responses to sympathetic nerve stimulation and to NA. Intestinal smooth muscle generally seems to be contracted by PGE_1 and PGE_2 (Bergström *et al* 1959 a, Bergström and Euler 1963 Horton and Main 1963) although relaxation has also been reported (Khairallah, Page and Türker 1967) Similarly the two compounds induce contraction of uterine muscle of some animal species, but relaxation in man (Bergström *et al.* 1959 a, Horton and Main 1963 Bygdeman 1964) The smooth muscle of the bladder is also contracted by PGE_1 (Naumada 1967) and in the eye the two compounds induce miosis (Wautzman and King 1967)

On the other hand several smooth muscle tissues respond with relaxation to PGE_1 or PGE_2. This has been demonstrated in the fallopian tube, in the

ureter and in tracheal muscle (Horton and Main 1963 Main 1964 Boyarsky and Labay 1968)

PGE_1 and PGE_2 have been shown to potentiate the mechanical responses to NA and to sympathetic nerve stimulation in several smooth muscle tissues. Thus PGE_1 enhances the contractile effect of NA on isolated rat and guinea pig seminal vesicles (Eliasson and Risley 1966) In the guinea pig *preparation PGE and PGE_2 magnify smooth muscle contraction* induced by hypogastric nerve stimulation (Naimzada 1969) The smooth muscle contractile responses to hypogastric nerve stimulation in the rat and guinea pig bladder and in guinea pig vas deferens are all increased by PGE_1 (Mantegazza and Naimzada 1965 Naimzada 1967)

On the other hand an antagonistic action of PGE_1 has been observed in many tissues. Thus in vas deferens, colon fundic strips and uterus of rats, and in uterus and tracheal chain of guinea pigs the responses to NA *in vitro* independently if relaxation or contraction, are antagonized by PGE_1 administered in subtreshold doses (Clegg 1966) Similarly the smooth muscle contractile response to sympathetic nerve stimulation is also counteracted by PCE in a number of tissues, such as rabbit vas deferens and oviduct and cat spleen (Mantegazza and Naimzada 1965 Brundin 1968 Hedqvist 1968)

The observations in a variety of smooth muscle tissues of antagonistic as well as agonistic actions of PGE_1 and PGE_2 were found rather intriguing and worth further consideration. To study this aspect three different sympathetically innervated muscle tissues were used, the cat spleen, the guinea pig vas deferens and the rabbit heart The methods used involved registration in the presence of PGE and PGE_2 of the mechanical responses to nerve stimulation and to NA, as well as analysis of the amount of NA overflowing in response to nerve stimulation with the aim to determine pre and postjunctional actions of the two compounds.

Experiments were carried out using cat spleens, guinea pig vasa deferentia, rabbit hearts and bovine splenic nerves.

Cat spleen The operation procedure was performed under general anaesthesia according to the method worked out at this laboratory (Hedqvist, Olivero and Stjärne 1968 Hedqvist and Stjärne 1969) The isolated spleen with intact vascular and nerve supply was transferred to a perfusion chamber maintained at 37° C, and perfused with a slightly modified Krebs-Henseleit's solution (NaCl 6 04 g, KCl 0 354 g, $MgSO_4$ 7 H_2O 0.294 g, $CaCl_2$ 0.282 g, Na pyruvate 0.524 g, Na l-glutamate 0.416 g, KH_2PO_4 0 163 g, $NaHCO_3$ 2.1 g, dextrose 2.08 g per 1000 ml H_2O) containing 2 % dextran and aerated with 5 % CO_2 in O_2. The flow rate was kept at 8—10 ml/min by means of a constant rate perfusion pump (Sigma motor) Ascorbic acid (20 μg/ml) was added to the perfusion fluid to protect noradrenaline (NA) in the medium, and hydrochloric acid was infused into the venous collecting system to maintain a suitably low pH in the perfusate. The effluent from the spleen was divided into 10 ml portions by means of an automatic fraction collector The splenic nerves were electrically stimulated by platinum electrodes. Trains of 200 pulses were applied by a Grass S4 stimulator operated at a frequency of 10/sec, a duration of 2—3 msec, and at supramaximal voltage, usually 10—15 V

Splenic capsular and trabecular contraction (splenic contraction) was measured as shortening of the organ, using a Harvard heart/smooth muscle transducer Changes in the perfusion pressure were measured with a Statham P23 transducer Both parameters were recorded on a Grass Model 5 Polygraph

In most experiments the NA stores of the spleen were labelled by intraarterial infusion of 30 μC of ^{3}H-dl-NA to facilitate monitoring of the outflow of total NA from the organ. The labelled NA was infused for 15 min at the beginning of the perfusion period. At least 30 min were then allowed to elapse before the main experiment was started.

Guinea pig vas deferens The animals were killed by a blow on the head and the vasa deferentia dissected out, freed from adjacent tissue and split longitudinally The preparation was mounted in a 20 ml bath at 37 C

with Tyrode solution of the following composition (NaCl 8 g KCl 0 2 g, CaCl 0.2 g, MgCl 0.1 g $NaHCO_3$ 1 g dextrose 1 g per liter of H O) and areated with 5 % CO_2 in O Electrical stimulation of the preparation was carried out with a Grass S4 stimulator coupled to a timer delivering 5 sec trains of rectangular pulses of 0 2—2 msec duration at frequencies of 20—40/sec and at supramaximal voltage, usually about 40 V each half or whole min The preparation was stimulated transmurally by two perspex—shielded electrodes placed on each side along the organ at a few mm distance (Birmingham and Wilson 1963) Contractions of the organ were recorded isometrically Hexamethonium (100 µg/ml) did not affect the contractile effect of stimulation, while guanethidine (1 µg/ml) abolished the stimulation response indicating postganglionic nerve stimulation.

Rabbit heart Rabbits were killed by a blow on the head and the hearts with intact sympathetic nerve supply were dissected out according to Hukovic and Muscholl (1962) and Löffelholz and Muscholl (1969) The preparation was perfused by Langendorff technique with Tyrode solution (NaCl 8 g, KCl 0 2 g CaCl 0.2 g $MgCl_2$ 0 2 g, $NaHCO_3$ 1 g, $NaH PO_4$ 0 05 g dextrose 1 g per liter of H O) aerated with 5 % CO in O_2 To the perfusion fluid were added ascorbic acid (40 µg/ml) to protect NA in the medium, and in most cases atropine (10^{-6} g/ml) The sympathetic nerves leaving the stellate ganglion were pulled through a plastic tubing with platinum ring electrodes in the wall Electrical stimulation of the nerves was carried out with Grass S5 stimulators, delivering 30 sec trains of rectangular pulses of 1 msec duration, at frequencies of 1 5 and 10/sec, and at supramaximal voltage usually about 30—40 V A strain gauge transducer was attached to the apex of the heart and myocardial contractile force and heart rate were recorded on a Grass Model 5 D Polygraph The effluent from the heart was collected in 2 min periods, immediately acidified and chilled. The NA content of representative samples was assayed fluorometrically as described below In a series of 6 control experiments the amounts of NA appearing in the effluent in response to 3 consecutive periods of nerve stimulation (300 pulses at 10/sec) at 15 min intervals were 112±19 ng 101±20 ng and 100±24 ng, (means ± S E.M)

Splenic nerves Bovine splenic nerves were obtained at the slaughter house within 30 min *post mortem* and immediately chilled with ice. The nerves were carefully dissected free from contaminating tissue and were in most cases delicated They were then placed in a nerve chamber with the midportion of the nerves kept in the same medium as was used in the cat spleen experiments (dextran excluded) The temperature of the chamber was kept constant at 37 C Stimulation of the nerves was carried out with

platinum electrodes and a Grass S4 stimulator operated at a pulse duration of 2—3 msec, using ascendant current, and at supramaximal voltage. Single pulses or short trains of rectangular pulses were delivered at 1 min intervals. The recording electrodes (platinum) were connected via a cathode follower to a DC preamplifier (Grass P 6 12 A, frequency response 0—10000 Hz) and a cathode ray oscilloscope (Tektronix 502)

Analysis The outflow of NA from the spleen was monitored by determining the radioactivity in the different crude perfusate fractions. 0.5 ml aliquots were counted in a 7 3 toluene absolute ethanol solution, containing 4 g of 2—5 diphenyloxazole and 100 mg of 1 4 bis-2(4-methyl—5-phenyloxazolyle) benzene per liter of toluene, in a Packard Liquid Scintillation Spectrometer Quenching was monitored by internal standards of H-dl-NA.

In the cat spleen and rabbit heart experiments representative perfusate samples were purified on alumina and their NA content assayed fluorimetrically according to Euler and Lishajko (1961) The NA values obtained in the different studies are expressed as free NA base and are not corrected for losses of about 25 % during the purification procedure. In control experiments the recovery of NA added to perfusate samples and carried through the entire procedure of purification and fluorimetrical determination was 70.4±2.2 % mean ±SEM, n = 12. Addition to the perfusate of PGE_1 or PGE_2 ($3\times10^{-}$ M) did not materially change the recovery of NA, being 73 9±2.0 % mean ±SEM, n = 6 with PGE_1 and 73 3±2.2 % mean ±SEM n = 6 with PGE_2

Agents used (doses used in the different studies refer to the salts unless otherwise stated) Atropine sulphate dextrane (Macrodex® M. 70000 Pharmacia) guanethidine sulphate (Ismeline® Ciba) hexamethonium chloride dl noradrenaline 7 H hydrochloride (New England Nucl. Corp) l noradrenaline bitartrate (used as free base) phenoxybenzamine hydrochloride (Dibenyline® Smith, Kline and French) prostaglandin E_1 [1] prostaglandin E_2 [1] tyramine hydrochloride.

[1] Kindly put at disposal by Professor S. Bergström, Karolinska Institutet, Stockholm, Sweden, and Dr J Pike, Upjohn Co., Kalamazoo, U.S.A.

III RESULTS AND DISCUSSION

A. Evidence for PGE_1 and PGE_2 acting as inhibitors of sympathetic neuromuscular transmission.

1 Effect of PGE_1 in the cat spleen (Paper 1) In the cat spleen experiments the vascular response to stimulation of the splenic nerves was counteracted in a dose—dependent manner by infusion of PGE (Fig 2) The pressor response to intraarterially administered NA was also reduced by PGE (Fig 1) On the other hand, the action of the compound on the outflow of NA in response to nerve stimulation was rather variable and showed no correlation between the administered dose and the effect obtained. In some cases PGE_1 caused a marked and reversible reduction of the efflux of NA in response to nerve stimulation. However in most experiments PGE_1 did not appreciably alter the outflow of NA, in spite of a clearcut depression of the vascular response This observation does not necessarily exclude a prejunctional action of the compound in these experiments. Certain adrenolytic drugs increase markedly the outflow of NA in response to sympathetic nerve stimulation (Brown and Gillespie 1957) So does an increased rate of flow through the organ (Hertting and Schiefthaler 1963 Follow Haggendal and Lisander 1968) By analogy PGE_1 which induces vasodilation and increased blood flow and counteracts the vascular response to NA (Horton and Main 1963 Steinberg *et al* 1963) would be expected to increase the outflow of NA in response to nerve stimulation by facilitating washout of NA released from the nerve terminals. Thus the absence of increased NA overflow seen in most experiments, is compatible with, and may be taken as further indication of an inhibitory prejunctional action of PGE leading to decreased transmitter release.

Blakeley *et al* (1968) using the cat spleen perfused with whole blood have reported that PGE increases the outflow of NA in response to nerve stimulation. They also found an increased rate of flow through the organ, which may thus contribute to the raised output figures for NA. However this cannot be the only explanation since PGE was also shown to increase the NA overflow after α-receptor blockade by phenoxybenzamine. Another and more reasonable explanation for increased transmitter overflow seems

to be improved microcirculation in the presence of PGE_1 since the compound was also found to inhibit the formation of platelet trombi in the penicillar arteries of the organ. In line with this assumption is the fact that the NA output figures in the cat spleen are several times higher when the organ is perfused with saline medium than with whole blood (Brown and Gillespie 1957 Blakeley Brown and Ferry 1963 Thoenen, Hürlimann and Haefely 1963 Hedqvist and Stjärne 1969) It is interesting to note that Davies and Withrington (1968, 1969) did not see any action of PGE on the effector responses either to nerve stimulation or to NA in the dog spleen perfused with whole blood

It may be concluded that PGE_1 counteracts the vascular response to nerve stimulation in the cat spleen. While this effect is mainly based on inhibition of the effector response to the NA released, it also appears to involve an inhibitory prejunctional action on NA release.

2 *Effect of PGE_2 in the cat spleen (Paper 2)* Low doses of PGE_2 were found to reduce the pressor as well as the splenic contraction responses to nerve stimulation. Higher doses of PGE_2 further depressed the splenic contraction response while the vascular response tended to increase again although it usually remained below that obtained during the preceding control stimulation. The effect of PGE_2 on the splenic contraction response to NA was weak and inconsistent. On the other hand the effect of PGE_2 on the vascular response to NA was found to be highly dose-dependent. Thus low doses of the compound reduced the pressor response while high doses produced a distinct potentation. This biphasic dose response curve may explain the above mentioned increase of the vascular response to nerve stimulation in the presence of high doses of PGE_2 as the result of a potentiating effect on released NA rather than facilitated release of NA from the nerves.

The effect of PGE_2 on the outflow of NA in response to nerve stimulation was much more consistent than that of PGE_1 In all doses used (6×10^{-8}—6×10^{-6} M) PGE_2 caused a clearcut decrease of the NA overflow response (Fig 1) The inhibition progressively increased with the dose of the compound, leading to NA output figures in the presence of PGE_2 6×10^{-6} M of the order of 20 per cent of those obtained during the preceding control stimulations. After the end of the infusion of PGE_2 the overflow response increased again For the lower doses of PGE_2 the time for complete restoration of the response was usually about 10 min and progressively increased with the dose used, often to exceed 30 min at 6×10^{-6} M.

In the dog spleen perfused with blood, PGE_2 does not alter the "splenic" responses to nerve stimulation or to NA (Davies and Withrington 1968,

1969) Species differences and/or differences in concentrations of PGE_2 used, may possibly account for the dissimilarity in effects produced in the cat and dog spleen.

The experimental data obtained in the cat spleen show that PGE_2 depresses the function of the sympathetic neuromuscular system of the organ, and that the compound exerts this action mainly prejunctionally.

3 *Interaction between PGE_2 and phenoxybenzamine in the cat spleen (Paper 5)* The interrelationship between the action of PGE_2 and phenoxybenzamine (PBA) on the outflow of NA in response to nerve stimulation will be discussed in detail in a later section and will therefore be only briefly mentioned here. While PGE_2 depresses the NA outflow in response to nerve stimulation PBA strongly potentiates this response, in the cat spleen (Brown and Gillespie 1957). In the present study exogenous PGE_2 was found to counteract this effect of PBA, leading to NA output figures close to those obtained during control stimulations in the absence of PBA and PGE_2 (Fig. 1). Since PBA blocks the mechanical motor response to nerve stimulation, the inhibitory effect of PGE_2 on NA outflow in this case can only be explained in terms of a prejunctional action. The fact that PBA has also been shown to abolish the outflow of endogenous PGE_2 in response to nerve stimulation in the spleen (Davies, Horton and Withrington 1967) raises the intriguing question whether the mechanisms of the potentiating effect of PBA on NA outflow in response to nerve stimulation could at least in part be due to "removal" of endogenous PGE_2 acting as brake on the transmitter release from sympathetic neurons.

4 *Effect of PGE and PGE in the guinea pig vas deferens (Paper 3)* At low concentrations PGE and PGE_2 were found to inhibit the contractions of the isolated guinea pig vas deferens elicited by transmural (postganglionic) nerve stimulation (Fig. 1). On the other hand higher concentrations, of the order of 100 ng/ml, potentiated the response to this type of stimulation. The contractile effect of NA was generally slightly enhanced even by doses found to inhibit the response to nerve stimulation.

The action of PGE on the hypogastric nerve—vas deferens preparation shows considerable species variation. In the guinea pig Mantegazza and Naimzada (1965) found that the contractile responses to adrenaline and to nerve stimulation were enhanced by PGE while it had no effect on the responses in rat and cat and in the rabbit preparation actually depressed the responses to both types of stimulation. In line with this last mentioned effect is the report that PGE both *in vivo* and *in vitro* reduces contractions

of the rabbit vas deferens induced by adrenaline (Holmes, Horton and Main 1963) In the guinea pig vas deferens Graham and Al Katib (1967) found that PGE_1 markedly enhanced the contractile effect of NA. In the same preparation PGE has also been reported to cause minor changes in the response to nerve stimulation varying from slight inhibition to moderate potentiation (Sjöstrand and Swedin 1968)

These data show that PGE_1 and PGE_2 alter the contractile effect of nerve stimulation or of exogenous catecholamines in the vas deferens, although there seems to be considerable species variation in the mode of response. In view of the present results it seems likely that in anyone species the dose of PGE_1 or PGE_2 used determines whether the action will be stimulatory or inhibitory

The NA overflow from the guinea pig vas deferens in response to nerve stimulation was not measured. However inhibition by PCE_1 and PGE_2 of the contractile response to nerve stimulation and potentiation by the same dose of the response to exogenous NA cannot be wholly explained by post junctional effects. By analogy with the results from the cat spleen it must be concluded that the two compounds in addition to having postjunctional effects also act prejunctionally decreasing the amount of NA released from the nerve terminals. A dual action of PGE_1 and PGE_2 might explain the inhibition of the response to nerve stimulation at low doses of PGE_1 and PGE_2, and potentiation by high doses. At a low dose the two agents act predominantly at the prejunctional level, reducing the release of transmit ter and thus the contractile response. At a higher dose the release of NA from the nerve terminals is probably still reduced, judging from the obser vations in the spleen but the progressively increasing potentiation of the smooth muscle stimulating effect of the NA released, in the high PGE and PGE_2 dose range, may thus lead to a net increase of the contractile response.

The observations on the guinea pig vas deferens discussed here have recently been confirmed by other authors, using the same preparation and PGE_2 (Ambache and Zar 1970) Additional support for a prejunctional action of PGE_1 and PGE_2 is the finding that after complete denervation of the guinea pig vas deferens the contractile response to direct electrical stim ulation is potentiated by PGE in doses, which in organs with intact nerve supply causes inhibition of the response to transmural nerve stimulation (Sjöstrand and Swedin 1970)

5 *Effect of PGE in the rabbit heart (Paper 4)* Stimulation of the sym pathetic nerves to the perfused rabbit heart for 30 sec periods at 1 5 and

10/sec produced a frequency—dependent increase in heart rate and in myocardial contractile force. Infusion of PGE_1 in doses ranging form 8×10^{-8} to 5×10^{-7} M markedly and reversibly reduced the chronotropic response. The inotropic response to nerve stimulation was also depressed by PGE_1 but to a lower extent than the chronotropic response

In contrast to the action on responses induced by nerve stimulation PGE_2 caused only weak and inconsistent effects on the chronotropic and inotropic responses to exogenous NA.

PGE_2 in the same doses as mentioned above markedly and reversibly depressed the outflow of NA to sympathetic nerve stimulation (Fig 1)

In connection with infusion of PGE_2 most fluorimetric analyses of NA in the effluent from perfused hearts were performed after stimulation at a frequency of 10/sec. However PGE_2 was found to inhibit the outflow of NA virtually to the same extent when the nerves were stimulated at 5/sec. At 1/sec stimulation the amount of NA in the effluent was too low to permit satisfactory evaluation. However judging from the inhibition of the effector response to nerve stimulation at 1/sec there is reason to believe that PGE at this frequency produced the same type of prejunctional interference as seen with higher frequencies of stimulation.

Recent observations made in the rabbit heart have shown that PGE in a similar manner as PGE_2 reduces the outflow of NA in response to nerve stimulation (Hedqvist and Wennmalm 1970) The chronotropic and inotropic responses to nerve stimulation are also reduced, while those to NA are only weakly and inconsistently affected by PGE_1 The only difference between PGE and PGE in the action on the rabbit heart seems to be that PGE more markedly reduces the inotropic response to nerve stimulation, while PGE appears to be more operative on the chronotropic response.

(*Concluding remarks* The data presented clearly demonstrate a prejunctional action of PGE and PGE_2 on the sympathetic neuromuscular system in the three tissues studied. This prejunctional effect apparently consists in direct inhibition of the process of NA release from the sympahtetic nerve terminals, as will be documented in the following section.

As to the relative activity in this respect there seems to be a certain species and or tissue difference Thus PGE_2 is at least more consistently effective than PGE in the cat spleen, while in the guinea pig vas deferens and in the rabbit heart PGE seems to be equally or more active This is in agreement with observations in other systems, according to which either PGE_1 or PGE_2 may be *quantitatively more efficient* although they produce qualitatively similar responses Horton and Main 1963 1966)

The results on the heart indicate that the two compounds exert their inhibitory action throughout the physiological range of impulse activity in sympathetic nerves (*cf* Folkow 1952 Celander 1954)

The demonstration that PGE and PGE_2 by a prejunctional action inhibit the sympathetic neuromuscular system in cat spleen guinea pig vas deferens, and rabbit heart makes it likely that this action of the two compounds is not confined to the animal species and tissues studied, but constitutes a principle generally present in sympathetically innervated tissues. Compatible with this view are several observations in other tissues, such as rat epididymal fat pad and rabbit vas deferens and oviduct (Berti and Usardi 1964 Mantegazza and Naimzada 1965 Brundin 1968)

The adrenal medulla, although homologue to sympathetic postganglionic nerves, does not seem to fit into this scheme. PGE_2 does not inhibit the catecholamine releasing effect of carbachol or acetylcholin in the isolated perfused adrenal gland or of splanchnic nerve stimulation *in vivo* (Hedqvist and Stjärne 1970)

B. Analysis of the prejunctional action of PGE_1 and PGE_2 in the sympathetic neuromuscular system.

Previous information concerning the mechanisms whereby PGE_1 and PGE_2 interact with the function of smooth and cardiac muscle seems to be exclusively related to postjunctional events. Thus hypopolarization or depolarization of the cell membrane has been supposed to explain enhancing or stimulating action (Khairallah Page and Türker 1967 Paton and Daniel 1967) The enhancing effect of PGE and PGE_2 has also been concluded to be mediated intracellularly by facilitation of excitation contraction coupling (Clegg, Hall and Pickles 1966) Antagonism of the smooth muscle response to NA has been explained by binding of NA to the adrenergic receptor (Clegg 1966) or by an intracellular action rendering the ionic calcium inactive (Strong and Bohr 1967)

The demonstration in the present experiments of a prejunctional action of the two compounds in the sympathetic neuromuscular system, prompted an attempt to study the target for this effect. However for technical reasons this is difficult since the chain of events from the propagated impulse in the postganglionic nerve fibre to extrusion of transmitter into the synaptic cleft may involve many steps and is only fragmentarily known It therefore must be kept in mind that the conclusive answer cannot be given at present and that data to be presented here provide only circumstantial evidence for or against some of the possible levels of action.

1 *Nerve conduction (Paper 6)* PGE injected retrogradely in the lingual artery does not inhibit contractions of the nictitating membrane induced by preganglionic nerve stimulation (Holmes, Horton and Main 1963) Nor is the ganglionic transmission in the superior cervical ganglion affected by PGE or PGE_2 (Kayaalp and McIsaac 1968) In the present study it was found that in bovine splenic nerves PGE_2 even in high concentration (3×10^{-4} M) did not alter the amplitude of action potentials elicited by electrical stimulation of the nerves (Fig 4) While these observations indicate that PGE and PGE_2 do not act as blockers of ganglionic transmission or of impulse conduction in sympathetic nerve trunks, they do not exclude the possibility that the two compounds may inhibit the propagation of nerve impulses in the fine fibres of the sympathetic ground plexus. Such intraterminal blocks have been suggested for drugs like guanethidine and bretylium (*cf* Iversen 1967) although they do not prevent the transmission of impulses in the postganglionic nerve trunk (Exley 1957 1960) However this action seems to be coupled to a selective accumulation in sympathetic nerves (Boura *et al* 1960 Bisson and Muscholl 1962) Since tritium labelled PGE_1 does not appear to accumulate in sympathetic nerves (Hansson and Samuelsson 1965) it does not seem likely that PGE and PGE_2 act by causing intraterminal block of nerve conduction.

PGE and PGE_2 do not affect the secretion of catecholamines from the adrenal medulla Miele 1969 Hedqvist and Stjärne 1970) In this tissue the resting membrane potential of the medullary cells is rather low and stimulation does not lead to reversal and overshoot but only to a minimal local depolarization Douglas, Kanno and Sampson 1967 Kanno and Douglas 1967) There does not seem to be any reason to believe that the resting membrane potential or the reversal and overshoot in response to stimulation in the sympathetic nerve terminals should differ qualitatively from that of other neurons. The differencies in electrophysiological properties between the two tissues might thus possibly explain why the release of NA from sympathetic nerves is inhibited by PGE and PGE_2 and the hormone secretion from the adrenal medulla is not.

2. *Stimulus secretion coupling (Paper 6)* Very little is known about the mechanism by which nerve impulses promote the release of NA from sympathetic nerves. However it seems established that the release process is critically calcium dependent (*cf* Ferry 1966 Simpson 1968) Thus the release of NA from the isolated rabbit heart as a result of stimulation is considerably reduced when the calcium content of the perfusion medium is lowered (Hukovic and Muscholl 1962) Similarly the release of NA from the

cat colon in response to nerve stimulation is prevented in calcium—free medium (Boullin 1966) In the cat spleen the amount of NA overflowing in response to nerve stimulation varies whit the calcium content in the per fusion medium (Kirpekar and Misu 1967) In several other preparations the effector response to sympathetic nerve stimulation is reduced or abol ished when calcium is omitted from the medium (Kuriyama 1964 Burn and Gibbons 1964 1965) Since the release of NA from sympathetic nerves in response to nerve stimulation is a calcium—dependent process, it seems possible that PGE_1 and PGE_2 may inhibit the release process by interfering with the availability of calcium. In view of this it is interesting that there seems to be, at the postjunctional level, a definite but so far unexplained interrelationship between PGE_1 and calcium. Thus in the frog and guinea pig heart PGE_1 increases the exchange of calcium (Arienti, Piccinini and Pomarelli 1967 Klaus and Piccinini 1967) On the other hand in rat stomach fundic strips the uptake of calcium is not increased by PGE while in this tissue the spontaneous loss of radiocalcium is affected by PGE_1 in a biphasic manner an initial acceleration later followed by retardation of the loss (Coceani *et al* 1969) In several tissues the action of PGE seems to vary with the calcium content in the medium. Thus, in the guinea pig heart the chronotropic and inotropic responses to PGE_1 are increased when the calcium concentration in the perfusion medium is lowered (Mantegazza 1965) Similarly the potentiating effect of PGE on the contractile effect of nerve stimulation in the guinea pig vas deferens, and the antilipolytic effect of the compound in rat epididymal fat, is mag nified by reducing the calcium content in the incubation medium (Mante gazza and Namyada 1965 Fassina and Contessa 1967) On the other hand, addition of calcium counteracts the inhibitory effect of PGE_1 on pla telet electrophoretic mobility (Emmons *et al* 1967) Similarly the short circuit current response to PGE_1 in the frog skin is reduced by excess cal cium (Fassina, Carpenedo and Santi 1969)

In the present study an increase of the calcium content in the medium perfusing the spleen was found to counteract the inhibitory action of PGE_2 and to restore to near normal values the amount of NA overflowing in response to nerve stimulation (Fig 1) This observation may indicate that PGE_2 interferes with transmitter release by inhibition of calcium influx induced by depolarization or by preventing calcium from reaching specific reactive sites in the interior of the neuron. Additional support for this view is given by the absence of action of PGE_2 on the NA releasing effect of TA, which is calcium—independent (Burn and Gibbons 1965 Lindmar Löffel holz and Muscholl 1967 Thoenen, Hürlimann and Haefely 1969) How-

ever the observation that the secretion of catecholamines from the adrenal medulla, which is calcium-dependent (Douglas and Rubin 1961 1963) is not affected by PGE_1 and PGE_2 (Miele 1969 Hedqvist and Stjärne 1970) might appear to make the calcium hypothesis less likely

3 *VI storage particles (Paper 6)* In recent years it has been increasingly clear that the NA store in postganglionic nerve endings cannot be regarded as a single homogenous pool (Trendelenburg 1961) Nerve stimulation has been assumed to release NA directly from the granules (Carlsson 1965) However the view has also been proposed that NA stored extragranularly possibly in the axonal membrane itself or closely associated with it might represent the small pool from which transmitter release occurs on arrival of nerve impulses (Euler 1966 1970 Stjarne 1966 Folkow Häggendal and Lisander 1968 Stjarne Hedqvist and Bygdeman 1969) Since PGE_1 and PGE inhibit the release of NA from the neurons induced by nerve stimulation it appeared desirable to study the effect of the two compounds on pharmacologically induced release of NA. For this purpose TA was chosen. The exact source from which NA is released by TA is not definitely known (*cf* Iversen 1967) *but it appears to be different from that from which NA is* released by nerve stimulation (Axelrod *et al* 1962) According to *in vitro* evidence TA accelerates the spontaneous loss of NA from nerve granules partly by a direct action, partly by inhibition of reuptake, (Euler and Lishajko 1968) The same mechanism may be relevant also for the *in vivo* effects of TA, since the temperature dependence of TA induced release of NA from the spleen is similar to that of spontaneous release of NA from isolated nerve granules (Wennmalm, Hedqvist and Stjärne 1970)

In the present study PGE_2 in a dose which caused a marked reduction of NA outflow to nerve stimulation, did not alter the efflux of NA induced by intraarterial infusion of TA into the isolated cat spleen (Fig 2) Moreover PGF and PGE (3×10^{-4} —3×10^{-3} M) did not affect the spontaneous loss of NA from incubated bovine splenic nerve granules (Hedqvist unpubl)

Thus processes directly associated with release of NA from nerve granules do not seem to be affected by PGE and PGE_2 However it is questionable whether NA release induced by nerve stimulation, even if it involves mobilization of granule bound NA, is immediately and directly dependent on the release from granules (for references, see above) The conclusion is so far negative Inhibition of spontaneous or TA induced release of NA from nerve granules does not seem to be the mechanism by which PGE and PGE depress the release of NA in response to nerve stimulation This indicates that the two compounds do not directly attack the nerve

granules themselves but rather some other link involved in the re[illegible] transmitter from sympathetic nerve terminals.

4 *NA synthesis (Paper I 2 6).* In the cat spleen experim labelled NA was infused in order to monitor the outflow of to[illegible] radioactive material released in response to nerve stimulation represent mostly unchanged NA (Hedqvist, Olivero and Str If newly synthesized NA is preferentially released by nerve stim Kopin *et al* 1968) an inhibitory action of PGE_1 and PGE_2 on t sis would be expected to cause a relative rise in the specific acti /gNA) of the NA in the effluent from the organ. However thi the case. PGE_1 and PGE_2 caused a closely parallel reduction of the of radioactive material and of fluorimetrically determined NA. Th present study gives no support for the possibility that inhibition o synthesis is of any major importance for the inhibitory action of the two compounds on sympathetic neurotransmission.

5 *Release of transmitter into the extracellular space.* Very little is known about the mechanisms by which NA, upon arrival of nerve impulses, is transported to the exterior of the axon. Nor do the present experiments provide any evidence for or against this process as possible candidate for action of PGE_1 and PGE_2. The striking differences in action of the two compounds on transmitter release from sympathetic nerves versus catecholamine secretion from the adrenal medulla, as well as the definite differences in secretory properties between the two tissues, may however justify a comment.

The feasibility for hormone secretion from the adrenal medulla by exocytosis seems to be well established (*cf* Douglas 1968). The evidence is that catecholamines secreted in response to splanchnic nerve stimulation or to acetylcholine appear in the effluent, accompanied by all soluble components of the medullary granules, in the same proportions in which they occur in the granules (Banks and Helle 1965, Douglas 1966, Blaschko *et al* 1967, Kirshner, Sage and Smith 1967). The exocytosis process has also been claimed to be all or none in character, leading to the extrusion of the total content of soluble components of individual granules (Viveros, Arqueros and Kirshner 1969).

Transmitter release from sympathetic nerves has also been postulated to occur by exocytosis (Douglas 1968). However the adrenal medulla and sympathetic nerves differ *in vivo* in catecholamine turnover as well as in sensitivity to drugs. Also the amine storage granules demonstrate certain

dissimilarities, such as in spontaneous amine—ATP release, response to drugs, and resistance to physical changes in the incubation medium (Stjärne 1964). Although stimulation of the sympathetic nerves of the spleen has been reported to involve the release of both NA and of presumably granular protein, there appears to be a large excess of NA in relation to protein in the effluent (De Potter *et al.* 1969). PBA induced potentiation of the outflow of NA in response to nerve stimulation does not cause increased efflux of granular protein (Moerman, personal communication). Similarly, after labelling the adenine nucleotides of catecholamine storage granules, catecholamine secretion from the perfused cat adrenal medulla, in response to carbachol, is accompanied by efflux of labelled nucleotides, while NA release from the isolated spleen, in response to nerve stimulation, is not. (Stjärne, Hedqvist and Lagercrantz 1970). Moreover, it has been enunciated that the amount of NA released by each nerve impulse corresponds to only a fraction of the NA content of individual nerve granules (Folkow, Häggendal and Lisander 1968, Stjarne, Hedqvist and Bygdeman 1969). On the basis of evaluation of the differences, as well as the similarities in secretory properties of sympathetic nerves and adrenal medulla, it has been postulated that exocytosis may be the mechanism whereby the process of transmitter release from sympathetic nerves is initiated, leading to breakthrough of the axonal membrane, and that other mechanisms take over for the continued transmitter efflux (Stjärne 1970).

Besides the differences in electrophysiological properties of sympathetic nerves and adrenal medulla, these dissimilarities in secretory properties might be sufficient reason why PGE_1 and PGE_2 affect transmitter release from the sympathetic nerves but not hormone secretion from the adrenal medulla.

In this context it is interesting that large amounts of PGE_2 are released from the spleen in response to nerve stimulation (Davies, Horton and Withrington 1967) accompanied by fading of the mechanical contractile response. The rate of fading progressively increases with the stimulation frequency and occurs in spite of an essentially unchanged NA content of the organ (Stjarne and Hedqvist to be published). This might be due to depletion of a small "transmitter pool". However, it might also represent active control of the release of transmitter exerted by endogenous PGE_2 secreted either from the smooth muscle cells or from other sources, possibly the nerve terminals themselves.

6. *Effect of PGE and PGE on the mechanisms of inactivation of transmitter released.* At least theoretically it is conceivable that PGE_1 and

PGE_2 could depress the motor response to nerve stimulation as well as the outflow of NA released by promoting its enzymatic inactivation or physical binding, in the nerves or extraneuronally

a) *Enzymatic degradation (Paper 1 2 6)* Considerable uncertainty remains about the relative importance of O-methylation or oxidative deamination in the inactivation of NA released from sympathetic nerves. Studies of the fate of intravenously administered labelled NA have shown that O-methylation is the main metabolic pathway for enzymatic inactivation of circulating NA (Axelrod *et al.* 1958) while deamination by monoamine oxidase represents the means for intraneuronal metabolism of NA not protected by binding (Kopin 1964) However neither metabolic route appears to be essential for the termination of the physiological action of NA released from the neurons in response to nerve stimulation (Brown and Gillespie 1957 Hedqvist and Stjärne 1969)

Theoretically PGE_1 and PGE_2 might reduce the effective outflow of NA by increasing the metabolic degradation of NA released. In the present study no direct analyses was performed of NA metabolites in the effluent from the cat spleen, in the presence of the two compounds. However the experiments seem to provide some evidence that the two compounds do not alter the rate of metabolic degradation of NA released from the nerve terminals. After labelling the NA stores with tritiated NA in order to monitor the efflux of NA from the spleen, PGE_1 and PGE_2 were found to cause simultaneous, closely parallel and reversible reduction of the efflux of total radioactive material and of fluorimetrically determined NA in response to nerve stimulation. Thus the conclusion may be drawn that PGE_1 and PGE_2 do not depress the outflow of NA released from sympathetic nerves by means of increased metabolic degradation.

b) *Physical binding (Paper 2)* Binding in the tissues represents a quantitatively important route for rapid removal of catecholamines from the circulation (Lund 1951 Whitby Axelrod and Weil-Malherbe 1961 Muscholl 1960 1961 Strömblad and Nickerson 1961) At physiological rates only small amounts of NA appear in the effluent from stimulated tissues (Celander 1954 Brown and Gillespie 1957) On the other hand the out flow can be greatly increased by drugs which depress uptake of NA into sympathetic nerves (Brown and Gillespie 1957 Brown 1965) These observations have led to the consideration that reuptake is the main mechanism for inactivation of NA released from sympathetic nerves (Paton 1960 Blakeley Brown and Geffen 1964 Stjärne 1964 Brown 1965 Haefely

Hürlimann and Thoenen 1965 Gillespie and Kirpekar 1965 Hedqvist and Stjarne 1966 1969 Geffen 1967 Folkow Häggendal and Lisander 1968)

The possibility that PGE_2 depresses NA outflow on nerve stimulation by facilitating reuptake of locally released NA, was studied in the cat spleen It was found that PGE_2 did not appreciably alter removal of tritium-labelled exogenous NA infused into the organ (Fig 3) Thus there is no reason to believe that action at this level can explain the observed prejunctional effects of PGE and PGE_2

7 *Physiological aspects on pharmacological interference with the effect of* PGE_1 and PGE_2.

a) *Interaction between* PGE_2 *and phenoxybenzamine (Paper 5)* Phenoxybenzamine (PBA) has been shown to cause a huge increase of the outflow of NA from the cat spleen in response to nerve stimulation (Brown and Gillespie 1957 Kirpekar and Cervoni 1963 Thoenen, Hürlimann and Haefely 1964 a, Hedqvist and Stjärne 1966) Since PBA inhibits the uptake of NA into sympathetic nerves (Hertting Axelrod and Whitby 1961 Axelrod, Hertting and Potter 1962, Farrant, Harvey and Pennefather 1964 Gillespie and Kirpekar 1965) inhibition of reuptake of NA released by nerve stimulation has been regarded as the main mechanism responsible for the increased outflow of NA in the presence of the drug (Thoenen Hürlimann and Haefely 1964 a) However cocaine and desmethylimipramine (DMI claimed to be at least equally potent and more specific as blocker of NA uptake (Iversen 1965 Gillespie and Kirpekar 1965 Malm for produce only a moderate increase or no change of NA efflux induced by nerve stimulation (Kirpekar and Cervoni 1963 Thoenen Hürlimann and Haefely 1964 b Geffen 1965) This has made it necessary to assume that the differences in effect are due to mechanical factors, the α-receptor blocking properties of PBA, by preventing the vascular responses to NA promoting washout of NA released while the intense vasoconstriction in the presence of cocaine and DMI counteracts effective washout of the NA released / Rosell Kopin and Axelrod 1963) In the case of phentolamine which is a rather weak inhibitor of NA uptake (Hertting Axelrod and Whitby 1961 almost the whole of its action on transmitter overflow may be related to the effect on blood flow (Hertting 1965) On the other hand α-receptor blockade *per se* has been reported to cause but a small increase of the NA outflow from the spleen in response to nerve stimulation (Thoenen Hurlimann and Haefely 1964 a)

This may justify the search for additional levels of action of PBA besides

inhibition of NA reuptake and blockade of α receptors, to explain the large outpouring of NA in the presence of the drug

In the dog spleen, nerve stimulation causes the simultaneous appearance of NA and PGE_2 in the effluent from the organ. In the presence of PBA is the outflow of NA markedly increased while that of PGE_2 is completely abolished (Davies, Horton and Withrington 1967) This observation seems particularly interesting since the present study provides good evidence that exogenous PGE_2 depresses the release of NA from sympathetic neurons in response to nerve stimulation and also counteracts the potentiating effect of PBA on this process, leading to NA output figures close to those obtained during nerve stimulations in the absence of PBA and PGE_2 (Fig 1)

It is thus conceivable that PBA, in addition to causing α receptor blockade and inhibition of NA uptake, promotes increased transmitter outflow in response to nerve stimulation by preventing the local release of endogenous PGE_2 possibly normally triggered by contraction of smooth muscle, and representing a braking mechanism controlling the continued release of NA from the sympathetic neurons. Such an action might well explain the striking differences between the effects of PBA, and of cocaine and DMI on the outflow of NA in response to nerve stimulation.

An interaction between PGE_2 and PBA is of particular interest because it provides support for the hypothesis that endogenous PG s of the E series may act as feed back inhibitors of neurotransmitter release from sympathe tic nerve terminals.

b) *Interaction between PGE and atropine (Paper 3 4 6)* Using crude prostaglandin extract, Euler (1934) found its action on rabbit blood pressure and intestinal smooth muscle to be resistant to administration of atropine. Similar results have been obtained with pure PGE Thus atropine does not inhibit the blood pressure reducing action or the contractile effect on intestinal smooth muscle of PGE_1 in the rabbit (Bergström *et al* 1959 a) Nor is the contractile response of the rat uterus to PGE_1 or the relaxation of isolated vascular strips caused by PGE altered by atropine (Paton and Daniel 1967 Strong and Bohr 1967)

In the present study atropine was found to counteract or in some cases to abolish the inhibitory action of PGE on the contractile response to post ganglionic nerve stimulation in the guinea pig vas deferens preparation, while the contractile effects of nerve stimulation or of exogenous NA were only very slightly affected. However atropine did not alter the effects of PGE_2 in spleen and heart. Atropine also appears to be devoid of effect on the inhibitory action of PGE_1 on NA release and on the effector responses

to sympathetic nerve stimulation in the rabbit heart (Hedqvist and Wennmalm 1970). Species differences or differences in nerve supply (*cf.* Sjöstrand 1965) may possibly account for this discrepancy.

C. Possible role of PGE_1 and PGE_2 in sympathetic neuromuscular transmission (Paper 1, 2, 3, 4, 5)

Already 1936 it was supposed that the strong smooth muscle stimulating properties of prostaglandin could be of importance for the emptying of the male accessory genital glands (Euler 1936). Further studies led to the hypothesis that prostaglandin acts by facilitating passive sperm transport in the uterus by a local action (Eliasson 1959). The discovery that prostaglandin is not a single substance but rather a family of biologically highly active compounds with a broad distribution in animal tissues has led to the proposal of several other functions.

PGs have been suggested to act as transmitters or transmitter modulators in the central nervous system (Horton and Main 1967). The experimental basis is that PGs are natural constituents of the brain and the spinal cord (Samuelsson 1964 b, Coceani and Wolfe 1965, Horton and Main 1967), that they are released from the central nervous system both spontaneously and in response to electrical and chemical stimulation (Coceani and Wolfe 1965, Ramwell and Shaw 1966, Ramwell, Shaw and Jessup 1966), and that they upon administration produce distinct effects (Horton 1964, Wanzuno, Bradley and Wolstencroft 1966, Hoffer, Siggins and Bloom 1969). Basically the same type of experimental evidence has led to the proposal that PGE_1 by an action on the adenyl cyclase—cyclic AMP system may modulate lipolysis and gastric secretion (Bergström 1967, Horton 1969).

PGE and PGE have been shown to be present in many sympathetically innervated tissues (Ramwell and Shaw 1967, Karim, Sandler and Williams 1967, Karim, Hillier and Devlin 1968). As to their cellular distribution *information is still incomplete*. In the spleen it appears that PGE_2 may be released from smooth muscle cells (Gilmore, Vane and Wyllie 1968). However some drugs have been reported to cause the release of PGE_2 from the spleen in the absence of smooth muscular contraction, indicating that PGE may be derived from other sources than the muscle cells (Gilmore, Vane and Wyllie 1969). PG or PG-like material has also been demonstrated in several types of nerve tissue (Kataoka, Ramwell and Jessup 1967, Karim, Hillier and Devlin 1968). Thus the possibility should no be overlooked that some PGE and PGE_1 may be located in sympathetic nerve ter-

minals, and become activated by mechanisms involved in transmitter release.

In the present study PGE and PGE_2 were found to markedly and reversibly inhibit the sympathetic neuromuscular transmission in the cat spleen, in the guinea pig vas deferens and in the rabbit heart. In the cat spleen the release of NA from the sympathetic neurons was markedly reduced by exogenous PGE_2 in concentrations comparable to those which may occur as result of release of endogenous PGE_2 from the dog and cat spleen in response to nerve stimulation (Davies, Horton and Withrington 1967 1968 Gilmore, Vane and Wyllie 1968) PBA causes a large increase of the outflow of NA from the spleen in response to nerve stimulation (Brown and Gillespie 1957) and simultaneously prevents the release of PGE_2 (Davies, Horton and Withrington 1967) Administration of PGE_2 was found to counteract the action of PBA on transmitter overflow leading to NA output figures close to those obtained in the absence of PGE_2 and PBA.

On the basis of their natural occurrence and availability for release, as well as the demonstrated effects of exogenous PGE and PGE_2 it is tempting to assume that endogenous PGE and PGE_2 may play a physiologically significant regulatory role in sympathetically innervated tissues, especially in view of the low concentrations needed to depress the effect of sympathetic nerve stimulation. This homeostatic action of PGE_1 and PGE_2 possibly but not necessarily triggered by the mechanical response to nerve stimulation, appears to be exerted by inhibition of NA release from the sympathetic neurons and, to some extent by inhibition of the effector response to NA released. However direct proof of this hypothesis cannot be given at present, and may probably be obtained only when specific inhibitors of PG synthesis and action are available.

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outflow in response to nerve stimulation. In the same preparation the NA releasing action of TA was not affected by PGE_2. The fact that NA release induced by nerve stimulation is critically calcium—dependent, while that by TA is not, indicates that PGE and PGE_2 might interfere with transmitter release by inhibition of calcium influx induced by depolarization, or by preventing calcium from reaching specific reactive sites in the interior of the neuron. However the observation that the calcium—dependent secretion of catecholamines from the adrenal medulla is not affected by PGE_1 and PGE_2 might appear to make the calcium hypothesis less likely.

In bovine splenic nerves PGE_2 did not alter the amplitude of action potentials elicited by electrical stimulation of the nerves. While this observation indicates that the compounds do not act as blockers of impulse conduction in sympathetic nerve trunks, it does not exclude the possibility that PGE_1 and PGE_2 may inhibit the propagation of nerve impulses in the fine fibres of the sympathetic ground plexus.

The striking difference in action of PGE and PGE_2 on transmitter release from sympathetic nerves versus catecholamine secretion from the adrenal medulla, as well as the definite differences in secretory properties between the two tissues were discussed, and it was tentatively suggested that the two compounds might act prejunctionally by interfering with the transport of NA into the extracellular space.

In the cat spleen a definite interaction was observed between PGE_2 and PBA. Since PBA is known to abolish the efflux of endogenous PGE_2 from the spleen in response to nerve stimulation and since the potentiating action of PBA on NA outflow to nerve stimulation in the same tissue seems difficult to explain only in terms of its α-receptor blocking and uptake inhibiting properties, it was suggested that part of the action of PBA on transmitter release from the spleen may be due to inhibition of local release of PGE_2 acting as an endogenous braking mechanism on transmitter release.

Atropine was found to counteract the inhibitory action of PGE_1 on the contractile response to nerve stimulation in the vas deferens preparation. However a similar action of atropine could not be established when PGE_2 was used in the spleen and heart experiments. It was suggested that species differences or differences in nerve supply might account for this discrepancy.

On the basis of previous information of their natural occurrence and availability for release from sympathetically innervated tissues, as well as the demonstrated effects of PGE_1 and PGE_2 in the present study it was suggested that the two compounds may play a physiologically significant regulatory role in sympathetically innervated tissues. This homeostatic ac-

tion of PGE_1 and PGE_2 appears to be mediated both by inhibition of NA release from the nerve terminals and by inhibition of the effector response to NA released.

The differences in tissue distribution of PGE_1 and PGE_2 as well as the quantitative differences between them, with respect to the observed predominance of pre or postjunctional effects, invites the more general speculation that the spectrum of prostaglandins, specific for each individual tissue, might serve the function of controlling different neuroeffector junctions, possibly including those with transmitters other than catecholamines, and make the motor responses rapidly or slowly adapting

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ACTA PHYSIOLOGICA SCANDINAVICA
SUPPLEMENTUM 346

LIMITING FACTORS FOR AEROBIC MUSCLE PERFORMANCE

The Influence of Varying Oxygen Pressure and Temperature

By

Lennart Kaijser

STOCKHOLM 1970

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SUPPLEMENTUM 346

From the Department of Clinical Physiology Karolinska Sjukhuset
Stockholm Sweden

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Lek. t. AB Thule Stockholm 1970

Contents

Part A.	INTRODUCTION	5
I	*Aim of study*	5
II	*Possible limiting factors for aerobic work performance*	6
	1 Dimensions of the respiratory and circulatory organs	6
	2. Pulmonary function	6
	3 Cardiovascular function	7
	4 Muscle metabolism	8
	5 Conclusions	9
Part B	PRESENT INVESTIGATIONS	10
I	*General procedures and methods*	10
	1 Modes of exercise	10
	2 The pressure chamber	11
	3 Risks and methodological problems during experiments at 3 atmospheres	13
	4 Catheterization procedures	14
	6. Definitions, symbols and abbreviations	20
II	*Control experiments on the determination of forearm work capacity and its reproducibility*	21
III	*Control experiments for the study of the adaptation to leg work during normoxia at 3 atmospheres*	26
IV	*Adaptation to exercise during oxygen breathing at 3 atmospheres*	29
	Introductory remarks	29
	1 *Adaptation to dynamic forearm work*	30
	2 *Adaptation to submaximal leg work*	41
	PRELIMINARY STUDY OF HEART RATE AND BLOOD LACTATE	42
	CARDIAC OUTPUT INTRAVASCULAR PRESSURES AND BLOOD GASES STUDIED BY MEANS OF HEART CATHETERIZATION	44
	3 *Adaptation to maximal leg work*	52
V	*Adaptation to exercise at decreased inspiratory oxygen pressure*	59

	Introductory remarks	59
	1 *Adaptation to dynamic forearm work*	60
	2. *Adaptation to maximal leg work*	66
VI	*Adaptation to dynamic forearm work at decreased muscle temperature*	76
Part C	GENERAL DISCUSSION	80
Part D	SUMMARY AND CONCLUSIONS	85
	ACKNOWLEDGEMENTS	88
	REFERENCES	89

CHAPTER I

Aim of the study

In spite of the great number of data available on respiratory and cardiovascular adaptation to exercise (Asmussen and Nielsen 1955 Asmussen 1965 Bevegård and Shepherd 1967) it remains to be established what factors normally limit aerobic work capacity Some authors seem to consider the cardiovascular system the limiting factor (*cf* Bevegård and Shepherd 1967) although evidence against this assumption is also found in the literature (Doll, Keul and Marwald 1968 Jöbsis and Stainsby 1968)

The present study is an attempt to supply further experimental data to elucidate the problem. The basic assumption was, that if the ability of the cardiovascular system to furnish the exercising muscles with oxygen is the main limiting factor for aerobic work capacity an increase in arterial oxygen content should increase the aerobic muscle performance. If, on the other hand, any step in the substrate metabolism and the electron transport chain is limiting the maximal oxygen utilization rate, an increase in arterial oxygen content should not significantly alter muscle performance.

A substantial increase in arterial oxygen content is possible only by oxygen breathing in a hyperbaric environment, when about 20 ml O_2/l blood is added in physical solution per atmosphere oxygen pressure. In the first part of the present study the influence on work performance of oxygen breathing at hyperbaric pressure (3 ata) was investigated.

To establish the relative importance of the oxygen amount supplied to the active muscle by the blood, a comparison with the effect of a decreased arterial oxygen content during exercise at decreased inspiratory oxygen pressure was also made. Furthermore, to test the significance of metabolic factors as limiting for muscle performance the effect of a decrease in temperature of the active muscles, presumed to retard the maximal metabolic rate, was studied.

The possibility exists that the circulatory system is more important as a limiting factor for aerobic work capacity when large muscle groups are utilized and a large demand is placed on the central circulation, than when work is performed with small muscle groups. Consequently it was considered of interest to study the adaptation to work with a small (the fore arm) as well as a large (the legs) muscle group.

CHAPTER II

Possible limiting factors for aerobic work performance

The energy for heavy muscle activity of short duration is derived from the hydrolysis of high energy phosphate, available to the contractile system in the active muscle cell (Lundsgaard 1930, Lohmann 1934, Margaria 1968). For a continuous muscle activity a resynthesis of high energy phosphate must occur. During limited periods of time, up to 1-3 minutes, the major part of the energy needed for the resynthesis may be yielded by anaerobic glycolysis. For longer exercise times the maximal rate at which exercise can be performed depends on the rate of aerobic metabolism.

The aerobic work capacity in the present study, defined as the capacity for work of 5-10 minutes duration, is dependent on oxygen supply by 1) ventilation of the lungs, 2) diffusion of oxygen between the lung alveoli and the blood in the lung capillaries, 3) the cardiac output, 4) blood perfusion of the muscle tissue, 5) diffusion from the capillaries to the muscle cells and 6) the substrate metabolism and the electron transport in the respiratory chain. A short survey will be given of the possible role of the different steps as rate limiting for aerobic muscle performance.

1. Dimensions of the respiratory and circulatory organs

Significant correlations have been demonstrated between the aerobic work capacity and dimensional measurements of the lungs and the cardiovascular system (Kjellberg *et al.* 1949, Åstrand 1952, Spostrand 1967, Åstrand *et al.* 1963, Holmgren and Åstrand 1966). These correlations are to be considered more as an indication of the morphological adaptation of the oxygen transport system to increased demands by the body than as evidence that cardiovascular or lung function limits the oxygen transport.

2. Pulmonary function

[illegible] at an intensity corresponding to the maximal oxygen uptake. [illegible] Healthy individuals [illegible] less than ½ of his maximal [illegible] Dripps, Freedman 1970). How-

ever the maximal voluntary ventilation is usually measured during 10-15 sec The largest lung ventilation which can be maintained for longer periods is far less (Zocche, Fritts and Cournand 1960 Freedman 1970) According to Freedman the maximal ventilation possible for 4 minutes is 70-75 per cent of the maximal voluntary ventilation. However this seems to be enough to yield an adequate alveolar ventilation during maximal work of 5-10 minutes duration

Diffusion

During exercise up to intensities corresponding to about 50 per cent of the maximal oxygen uptake the lung diffusing capacity increases, as first shown by M Krogh (1915) Differing opinions prevail regarding the increase in diffusing capacity above the work intensity mentioned (cf. Boje 1933 Filley MacIntosh and Wright 1954 Riley *et al.* 1954 Linderholm 1959 Freyschuss and Holmgren 1965 Holmgren 1965) However the arterial oxygen saturation does not even during exhaustive work, decrease significantly (Lilienthal *et al* 1946 Holmgren and Linderholm 1958 Asmussen and Nielsen 1960 Holmgren and McIlroy 1964) Thus, under ordinary atmospheric conditions, the diffusing capacity of the lungs seems not to limit the maximal oxygen uptake.

3. Cardiovascular function

Central circulation

An increased supply of oxygen to the working muscles is brought about by an increased blood flow and an increased oxygen extraction per unit blood (see reviews by Asmussen and Nielsen 1955 Asmussen 1965 Bevegård and Shepherd 1967)

In spite of the ample knowledge on the adaptation of blood flow to exercise the importance of the cardiovascular system as a limiting factor for the maximal rate of oxygen uptake is not well delineated. Many authors consider it of main importance (cf Bevegård and Shepherd 1967) However not even during heavy exercise with large muscle groups have any signs of impaired function of the central circulation been reported in healthy subjects as judged from arterial pressure or stroke volume. (Holmgren 1956 Åstrand *et al* 1964 Ekblom and Hermansen 1968)

Peripheral circulation

Krogh (1929) stated that the volume of capillary blood in relation to the

tissue volume and the diffusion of oxygen from blood to tissue was probably the crucial factor limiting tissue oxygen uptake. Chance *et al* (1962) have calculated that cell respiration does not decrease until the Po_2 is less than about 1 mm Hg. Staunsby and Otis (1964) and Staunsby (1966) calculated the lowest oxygen tension of the venous blood ("critical oxygen tension") to furnish the interior of the muscle cell with oxygen at a sufficient pressure to be about 10 mm Hg in dog leg muscle preparations. In man Doll *et al* 1968 found an oxygen tension in the femoral vein during heavy exercise of about 20 mm Hg. Consequently oxygen transport by the blood circulation should not limit the maximal oxygen uptake in the active muscle. However the venous blood may not come exclusively from active muscle tissue but partly from tissue with a lower oxygen utilization rate. In that case the described findings are less valid as evidence, that the circulation does not limit the aerobic work capacity.

4 Muscle metabolism

Using the microfluorimetric technique on the exposed *sartorius* muscle of pithed *bufo marinus* Chance and Jobsis (1959) concluded that by electrical stimulation it was possible to increase the oxidative phosphorylation to a rate limited by the blood flow. On the other hand in experiments on electrically stimulated dog *gastrocnemius* and *gracilis* muscles with the same fluorimetric technique Jobsis and Stainsby (1968) concluded that the blood circulation did not limit the respiratory metabolic activity. In that case the oxygen utilization system inside the muscle should be the limiting factor for aerobic performance.

Higher activities of respiratory enzymes are generally found in muscles performing prolonged heavy work than in less active muscles (Paul and Sperling, 1967; Lawrie 1953). Holloszy (1967) showed an increased activity of respiratory enzymes in the hind limb muscles of rats after physical training. The described relations suggest that the oxygen utilization system may be a limiting factor for oxygen uptake.

Metabolic changes in the internal milieu of the active muscle cell may contribute to limit the maximal rate of cell respiration. One obvious intracellular change during muscle activity is the increased H^+ concentration. Evidence for a pH dependence of the oxidative phosphorylation of importance in the physiological pH range has been given by Myers and Slater (1957). Also the function of the contractile system seems to be impaired by a pH decrease of the same order of magnitude as that inside the muscle cell during maximal exhaustive exercise (Bendall 1960).

5 Conclusions

As no significant decrease in arterial oxygen saturation is seen during exhaustive exercise, *pulmonary function* cannot possibly be a limiting factor for aerobic work capacity

Neither seems available data in literature prove the often repeated statement that oxygen uptake is limited by the *cardiovascular function*

Increasing evidence, however points at the possibility that the *oxygen utilization system* in the muscle cell should limit aerobic work capacity

Part B. PRESENT INVESTIGATIONS

CHAPTER I

General procedures and methods

1 Modes of exercise

Dynamic forearm work

For the study of work with a small muscle group, rhythmic dynamic forearm work on a spring loaded hand ergometer was chosen. This mode of exercise makes it possible to quantitate the work intensity and to sample blood from veins, which during work almost exclusively drain muscle tissue (Cooper Edholm and Mottram 1955 Coles *et al* 1958 Idbohm and Wahren 1964

The same kind of ergometer was used in all experiments. On one of the ergometers, the handle was connected to a linear motion potentiometer the output of which could be visualized on an oscilloscope (Tectronix, type 30). Hence the time course of the individual contractions could be followed. The number of contractions was counted with a mechanical counting device.

In all experiments (except a pilot study see below) a constant contraction distance (10 mm) and a constant contraction frequency (60 contractions/min) was used. The work intensity was varied by varying the length or the number of springs. The shortest spring length, measured in the mid contraction position, was 36 cm. Hence the largest variation from mean length and mean tension during one contraction was ±4.5 per cent.

All work tests were performed in the sitting position. The active forearm rested on a pillow on a table in the horizontal position, the elbow flexed at about 90°, the back of the hand upwards. The height of the table was adjusted to correspond to the level of the fourth intercostal space.

No standardized procedure for the determination of dynamic forearm work capacity is described earlier. Hence it was considered eligible in certain experiments to study the possibility of its determination (Chapter II).

Dynamic leg work

Dynamic leg work was performed on a mechanically braked cycle ergometer (Cykelfabriken Monark, Varberg, Sweden). The pedalling fre-

quency governed by a metronome, was in all tests 60 cycles/min. The choice of the mechanically braked instead of an electrodynamic cycle ergometer was due to the potential fire hazards with electrical equipment when handling oxygen at hyperbaric pressures.

The work capacity of the subjects was prior to the experiments determined as the work intensity which they could perform in relative steady state at a heart rate of 170 beats/min (Sjostrand 1947 Wahlund 1948)

The maximal work experiments were performed as exhaustive work for durations similar to those used in the Tornvall test (Tornvall 1963) According to Tornvall the performance time for exhaustive work is well correlated to the maximal oxygen uptake.

In some of the maximal work experiments also the oxygen uptake was measured

When two work tests were performed on the same day at least 4 hours elapsed between them.

2. The pressure chamber

Forearm and leg exercise during oxygen breathing at hyperbaric pressure as well as leg exercise with decreased oxygen pressure in the inspiratory air was performed in a pressure chamber Forearm work at decreased inspiratory oxygen pressure was performed during breathing of hypoxic gas mixtures. Decreased ambient pressure was preferred to hypoxic mixtures during the low oxygen leg work experiments as it offered the possibility of disconnecting the volunteer from the mouthpiece without much affecting P_{O_2} in the inspiratory air

The pressure chamber consisted of a main chamber internal length 320 cm and internal height 210 cm, and connected to the main chamber an air-lock chamber of smaller dimensions. The total volume was 16 3 m^3 During the experiments a communication was always open between the two chambers. The exercise test was performed in the main chamber and the instruments for blood gas analysis were placed in the air-lock chamber

For the *hyperbaric experiments* the chamber was pressurized from a compressed air supply The volunteers breathed oxygen via a low resistance valve (Collins Triple J dead space about 180 ml) from a Douglas bag, which could be continuously refilled from an oxygen supply placed outside the chamber In order not to increase the oxygen concentration in the chamber air the expiratory air was collected from the exhaust side of the breathing valve in a second Douglas bag. This bag could be continuously evacuated via a connection to the outside of the chamber Alternatively

times are needed however before lung function changes can be detected

The potential risk of oxygen toxicity called for special care to be taken. At 3 ata toxical manifestations of oxygen seldom occur during exposure periods of less than 60 minutes (Foster 1965) The interindividual and also the day to-day intraindividual variation is large however (Donald 1947) Furthermore toxical symptoms are considered to appear earlier during exercise than at rest.

The present investigations and the pilot studies include 75 oxygen exposure periods at rest and 65 oxygen exposure periods during work at 3 ata. In a few pilot experiments volunteers were exposed to oxygen for periods up to 30 minutes at rest (range 5-30 minutes only 10 exposure periods were longer than 15 minutes) During exercise the exposure periods ranged between 6 and 13 minutes.

In the main experiments no instance of oxygen toxicity was experienced However in a preceding pilot experiment a member of the laboratory staff developed convulsions after 19 minutes of oxygen breathing at 3 ata at rest Furthermore in another pilot experiment on submaximal leg exercise another member of the laboratory staff complained of dizziness after 13 minutes of O breathing at 3 ata. Exercise and oxygen breathing were discontinued as the symptoms could have been attributed to oxygen toxicity In both instances the subjects recovered without further complications.

From the author's experience and reports in the literature it was considered advisable to keep the times for continuous oxygen exposure below 30 minutes at rest and 12 minutes during work.

4 Catheterization procedures

Arterial catheterization was performed percutaneously by the Seldinger technique Seldinger 1953 In most experiments and in all experiments in which arterial pressures were measured, the brachial artery was catheterized A teflon catheter was used, with an outer diameter of 1 45 mm and an inner diameter 1 0.5 mm, and with the catheter tip adapted to a nylon guide line diameter 0.8 mm The length of the catheter was 60 cm. In some experiments, when no pressure recordings were performed the radial artery was preferred For the radial artery a teflon catheter was used with an outer diameter of 1 15 mm and an inner diameter of 0 75 mm and the tip adapted to a nylon guide line with a diameter of 0 6 mm.

The *deep venous system* in the forearm (forearm exercise experiments) was catheterized with the same kind of catheters as used for the brachial

artery Via a puncture of vena mediana cubiti the catheter was advanced 4-8 cm in the distal direction into the deep venous system The catheter position was checked by palpation.

The *right heart* and the *pulmonary artery* were catheterized with a Gensini Positrol catheter size 6.5 (United States Catheter & Instrument Corp.) The catheter was introduced percutaneously via the cubital vein in the left arm by the Seldinger technique and advanced under fluoroscopic guidance to the pulmonary artery The catheter tip was placed about 3 cm out in the right branch of the pulmonary artery and kept in position with a cutaneous suture. When the volunteer was sitting on the cycle ergometer in the pressure chamber the catheter position was checked by studying the shape of the pressure recording

The *femoral vein* in the right leg (maximal leg exercise experiments) was catheterized with a teflon catheter of the same outer and inner dia meter as used for the brachial artery It was introduced percutaneously advanced about 5-6 cm in the proximal direction and sutured to the skin.

5. Methods

Blood analysis

Po_2 was measured with a polarographic electrode and Pco_2 with a glass electrode according to Severinghaus (Instrumentation Laboratory blood gas analyzer mod. 113) in the pressure chamber The chamber pressure at the time of analysis was the same as that at which the samples were obtained. The electrodes were kept at a constant temperature of 37 C.

With the exception of the experiments described in Chapter VI no correction was made for the difference in temperature between the measur ing instrument and the blood at the site of sampling The reasons for this were multiple. 1 Most conclusions were based on comparisons between work at corresponding loads and almost corresponding durations at two experimental conditions (*e g* 1 ata and 3 ata) The temperature differences between the two conditions may be assumed to be small. 2 The quite short exercise periods resulted in moderate temperature increases. In control experiments the rectal temperature increased by 0 1 -0.2 C during leg work at a heart rate of 120 beats/min. During maximal work for 6 minutes the rectal temperature increased by 0 5 1 0 C, and the temperature in the femoral vein by 0.9 1 5 C reaching 37.3 -38 0 C at exhaustion. The temperature in the deep vein of the forearm increased by 0 2 -0 6°C during exhaustive forearm work, the final temperature being 32.9 36 3 C

The electrodes were calibrated before and after the blood gas deter minations. When series of blood samples were taken the calibration was

checked at about 15 minutes intervals. For blood samples obtained during oxygen breathing a calibration gas containing approximately 80 per cent O 5 per cent CO_2 and 15 per cent N_2 was used, for samples taken during air breathing a calibration gas containing approximately 15 per cent O_2 5 per cent CO 80 per cent N_2. The linearity of the instrument for the analysis of O pressures up to about 2 200 mm Hg and CO pressures up to about 120 mm Hg was checked by analysing gas mixtures of known Po and Pco at 3 ata (Figs. 1 and 2). The same correction factor for the

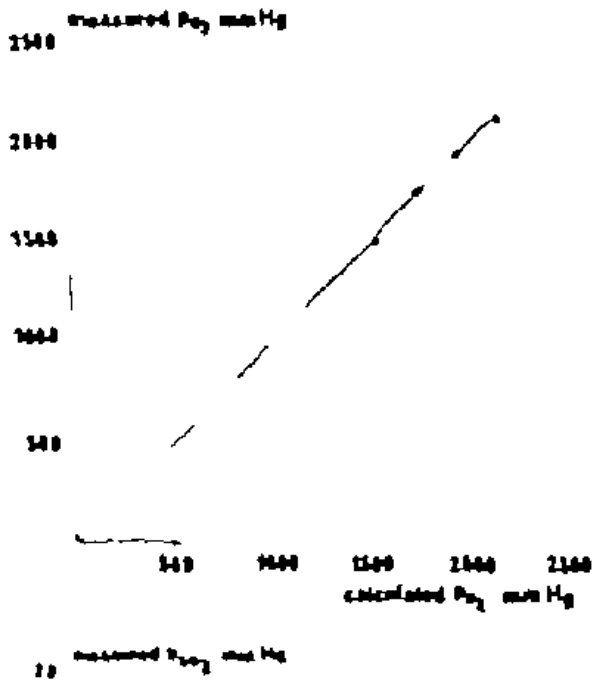


Fig. 1 Linearity for Pox measurements at 3 ata. Each filled circle represents a dublicate determination. × (the fifth point from below) denotes the calibration gas. Test gases analysed by the Scholander technique.

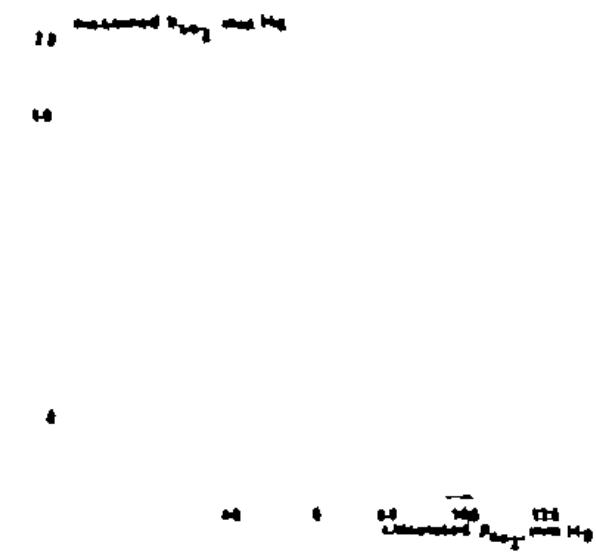


Fig. 2 Linearity for P on measurements at 3 ata. Each filled circle represents a dublicate determination. × (the fourth point from below) denotes the libration gas.

difference between calibration with gas and with tonometered blood 1 015 was used in all experiments. The factor was determined at 1 ata.

The *Haemoglobin concentration* was determined spectrophotometrically as oxyhemoglobin

The *oxygen saturation* was considered 100 per cent when the oxygen tension exceeded 350 mm Hg At lower oxygen tensions the saturation was determined spectrophotometrically according to the method described by Drabkin (1950) as modified by Holmgren and Pernow (1959)

The *oxygen content* was calculated from the hemoglobin oxygen saturation, the hemoglobin concentration and the oxygen tension, assuming 0 031 ml O in physical solution per mm Hg and liter of blood at the temperature of the Po_2 electrode.

pH was measured with a micro-Astrup equipment outside the chamber The syringes with blood samples were chilled with ice till the end of the experiment and analysed immediately thereafter

Lactate was analysed by an enzymatic method according to Hohorst (1962) Lundholm Mohme Lundholm and Varnos (1963) as modified by Wahren (1966)

Pyruvate was analysed by the enzymatic method described by Segal Blair and Wyngaarden (1956) and Bücher *et al.* (1962)

Blood temperature in the study of the effects of muscle temperature variations was measured at the site of blood sampling with a thermocouple introduced in the deep vein via the catheter and registered by a mirror galvanometer (Ellab TE 3) The same instrument was used for intravascular temperature measurements in control experiments (see under Po_2 and Pco_2)

Gas analysis

The *oxygen uptake* during air breathing and the *CO_2 elimination* were measured by sampling expired air in Douglas bags. Gas concentrations were analysed by the Scholander technique. During oxygen breathing no direct measurements of oxygen uptake were made. Possible techniques for its measurement should premise quite long breathing times to reach a steady state in gas exchange, and this would introduce the risk of toxic effects of oxygen. In the heart catheterization study the oxygen uptake during oxygen breathing was calculated from the cardiac output and the arterio-venous oxygen difference.

Heart rate intravascular pressures and cardiac output

The *heart rate* was measured by ECG recorded on an ink jet recorder (Mingograf 81 Elema-Schönander) In most exercise tests the leads CH_5 and CH were recorded

Intravascular pressures were measured with capacitative transducers (EMT 34 and 35 Elema-Schönander) and recorded on the Mingograf 81

As reference point the articulation of the fourth rib with the sternum was taken.

The cardiac output was determined by the dye dilution technique 5 mg of Indocyanin green (Cardio-green Hynson Westcott & Dunning) was injected from a 1 ml syringe into the catheter system and rapidly flushed into the pulmonary artery Arterial blood was continuously withdrawn at a rate of 18 ml/min from the brachial artery through a cuvette densitometer Gilford mod. 103-IR) Dye curves were recorded on a pen recorder Electro Instruments) The time for 10 cm deflection for a square-wave transient fed into the recorder was 0 23 second. One calibration curve was made for each subject using pooled blood withdrawn during light and heavy work. For each calibration curve three dye concentrations were used. The response was linear within the concentration range applied

During air breathing, the cardiac output was also determined according to the direct Fick technique A comparison was made between the two methods Fig 3) Above 18 l/min slightly higher values of cardiac output

TABLE 1 Errors for a single determination as estimated from duplicate determinations.

Variable	Range	Number of duplicate determinations	Mean value	Error $\sqrt{\frac{\Sigma d^2}{2n}}$	Error in per cent of the mean value
Hemoglobin conc. g/100 ml	12.0—16.0	200	14.85	0.110	0.74
Oxygen saturation, %	10—30	63	19.2	0.413	2.30
	40—70	32	47.7	0.483	1.01
	90—100	61	95.8	0.157	0.48
Pressure mm Hg					
Pulmonary artery	60—70	16	68.3	0.625	0.92
Aorta	1500—1600	10	1520.5	22.3	1.5
Pressure mm Hg					
Pulmonary artery	30—40	1	37.5	0.475	1.3
Brachial artery	110—120	11	116.9	1.45	1.2
pH	7.25—7.50	205	7.279	0.006	—
Lactic acid	0—3	76	1.36	0.069	5.1
mM/l	3—5	60	5.25	0.117	2.2
	7—18	124	10.47	0.185	1.8
Pyruvic acid	0.1—0.2	122	0.084	0.006	7.1
mM/l	0.150—0.250	113	0.203	0.010	4.9
	0.250—0.400	47	0.309	0.019	6.2

were attained by the dye method The difference may be caused by the higher hematocrit during heavy exercise (Åstrand *et al* 1964)

Statistical methods

Statistical calculations were performed according to Snedecor (Snedecor 1959) The following symbols for probability (P) levels of significance were used.

0 05>P>0 01	probably significant *
0 01>P>0 001	significant **
P<0 001	highly significant ***

When not otherwise stated, values given in the text are mean values for the group studied

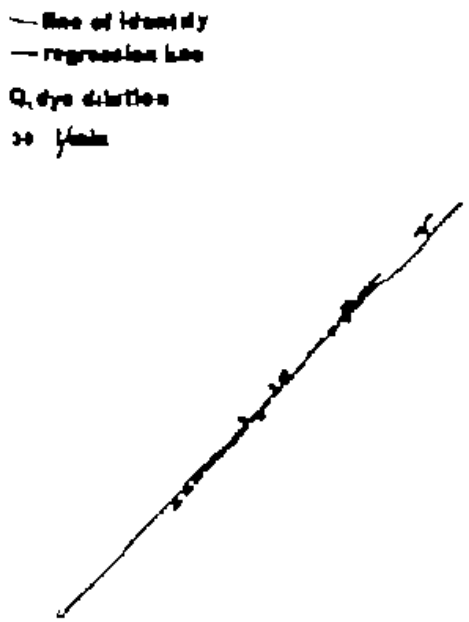


Fig 3 Correlation between cardiac output with the dye and the Fick method. $Q_{dye} = 1.11 \times Q_{Fick} - 1.54 \quad r = 0.98$.

6. Definitions, symbols and abbreviations

Chamber pressure

Environmental pressure is expressed in hyperbaric experiments in atmospheres absolute pressure (ata) in hypobaric experiments in mm Hg

630 mm Hg corresponds to an altitude of about 1,500 m.

560 mm Hg corresponds to an altitude of about 2,500 m.

Air at 560 mm Hg has approximately the same P_{O_2} as a gas mixture containing 15 % O_2 at 760 mm Hg

Blood and gas symbols

For blood and gases the symbols and abbreviations are those proposed by Pappenheimer *et al.* (1950)

Physical working capacity

W_{170} = work at heart rate 170 in steady state during leg work on a cycle ergometer

W_{max} = maximal work intensity a subject can sustain for 6 minutes with the muscle group and mode of exercise stated.

CHAPTER II

Control experiments on the determination of forearm work capacity and its reproducibility

It is of importance to define the work intensity not only in absolute values, but also in relation to the work capacity of the subject for the specific mode of work studied. During steady state leg work the heart rate gives information on the relative work intensity During forearm work no such easily measured indicator of the relative load is obtainable.

Grosse Lordemann and Müller (1937) and Müller (1938) found that the relation between maximal performance time for exhaustive work and work intensity on a cycle ergometer could be expressed by the equation $\log T = a \times \log N + b$, where T = the performance time to exhaustion and N = the work intensity Tornvall (1963) studied this relation in greater detail and showed that for exercise times between 1 and 18 minutes log performance time was linearly related to log work intensity He suggested a test of physical work capacity in which the maximal performance time was measured at one arbitrarily selected heavy work load. From the correlations stated above the work capacity could then be expressed as the maximal work load which the subject could sustain for 6 minutes, $W_{max'}$ This was shown to be well correlated to the maximal oxygen uptake ($r = 0.94$)

To see whether the same kind of determination of work capacity could be used for dynamic forearm work on a hand ergometer the relation between work intensity and maximal performance time for exhaustive dynamic work of durations between 1 and 25 minutes was studied. The reproducibility of the measured performance times at various work levels was also determined.

The work intensity can be varied by varying the spring tension, the contraction distance or the contraction frequency The mode by which the work intensity is varied may influence the measured value of forearm work capacity by influencing blood flow or mechanical efficiency (*cf* Wahren 1966) For that reason the performance times at constant work intensities achieved with different contraction frequencies were measured in pilot experiments.

For volunteers not familiar with the hand ergometer the reproducibility

of the performance time at a given work load is low. This may be because they often perform the contractions in a manner that differs from test to test. The hand is kept in a contracted position for an unnecessarily long time if it is not relaxed completely between the individual contractions, and hence static work of a varying and unknown magnitude is added to the dynamic work. To minimize this source of error the subjects were instructed to initiate the contraction as fast and to relax as rapidly as possible after the end point of the contraction path was reached. The subjects were also given plenty of time to practice with the ergometer before the investigation. The influence of different relations between contraction and relaxation time was also studied in pilot experiments.

Subjects

Four male volunteers of average physical fitness, two students and two members of the laboratory staff aged 18-32 years, took part in the investigation.

Procedure

Dynamic forearm work to exhaustion was performed on a spring loaded hand ergometer (see Chapter 1).

Pilot experiments

Performance times at constant work intensities ranging between 17.5 and 30.0 kpm/min, achieved with different contraction frequencies, 40-80 contractions/min, were measured in two of the subjects. In these experiment the contraction distance was kept constant 40 mm.

A comparison between the performance times when the contraction was maintained for 150 msec and for 500 msec was performed in one subject. This could be done by watching the time course of the contraction on the oscilloscope. Ten tests of each mode of work were made.

Main experiments

All experiments were performed with 60 contractions/min and a contraction distance of 40 mm. Each of the four subjects performed exhaustive work at four work intensities with each hand. To determine the reproducibility each subject performed a repeated work test at one higher (performance time 4 minutes) and one lower performance time 4-8 minutes) work intensity.

Results

Pilot experiments

The average performance time was 22 1 per cent shorter (SE 9 1 $P < 0.05$) with 80 contractions/min compared to 40 contractions/min (Fig 4)

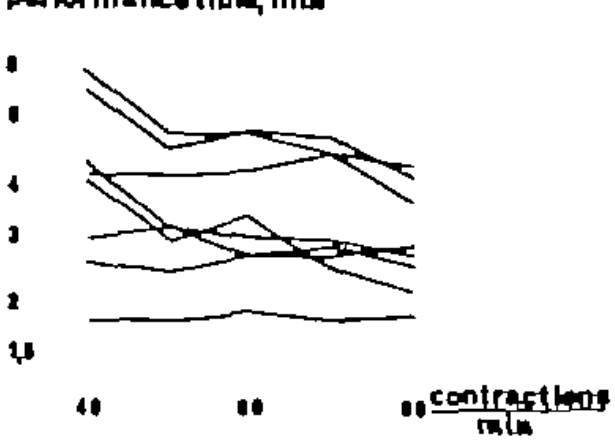


Fig 4 The effect of varying contraction frequency on performance time t exhaustion. Logarithmized y-axis.

Between 50 and 70 contractions/min no significant differences were found.

The performance time when the contraction was maintained for 500 msec in each contraction cycle was for times between 2 and 4 min 65 0 per cent (SD 5.5) and for times between 4 and 8 min 57 0 per cent (SD 5 1) of the performance time with the contraction maintained for 150 msec.

Performance time/work intensity relation

Within the time range 1-8 min, log work intensity was well correlated to log performance time (Fig 5) The coefficient of correlation for the individual subjects varied between 0.98 and 0 99 The calculated W_{max} ranged between 13.3 and 23.9 kpm/min, average value 17 9 kpm/min for the right and 15 0 for the left hand (Table II)

When the work intensity was decreased and performance times longer than about 8 minutes were achieved, the time increased far more than was calculated from the log/log correlation. At a work intensity of about 80 per cent of W_{max} the time was often doubled by a 3-6 per cent decrease in work load. When the performance time exceeded 25 minutes the test was in most cases interrupted by the experimenter All subjects reached this point at a load 0,5 kpm/min lower than the lowest load presented in Fig 5

Reproducibility

The reproducibility calculated from a duplicate determination as the standard error of a single determination was 10.9 per cent in the performance time range 2-4 min and 9 2 per cent in the range 4-8 min.

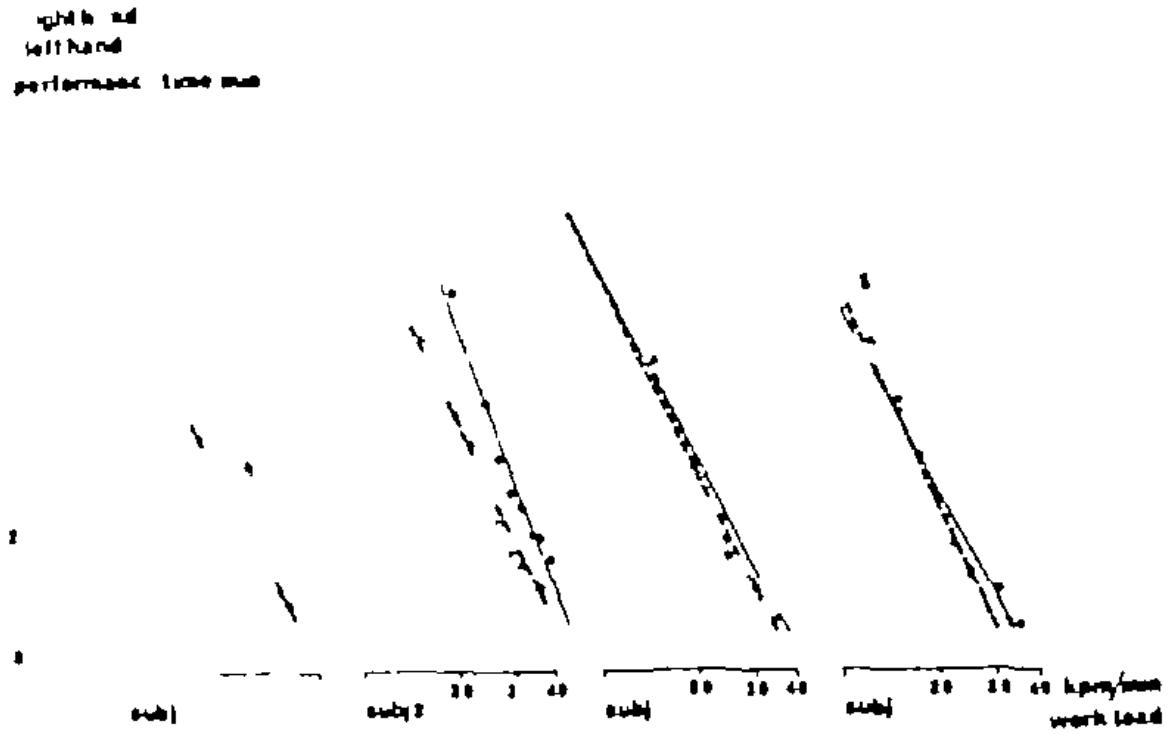


Fig. 5. Relationship between log performance time and log work load for the individual subjects.

Discussion

The energy for heavy exhaustive work of short duration derives mainly from anaerobic sources whereas for exercise times longer than about 1-2 min aerobic processes are most important. The aerobic work output decreases very little with increased work time (Hill 1927, Margaria 1964).

From this it can be assumed that, if exhaustive exercise is performed at a heavy load, log maximal performance time will be approximately linearly correlated with log work intensity. On the other hand, when the work intensity is increased and a larger proportion of the energy is derived from oxidative regeneration of ATP, the performance time will increase more with decreased work intensity than calculated from the above mentioned correlation. This is actually what was found in the present study. Within the performance time range 1-8 min, a good estimation of W_{max} can be obtained from intra- or extrapolation on a log/log chart between performance times determined at two work intensities. For intensities lower than about ¼ of W_{max}, the relation between a change in work intensity and performance time was almost of the same order of magnitude as the decrease in maximal aerobic work output with increased performance times calculated by Margaria 1964. From this it can be assumed that

TABLE II Performance time/work intensity relationship and forearm work capacity for the individual subjects. t = performance time to exhaustion W = work intensity

	right hand	left hand
Subject 1	$\log t = -2.39 \times \log W + 3.85$	$\log t = -1.98 \times \log W + 3.06$
r	0.99	0.99
W_{max} kpm/min	19.3	14.1
Subject 2	$\log t = -2.90 \times \log W + 4.76$	$\log t = -2.21 \times \log W + 3.54$
	0.99	0.98
W_{max} kpm/min	23.9	17.9
Subject 3	$\log t = -2.0 \times \log W + 3.12$	$\log t = -2.12 \times \log W + 3.25$
	0.99	0.98
W_{max} kpm/min	15.0	14.6
Subject 4	$\log t = -1.99 \times \log W + 3.07$	$\log t = -2.14 \times \log W + 3.30$
	0.99	0.99
W_{max} kpm/min	15.3	15.5

the maximal aerobic work capacity of the forearm is of the order of magnitude ⅗ ¾ of W_{max}

Conclusions

1 It is possible with reasonably good reproducibility to determine a subject's forearm work capacity as W_{max} by intra or extrapolation between two values of maximal performance time in the range 1-8 min This statement is valid provided that the subject has been allowed to become familiar with the exercise technique.

2. The aerobic work capacity can be assumed to be about ⅗-¾ of W_{max}

3 Measurement of maximal performance time seems to provide a fairly good estimate of the aerobic work capacity if work intensities yielding performance times of about 5 min are chosen as evidenced by Tornvall (1963)

CHAPTER III

Control experiments for the study of the adaptation to leg work during normoxia at 3 atmospheres

When exercise is performed during oxygen breathing in a hyperbaric milieu factors other than the increased oxygen tension, *e.g.* the increased gas density may influence the adaptation to work. The exercise heart rate during air breathing at 4.5 ata was reported to be decreased compared to air breathing at 1 ata (Hesser, Fagraeus and Linnarsson 1968). It cannot be concluded at present whether this is an effect of the increased nitrogen pressure or whether other factors are more important. Thus in experiments during saturation exposures to helium-oxygen mixtures at 31.3 ata, yielding an inspiratory oxygen pressure slightly above the normal atmospheric a heart rate decrease of a corresponding magnitude was recorded (Salzano, Rausch and Saltzman 1970). The investigations mentioned also report a decreased lung ventilation during exercise at increased ambient pressure. The reason for this is probably the increased air density (Millic-Emili and Tyler 1963). The O_2 uptake at corresponding loads was slightly higher at increased pressure. This was considered due to a larger work of breathing.

Before discussing the effect of oxygen breathing at 3 ata, it was considered necessary to investigate whether the heart rate and the lung ventilation change during normoxic exercise at the same ambient pressure.

Subjects

Four male volunteers, aged 22–39 years, were studied. Three of them belonged to the laboratory staff, two were students. Their average physical working capacity measured as W_{170} was 1170 kpm/min (range 1000–1350 kpm/min).

Procedure

Exercise was performed in a mechanically braked cycle ergometer in the pressure chamber. The subjects started to work for 6 minutes at a load yielding a heart rate of about 130 beats/min and continued for 6 minutes

at a load yielding a heart rate of about 160 beats/min Comparisons were made between air breathing at 1 ata and breathing a gas mixture containing about 8 per cent O and 92 per cent N_2 yielding an inspiratory Po_2 of about 190 mm Hg at 3 ata.

Measurements

The heart rate was recorded at rest and continuously during work. Lung ventilation, respiratory frequency O uptake Co_2 elimination and the pressure variations in the mouthpiece were recorded during the last minutes of each exercise period.

TABLE III Heart rate, respiratory rate, tidal volume, total lung ventilation and mouthpiece inspiratory to expiratory pressure variation during 8 per cent O_2 breathing (PIo_2~190 mm Hg) at 3 ata and air breathing at 1 ata.

	Air 1 ata Mean ± S D			8 % O_2, 3 ata Mean ± S D			Differences, 3 ata—1 ata Mean ± standard error of the mean	
Load, kpm/min, mean range	rest	load I 620 500—750	load II 1110 1000—1200	rest	load I 620 500—750	load II 1110 1000—1200	load I	load II
Heart rate, beats/min	80 ±10	126 ±7	172 ±10	81 ±10	126 ±9	170 ±11	0 ±1	—2 ±1
Respiratory rate, breaths/min		20 ±4	31 ±4		19 ±5	27 ±6	—1 ±1	—4 ±1
V_T L BTPS		2.4 ±0.35	2.67 ±0.40		2.28 ±0.17	2.91 ±0.42	0 04 ±0.15	0.24 ±0.19
V_E L BTPS/min		44.96 ±7 73	81 64 ±16.13		45 14 ±6 07	75.34 ±9.23	—1.82 ±1 60	—6.30 ±4.88
Mouthpiece pressure variation, mm Hg		1.5 ±0 2	3.3 ±1 7		3.3 ±0 7	7 7 ±2.5	1.8 ±0 8	4 4 ±0.9
Vo_2 L STPD		1581 ±98	2617 ±214		1504 ±222	2518 ±251	—77 ±76	—99 ±173
Vco_2 L STPD		1452 ±85	2739 ±335		1421 ±104	2621 ±139	—31	—118 ±112

Results

The results are summarized in Table III.

No significant differences between 3 and 1 ata were found in heart rate lung ventilation respiratory frequency O_2 uptake or CO_2 elimination neither at the lower nor at the higher work load ($P>0.05$). At the higher load, however the average respiratory frequency was slightly lower 4 breaths/min at 3 ata ($P<0.05$). The average inspiratory to expiratory pressure variation in the mouthpiece, that is the pressures needed to overcome the resistance in the breathing equipment, was 7.7 mm Hg at 3 ata compared to 3.3 mm Hg at 1 ata during expiratory air sampling at the higher work load with a lung ventilation of about 75 litres/min.

Conclusions

The ambient pressure used in the present study 3 ata, was apparently too low in itself to cause significant changes in heart rate, lung ventilation or O uptake. The pressure variations in the breathing mouth piece were also at rather large lung ventilations moderately increased at 3 ata.

CHAPTER IV

Adaptation to exercise during oxygen breathing at 3 atmospheres

Introductory remarks

Nielsen and Hansen (1937) reported that the maximal oxygen uptake measured by the Douglas bag technique increased considerably when the oxygen fraction of the inspiratory air was augmented. The increase in oxygen uptake was larger than the calculated increase in arterial oxygen content. The CO production, on the other hand, did not increase. Margaria *et al.* (1961) found by the closed circuit spirometer technique a 10 per cent increase in maximal oxygen uptake during oxygen breathing Asmussen, von Döbeln and Nielsen (1948) Miller (1952) and Bannister and Cunningham (1953) reported an improved performance time for exhaustive work during 66-100 per cent oxygen breathing Miller however stated that the increase was very small and concluded that the major part of the effect could be attributed to suggestion. Asmussen and Nielsen (1955) reported a decrease in heart rate at corresponding work intensities of about 6 per cent during oxygen breathing whereas no measurable difference in cardiac output could be detected

Several authors, for example Asmussen and Nielsen (1946 1958) Bannister and Cunningham (1953) and Dejours (1959) have described a reduction in pulmonary ventilation during heavy exercise when breathing oxygen, whereas the effect is more uncertain during light exercise.

To sum up oxygen breathing during exercise at atmospheric pressure seems to reduce the pulmonary ventilation, at least at heavy loads. On the other hand the effect on the cardiovascular adaptation and aerobic work performance is small or even doubtful (Miller 1952)

The cardiovascular effects of hyperbaric oxygen breathing have been studied mainly at rest. Whalen *et al.* (1965) found a decrease in heart rate and cardiac output with unchanged stroke volume and arterial pressure. Decreases in cerebral, renal and forearm blood flows have also been reported (Lambertsen *et al* 1953 Jacobson, Harper and McDowall 1963 Rennie and Knox 1964 Hahnloser *et al.* 1966 Bird and Telfer

1965) However little is known about the adaptation to exercise during oxygen breathing at hyperbaric pressure. In the dog Waxman *et al.* (1968) reported a 17 per cent decrease in cardiac output at 3 ata compared to at corresponding work loads during air breathing at 1 ata. Weglicki *et al* 1966) found a decreased lactate production in dogs exercising during and after oxygen breathing at 3 ata. The work intensities studied were low however

In man the heart rate during moderately heavy exercise has been shown to decrease slightly whereas the arterial blood pressure is unchanged while breathing oxygen at 2 ata as compared to air breathing at 1 ata (Salzano *et al* 1967) Also the lung ventilation has been found to decrease during oxygen breathing at 2 ata (Lambertsen *et al* 1959) as well as 3 ata (Telfer and Jennett 1965) compared to air breathing The decrease, 12 15 per cent, during moderately heavy exercise, is of the same order of magnitude as that found during oxygen breathing at atmospheric pressure (Asmussen and Nielsen 1946)

When studying exercise during hyperbaric oxygen breathing it is important to state whether the effects recorded are the result of an increased arterial oxygen supply or of toxic metabolic changes. Numerous enzymes, the sulfhydryl enzymes in particular are inhibited by high pressures of oxygen (Haugaard 1946) Stadie, Riggs and Haugaard (1954 a, b) and Dickens (1946 a, b) found *in vitro* that tissue respiration was inhibited by high oxygen pressure the brain being more rapidly affected than other tissues. Chance Jamieson and Coles (1965) and Chance, Jamieson and Williamson (1966) demonstrated in brain tissue an inhibition in the electron transfer pathways and the oxidative phosphorylation. The comparatively low sensitivity of muscle tissue (Dickens 1946 b) to high oxygen pressure and the high oxygen utilization rate in the active muscle makes it less probable however that toxic effects of oxygen might change the muscle metabolism during short exposure times at 3 ata.

The ambient pressure in the present study 3 ata, was chosen to yield a substantial increase in arterial O content and still permit exposure times long enough for meaningful measurements without toxic manifestations. Some of the results have been presented in preliminary reports (Kaijser 1969 a, b 1970)

I. Adaptation to dynamic forearm work

Subjects

Seven male volunteers, aged 21 27 years, and three female volunteers, aged 25-31 years, were studied. Five of the subjects were students, two were

conscripts and the three female subjects were nurses at the laboratory. Prior to the experiment their forearm work capacity was determined as W_{max}. The average value for the male subjects was 14 kpm/min (range 10-18 kpm/min) for the females 8.5 kpm/min (range 8-9 kpm/min). The values are, according to the author's experience, normal for subjects with sedentary occupations, who do not take part in competitive sports engaging the forearm muscles.

Procedure

Two sets of experiments were performed. In both the subjects were studied during oxygen breathing at 3 ata and for comparison during air breathing at 1 ata.

In the *first investigation* the influence of varying loads was studied. Six male volunteers worked at stepwise increasing work loads 4 minutes at 50 per cent of W_{max}, 4 minutes at 100 per cent of W_{max}, and until exhaustion at 150 per cent of W_{max}. The aim in the selection of work loads was that the first load should be well below the maximal aerobic capacity of the forearm and the last load should lead to exhaustion within about 2 minutes.

In the *second investigation* the adaptation to a somewhat longer work time at a constant load was investigated. Three of the male and the three female subjects were studied during 20 minutes work at ⅔ of W_{max}. After 20 minutes exercise the work load was increased to that corresponding to W_{max}, and the subjects worked until exhaustion. In general, the subjects were exhausted after 2–3 minutes.

During all experiments arterial blood was sampled from a catheter in the brachial artery of the resting arm, and muscle vein blood from a catheter in a deep vein of the active forearm.

Measurements

P_{O_2} was measured in arterial blood, and O saturation, P_{CO_2}, pH, lactate and pyruvate concentrations in arterial and venous blood. In the study of stepwise increased load, blood was sampled at rest and during the last 30 seconds of each work load. In the study of constant load, blood was sampled at rest and after 5, 10, 15 and 20 minutes of work, as well as at exhaustion. Also the performance time to exhaustion at the highest load was measured.

For methodological details see Chapter I.

Results

1 *Stepwise increased load*

The results are summarized in Table IV and Fig 6

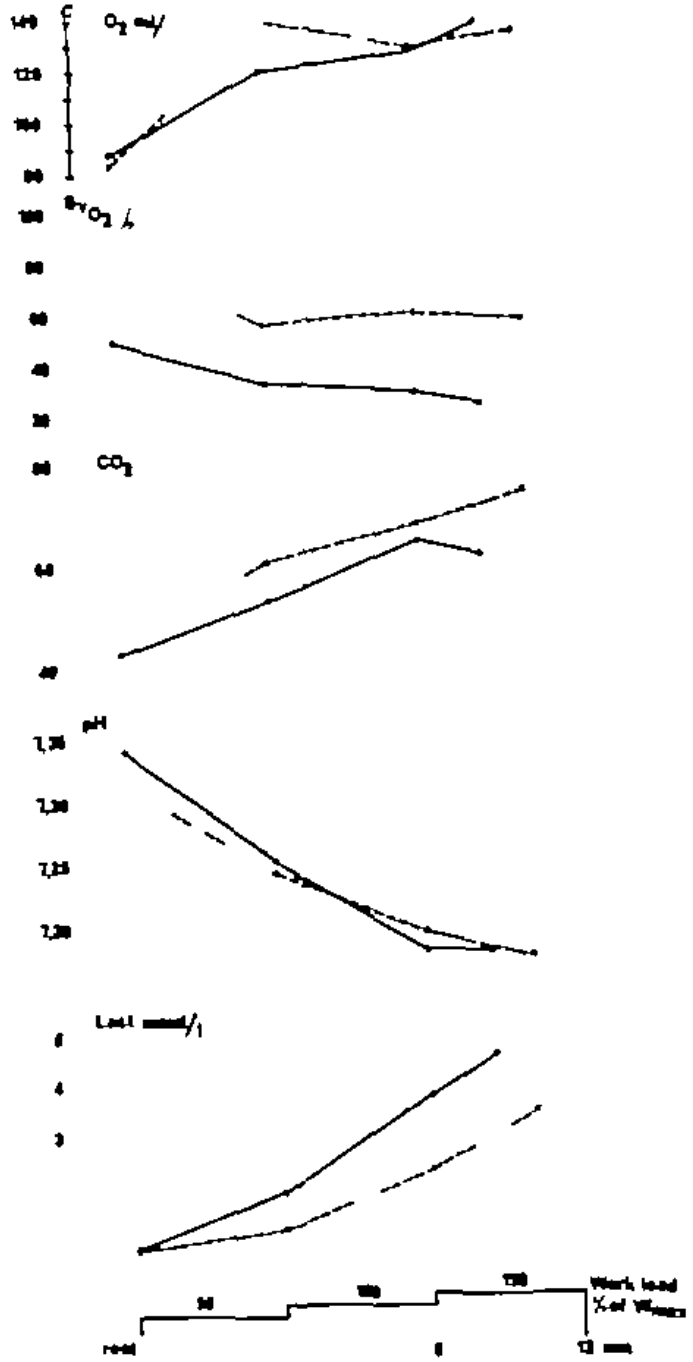


Fig 6 Arterial-deep venous O_2 difference and O_2 saturation Pco_2, pH and lactate concentration in the deep vein of the active forearm during work stepwise increased loads. Average values (n = 6) at rest, after 4 min at the first and the second load, and at exhaustion. Continous lines denote air breathing at 1 ata, dashed lines oxygen breathing at 3 ata.

Air breathing at 1 ata

Arterial blood In arterial blood no significant changes in *Po* *Pco*$_2$ *pH* or *pyruvate* concentrations appeared during the exercise period. The *lactate* concentration increased slightly being 1 11 mmoles/l at exhaustion compared to 0 68 at rest ($P<0.01$)

Table IV. A. Data obtained during forearm work at stepwise increased loads during oxygen breathing at 3 ata and air breathing at 1 ata. Blood was sampled from the brachial artery and the deep vein of the active forearm at rest, after 4 min at the two lower loads and at exhaustion at the highest load. Mean ± SD. n = 6.

	1 ata	O_2, 3 ata	
Load, % of max. kpm/m	rest	30 7 (range 5–10	100 1	150 21	rest	30 7	100 1	150 21
Performance loss, per cent				110 40				153 A
P_{O_2} mm Hg	99.3 7	96.8 7.8	95.7 8	96.3 11.0	940 82	1956 91	1900 97	1982 40
S_{O_2} %	97.8 1.7	98 1.2	98. 0.4	99.0				
S_{O_2} %	80 20.	84.5 16.	81.1 15.4	87.2 14.3	85.9 18.3	96.7 13.7	92 12.6	96.7 16.7
$C_{a-v}O_2$ ml/l	34.6	120 28	129.1 29.0	140.4 26.9	80.0 30.7	142.3 38.	133.1 22	137.9 30.2
CO_2 mm Hg	37.3 .2	37 0.8	37.3 3.7	36.2 6.2	37.8 2.5	38.2 5.7	36.2 6.4	34.5 8.9
CO_2 mm Hg	3.2 6.2	54.8 9.6	64.0 1	63.3 19.4	7.2 7.6	63. 2	73.0 8.8	77.5 18.1
Lactate$_a$, moles/l	0.68 .88	0.72 0.17	80 .2	1.11 0.28	0 10	0.64 0.1	0.76 0.25	0.96 0.1
Lactate moles/l	83 0.20	1.99 0.99	3.82 0.87	76 98	0.77 0.18	.22 0.25	2.46 0.82	3.62 0.77
pyruvate moles/l	0.060 0.037	0.058 +0.030	0.089 +0.35	079 ±0.037	082 090	0.064 +0.028	077 +0.037	0.082 +0.32
pyruvate$_v$, moles/	083 0.048	0.134 +0.02	0.140 +0.028	0.199 +0.02	1 +0.046	093 +0.040	0.13 +0.30	76 +0.05
pH	7.389 0.034	7.378 +0.6	7.376 +0.023	7.36 +0.029	7.394 +0.034	38 +0.023	389 +0.078	386 +0.030
pH	7.342 0.044	7.298 +0.051	7.84 +0.037	7.185 +0.037	7.313 +0.032	7.266 +0.022	303 +0.39	7.82 ±0.032

B. Difference between oxygen breathing at 3 ata and air breathing at 1 ata. Mean ± standard error of the mean. n = 6.

	O_2, 3 ata – 1 ata			
	rest	30	100	150
P_{O_2} mm Hg	940 82	1956 91	1900 97	1882 40
S_{O_2} %				
S_{O_2} %	85.9 18.3	96.7 13.7	92 12.6	96.7 16.7
$C_{a-v}O_2$ ml/l	80.0 30.7	142.3 38.	133.1 22	137.9 30.2
CO_2 mm Hg	37.8 2.5	38.2 5.7	36.2 6.4	34.5 8.9
CO_2 mm Hg	7.2 7.6	63. 2	73.0 8.8	77.5 18.1
Lactate$_a$, moles/l	0 10	0.64 0.1	0.76 0.25	0.96 0.1
Lactate moles/l	0.77 0.18	.22 0.25	2.46 0.82	3.62 0.77
pyruvate moles/l	082 090	0.064 +0.028	077 +0.037	0.082 +0.32
pyruvate$_v$, moles/	1 +0.046	093 +0.040	0.13 +0.30	76 +0.05
pH	7.394 +0.034	38 +0.023	389 +0.078	386 +0.030
pH				

	O_2, 3 ata – 1 ata		
	30	100	150
P_{O_2} mm Hg	1956 91	1900 97	1882 40
S_{O_2} %			
S_{O_2} %	96.7 13.7	92 12.6	96.7 16.7
$C_{a-v}O_2$ ml/l	142.3 38.	133.1 22	137.9 30.2
CO_2 mm Hg	38.2 5.7	36.2 6.4	34.5 8.9
CO_2 mm Hg	63. 2	73.0 8.8	77.5 18.1
Lactate$_a$, moles/l	0.64 0.1	0.76 0.25	0.96 0.1
Lactate moles/l	.22 0.25	2.46 0.82	3.62 0.77
pyruvate moles/l	0.064 +0.028	077 +0.037	0.082 +0.32
pyruvate$_v$, moles/	093 +0.040	0.13 +0.30	76 +0.05
pH	38 +0.023	389 +0.078	386 +0.030
pH			

	O_2, 3 ata – 1 ata		
	30	100	150
P_{O_2} mm Hg	1016 91	960 97	942 40
S_{O_2} %			
S_{O_2} %	10.8 13.7	6.0 12.6	10.8 16.7
$C_{a-v}O_2$ ml/l	107.6 38.	98.4 22	102.9 30.2
CO_2 mm Hg	1.4 5.7	−1.6 6.4	−3.2 8.9
CO_2 mm Hg	56.0 2	65.8 8.8	70.3 18.1
Lactate$_a$, moles/l	0.56 0.1	0.68 0.25	0.88 0.1
Lactate moles/l	.14 0.25	2.38 0.82	3.54 0.77
pyruvate moles/l	0.056 +0.028	069 +0.037	0.074 +0.32
pyruvate$_v$, moles/	085 +0.040	0.05 +0.30	68 +0.05
pH	36 +0.023	381 +0.078	378 +0.030
pH			

	O_2, 3 ata – 1 ata		
	30	100	150
P_{O_2} mm Hg	1016 91	960 97	942 40
S_{O_2} %			
S_{O_2} %	10.8 13.7	6.0 12.6	10.8 16.7
$C_{a-v}O_2$ ml/l	107.6 38.	98.4 22	102.9 30.2
CO_2 mm Hg	1.4 5.7	−1.6 6.4	−3.2 8.9
CO_2 mm Hg	56.0 2	65.8 8.8	70.3 18.1
Lactate$_a$, moles/l	0.56 0.1	0.68 0.25	0.88 0.1
Lactate moles/l	.14 0.25	2.38 0.82	3.54 0.77
pyruvate moles/l	0.056 +0.028	069 +0.037	0.074 +0.32
pyruvate$_v$, moles/	085 +0.040	0.05 +0.30	68 +0.05
pH	36 +0.023	381 +0.078	378 +0.030
pH			

	O_2, 3 ata – 1 ata		
	30	100	150
P_{O_2} mm Hg	1016 91	960 97	942 40
S_{O_2} %			
S_{O_2} %	10.8 13.7	6.0 12.6	10.8 16.7
$C_{a-v}O_2$ ml/l	107.6 38.	98.4 22	102.9 30.2
CO_2 mm Hg	1.4 5.7	−1.6 6.4	−3.2 8.9
CO_2 mm Hg	56.0 2	65.8 8.8	70.3 18.1
Lactate$_a$, moles/l	0.56 0.1	0.68 0.25	0.88 0.1
Lactate moles/l	.14 0.25	2.38 0.82	3.54 0.77
pyruvate moles/l	0.056 +0.028	069 +0.037	0.074 +0.32
pyruvate$_v$, moles/	085 +0.040	0.05 +0.30	68 +0.05
pH	36 +0.023	381 +0.078	378 +0.030
pH			

No difference in *hemoglobin* concentration was found between arterial and venous blood The hemoglobin concentration was higher at exhaustion, 14 38 g/100 ml than at rest 13 36 ($P<0.05$) or at the lowest load, 13 87 ($P<0.05$)

Deep vein blood The *O_2 saturation* was 50 1 per cent at rest. During work at the lowest load it decreased to 34 5 per cent ($P<0.01$) At exhaustion it had decreased further to 27 2 per cent ($P<0.05$)

As no major changes in arterial O_2 saturation occurred the *arterio-venous O difference* increased from an average of 87 7 ml/l at rest to 120 9 ml/l at the lowest work load ($P<0.01$) At the highest load it had increased further to 140 4 ml/l ($P<0.05$)

Pco increased from an average resting value of 43.2 mm Hg to 54 8 mm Hg at the lowest and 63 5 mm Hg at the highest load The differences between rest and the lowest and between the lowest and highest work loads were significant ($P<0.01$)

The deep vein lactate concentration was 0 85 mmoles/l at rest. At the first work load it had increased to 1 99 mmoles/l ($P<0.05$) It increased markedly with further increase in work intensity being 4 76 mmoles/l at exhaustion.

The deep vein pyruvate concentration was at rest 0 083 mmoles/l At the lowest work load it had increased to 0 134 mmoles/l ($P<0.05$) It increased further to 0 181 mmoles/l ($P<0.01$) at exhaustion. The lactate/pyruvate ratio in venous blood was almost unchanged at the lowest load, 16 4 (S D 7.8) compared to 12.4 (S D 4.5) at rest. It increased at the second load to 30 0 (S D 8 3) but was in four out of five subjects slightly lower at exhaustion, average value 23 5 (S D 4 9)

pH decreased with increased work load from 7 342 at rest to 7 185 at exhaustion ($P<0.001$)

The average *performance time* to exhaustion at the highest work load was 110 seconds.

Oxygen breathing at 3 ata. At rest

After oxygen breathing for 4 minutes the average *arterial Po* was 1840 mm Hg The *deep vein O saturation* was substantially higher than during air breathing in all subjects. The average saturation was 85 9 per cent. The arterio-venous O_2 difference was 80 0 ml/l. It was not significantly different from that during air breathing ($P>0.05$) Compared to air breathing the arterial *Pco_2* and *pH* were unchanged ($P>0.05$) No diffe-

rences in deep vein Pco_2 *pH lactate* or *pyruvate* concentrations between oxygen breathing at 3 ata and air breathing were found ($P>0.05$)

Oxygen breathing at 3 ata During exercise

Arterial blood Po_2 at the end of the first load was 1958 mm Hg the amount of physically dissolved oxygen being about 60 ml/l. Po_2 decreased slightly during the exercise period being 1892 mm Hg at exhaustion ($P<0.05$) No significant differences in arterial *Pco pH lactate* or *pyruvate* concentrations were found between oxygen breathing at 3 ata and air breathing at 1 ata.

Deep vein blood The *O saturation* was decreased during exercise as compared to rest ($P<0.05$) At the lowest work load it was 56.7 per cent. It remained on the same level at all work loads, being 59.7 per cent at exhaustion. The O_2 saturation was in all subjects, at all work loads, substantially higher during oxygen than air breathing The difference compared to air breathing was higher at exhaustion than at the lowest work load ($P<0.05$)

The *arterio-venous O difference* was at the lowest work load 21.4 ml/l higher during oxygen than air breathing ($P<0.001$) It did not change significantly with increased work intensity At exhaustion the arterio-venous O difference was 137.9 ml/l. This value did not differ from that during air breathing ($P>0.05$)

Pco_2 was generally higher during oxygen than air breathing at low and high work loads.

The lactate concentration was lower during oxygen than air breathing It increased more, however from the second load to exhaustion, and the value at exhaustion was not significantly lower than during air breathing

The *pyruvate concentration* was lower than during air breathing at the lowest load ($P<0.05$) but did not differ at the higher loads.

The lactate/pyruvate ratio did not differ significantly from that during air breathing at any level of work.

The performance time to exhaustion at the highest load was prolonged in two subjects and almost unchanged in four during oxygen breathing at 3 ata as compared to air breathing The average performance time was 153 seconds during oxygen breathing The average difference compared to air breathing, 43 seconds, was not statistically significant ($P>0.05$)

2. *Prolonged work at constant load*

The results are summarized in Fig 7

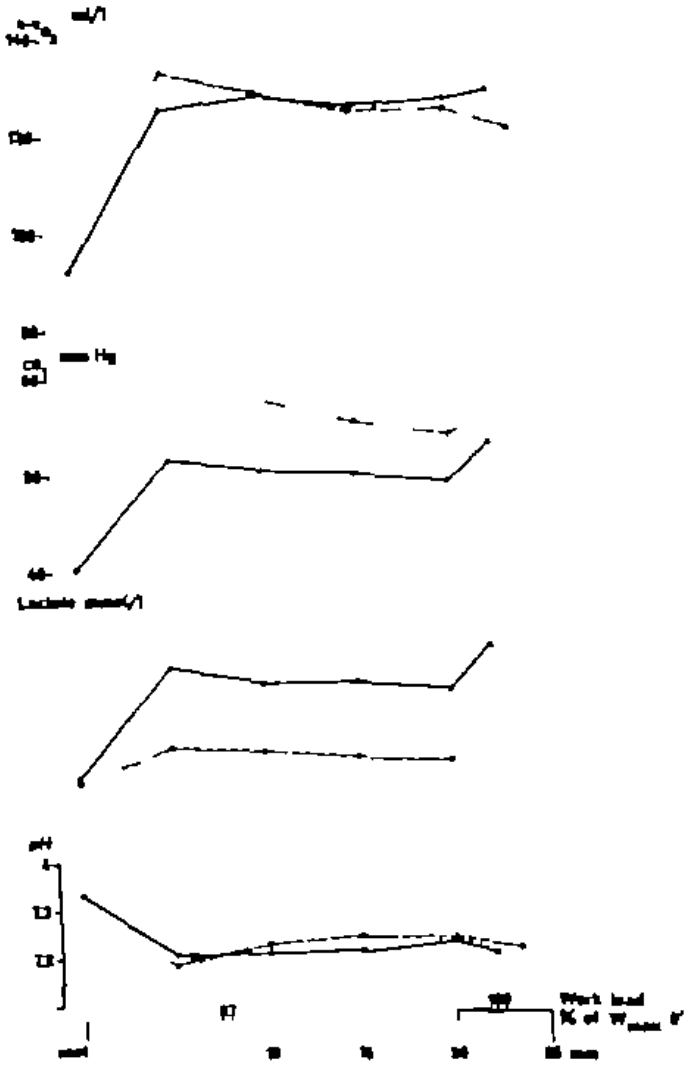


Fig. 7 Arterial-deep venous O_2 difference and P_{CO_2}, lactate concentration and pH in the deep vein of the active forearm during 20 min work at a load corresponding to 67 per cent of W_{max}, followed by work to exhaustion at 100 per cent of W_{max}. Average values ($n = 6$) at rest, after 5, 10, 15 and 20 min at the lower load and at exhaustion at the higher load. Continuous lines = air breathing at 1 ata, dashed lines = oxygen breathing at 3 ata.

Air breathing at 1 ata

Arterial blood The *lactate concentration* increased slightly from 0.47 mmoles/l (SD 0.20) at rest to 1.06 mmoles/l (SD 0.59) after 20 minutes work and 1.05 mmoles/l (SD 0.30) at exhaustion. The difference between exhaustion and rest is significant ($P < 0.01$). No other significant changes occurred during work.

Deep vein blood The *O_2 saturation* decreased significantly from 48.8 ml/l at rest to 31.5 ml/l after 5 minutes work ($P < 0.05$). It remained on the same level throughout the work at $\frac{2}{3} \times W_{max}$ and did not either change during the terminating exhaustive work ($P > 0.05$). The *arterio-venous O_2 difference* was 125.2 ml/l after 5 minutes' work. It was unchanged after 20 minutes, 127.4 ml/l, as well as at exhaustion, 123.3 ml/l ($P > 0.05$).

P_{CO_2} was 40.0 mm Hg at rest. It increased to 51.7 mm Hg after 5 minutes work ($P < 0.05$) and remained on the same level to the 20th

minute. It increased slightly to 53 7 mm Hg at exhaustion after the increase in work load ($P<0.05$)

The *lactate concentration* was 3 05 mmoles/l after 5 minutes. It was not significantly changed after 20 minutes, 2 60 ($P>0.05$) At exhaustion it had increased in four subjects and remained almost unchanged in two the average value being 3 50 mmoles/l and not significantly different from that after 20 minutes ($P>0.05$) The veno-arterial concentration difference was not changed between 5 and 20 minutes ($P>0.05$)

The *pyruvate concentration* was 0 068 mmoles/l at rest. It increased to 0 121 mmoles/l after 5 minutes work ($P<0.05$) and remained unchanged throughout the work period.

The *pH* decreased from 7 384 at rest to 7 212 after 5 minutes work ($P<0.01$) It increased slightly during the constant load work, being 7 245 after 20 minutes ($P<0.05$) At exhaustion it was 7.226 which is not significantly lower than after 20 minutes.

The *performance* time to exhaustion after increasing the load was 1 '8 seconds (SD 68)

Oxygen breathing at 3 ata

Arterial blood Po_2 was 1778 mm Hg (SD 74) at rest. After 5 minutes' work it was 1837 mm and it remained on the same level throughout the work period. The *lactate concentration* was 0 44 mmoles/l at rest and increased slightly to 0 66 mmoles/l ($P<0.05$) at exhaustion. No other changes of importance occurred in the arterial blood

Deep vein blood The *O saturation* at rest, 76 3 per cent, was significantly higher than during air breathing ($P<0.01$) It decreased to 59.5 per cent after 5 minutes' work and did not change significantly during work ($P>0.05$) During the whole work period it was significantly higher during oxygen than air breathing The *arterio-venous O difference* did not differ significantly from that during air breathing neither at rest nor at the beginning nor at the end of the work period ($P>0.05$)

Pco was after 5 minutes work 58 2 mm Hg, that is 6.5 mm Hg higher than during air breathing ($P<0.05$) It decreased slightly throughout the work period to 52 0 mm Hg after 20 minutes ($P<0.05$) and did not increase significantly during the exhaustive work ($P>0.05$)

The *lactate concentration* was 1.37 mmoles/l after 5 minutes' work. It had not changed significantly after 20 minutes ($P>0.05$) During the constant load work it was slightly lower during oxygen than air breathing ($P<0.05$) During the terminating exhausting work it increased signi-

ficantly ($P<0.05$) and the value at exhaustion, 2.46 did not differ significantly from that during air breathing ($P>0.05$). The veno-arterial concentration difference was not significantly changed between 5 and 20 minutes ($P>0.05$).

The *pyruvate concentration* was at rest 0.072 mmoles/L. It had increased slightly after 5 minutes work ($P<0.05$) and remained unchanged throughout the work period, being 0.099 mmoles/l at exhaustion. The pyruvate concentration during work was slightly lower during oxygen than air breathing ($P<0.05$).

pH was 7.304 (SD 0.029) at rest. After 5 minutes it had decreased to 7.219 ($P<0.05$). It thereafter increased significantly to 7.256 after 20 minutes ($P<0.05$). It decreased slightly to 7.237 at exhaustion at the higher load ($P<0.05$). There was no difference compared to 1 ata.

The *performance time* at the higher work load was increased in 3 decreased in 2 and unchanged in 1 subject. The average performance time, 172 sec (SD 98) did not differ significantly from that during air breathing ($P>0.05$).

Discussion

Stepwise increased load

Submaximal work. At the lowest work load, which from an oxygen uptake point of view was submaximal, the arterio-venous O difference was about 15 per cent higher during oxygen than air breathing. The increase corresponds to about 1/3 of the increase in arterial oxygen content. If the oxygen uptake in the active muscle is assumed to be the same at corresponding submaximal work intensities during oxygen and air breathing, the forearm blood flow can be calculated to be about 15 per cent lower during oxygen breathing.

The difference in calculated blood flow is caused by a difference in vascular resistance, as oxygen breathing at 3 ata does not change the arterial pressure significantly during resting conditions (see heart catheterization study p. 47). The difference in resistance may partly be caused by a direct effect of the oxygen pressure on the arteriolar tonus (Carrier Walker and Guyton 1964).

Numerous other factors probably contribute to the difference in blood flow and their relative importance cannot be established from the present data. Of interest is the observation that pyruvate has a vasodilating effect (Molnar *et al.* 1963) although the direct effect is considered weak in physiological concentrations. The difference in calculated blood flow between oxygen and air breathing is in the present study correlated to the

difference in deep vein pyruvate concentration ($r = 0.90$). However the number of observations are too few to permit definite conclusions about its role in blood flow regulation.

Maximal work. During air breathing the arterio-venous O difference increased slightly with increased work intensity. Corresponding findings are reported in studies of the femoral venous oxygen saturation during work of increasing intensity (Donald *et al.* 1957, Pernow *et al.* 1965). Wahren (1966) on the other hand did not find any increase in arterial-deep [illegible] O_2 difference with increasing work load during dynamic forearm work probably because only rather low work intensities were studied.

During oxygen breathing the arterio-venous O_2 difference did not increase with increased work load. At exhaustion the same arterio-venous O_2 difference was found during oxygen and air breathing. If the oxygen uptake in the active muscles is limited by the local blood circulation the increased blood oxygen content during oxygen breathing should have made possible an increased O uptake by an increased O_2 extraction per unit blood volume. Consequently the unchanged arterio-venous O difference during oxygen breathing indicates that the oxygen uptake is unchanged by oxygen breathing. Hence the oxygen supply by the circulation should not limit oxygen uptake. This is further evidenced by the lack of significant increase in performance time during oxygen breathing.

The deep vein blood was at exhaustion more than 50 per cent O_2 saturated, *i.e.* more than twice as high as during air breathing with a calculated Po_2 of 40-50 mm Hg during oxygen compared to about 20-25 mm Hg during air breathing. Hence not either diffusion of oxygen from the blood to the interior of the muscle cell seems to limit the maximal oxygen uptake during work with small muscle groups.

In spite of the evidence mentioned of a sufficient oxygen supply in the muscle tissue, the deep vein lactate concentration was quite high during oxygen breathing, actually not significantly lower than during air breathing at the time for exhaustion. Apparently a substantial lactate production—or a high lactate/pyruvate ratio—should not be taken as evidence of anaerobic conditions in active muscles in the sense of lack of oxygen (*cf.* Doll *et al.* 1968, Jöbsis and Stainsby 1968). It may rather be taken as an indicator that the limit for the capacity of the oxidative metabolism is approached. With normal arterial O_2 saturation and normal circulation this limit seems to be set by the oxygen utilization system in the muscle cell.

Among factors contributing to limit the maximal aerobic capacity of a muscle, the intracellular pH must be considered. As the deep vein pH at

exhaustion was the same during oxygen and air breathing, and as the deep vein lactate concentration did not significantly differ during oxygen breathing from that during air breathing the intracellular pH was probably the same under the two conditions.

Prolonged work

When, in the present study the subjects worked for 20 minutes at a load assumed to be slightly below that corresponding to the maximal aerobic work capacity no change in arterio-venous O difference with time occurred. The increase in deep vein pH during oxygen breathing closely paralleled corresponding changes during air breathing No significant change in lactate concentration or veno-arterial difference with time ocurred This is in accordance with earlier findings by other authors for the time intervals and work intensities studied (Bang 1936 Saiki Margaria and Cuttica, 1967)

When exhaustive work was performed following the submaximal the lactate concentration increased significantly during oxygen breathing The lactate concentration at exhaustion was slightly lower than during the earlier experiment, probably because of the somewhat longer work time (Åstrand *et al* 1963) The findings render it probable that enzyme inhibition induced by the high oxygen pressure did not change the muscle metabolism significantly under the conditions studied The results seem not to confirm the findings by Weglicki *et al.* (1966) in the dog and Banister *et al* (1969) during leg work in man that the lactate production is markedly inhibited by high oxygen pressure. However at least the result by Banister *et al* may be explained by the long oxygen exposure time at rest preceding the work period.

In agreement with the findings during the shorter work at stepwise increased loads the performance time was not significantly prolonged by oxygen breathing

Comment

Although oxygen breathing did not increase the work performance time significantly there are obviously interindividual variations in the influence of oxygen breathing on performance time. This is partly caused by the error of measurement. However the relation between circulatory and metabolic capacities may differ between the subjects. This statement may be supported by the apparent correlation between the changes in performance time and maximal arterio-venous O difference induced by oxygen breathing (Fig 8)

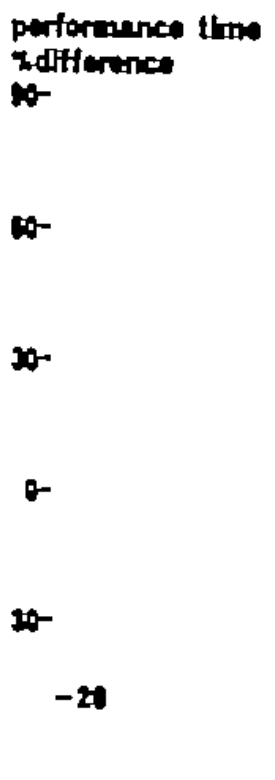


Fig. 8. Relation between the individual changes in performance time and in maximal arterio-venous O_2 difference during submaximal forearm exercise breathing air at 1 ata. Data from different sets of experiments (see text): pilot experiments (filled circles) and experiments according to W_{max} (open circles). $n = 14$, $r = 0.62$, $SD = 24.0$, $P < 0.05$.

Conclusions

1) During oxygen breathing at 3 ata the arterial O_2 content is increased about 30 per cent. During submaximal forearm work about ⅓ of this increase was utilized.

2) The aerobic work capacity was not influenced by the increased blood oxygen content at 3 ata, as evidenced by the unchanged maximal arterio-venous O_2 difference and performance time. Hence oxygen supply by the local blood flow is not limiting for maximal local O_2 uptake, apparently not either under normal atmospheric conditions.

3) At exhaustion during oxygen breathing at 3 ata the deep vein lactate concentration was not significantly lower than during air breathing in spite of the much higher P_{O_2}. This indicates that a large lactate production cannot be taken as evidence for lack of oxygen in the active muscles.

4) The deep vein pH reached at exhaustion the same level during oxygen breathing at 3 ata as during air breathing. Hence tissue acidity may be a limiting factor for aerobic muscle performance.

2. Adaptation to submaximal leg work

As little is known about the adaptation to exercise at hyperbaric oxygen pressures, it was considered necessary to make a comprehensive study of the adaptation to submaximal work before maximal work tests were performed.

The heart catheterization procedure may influence the cardiovascular adaptation to exercise For that reason the heart rate reaction during exercise, breathing oxygen at 3 ata, was studied in preliminary experiments without heart catheters. During work under normal atmospheric conditions a correlation is found between heart rate and arterial lactate concentration. Therefore, also blood lactate was determined.

The cardiovascular and ventilatory adaptation to exercise was then studied in greater detail by means of heart catheterization in the main experiment.

Preliminary study of heart rate and blood lactate

Subjects

Eight male volunteers, aged 19-31 years, were studied. Their average work capacity determined as W was 1240 kpm/min (range 1150-1400 kpm/min) By the author's experience they were of average physical fitness.

Procedure

Exercise was performed during oxygen breathing at 3 ata and for comparison during air breathing at 1 ata. Each subject exercised at a lower load selected to yield a heart rate of about 120 beats/min for 5 minutes, rested for 10 minutes and exercised at a higher load selected to yield a heart rate of about 160 beats/min for 5 minutes. During the oxygen breathing tests resting values were recorded after 4 minutes of oxygen breathing and each exercise period was preceded by about 4 minutes of oxygen breathing

Measurements

The heart rate was recorded at rest and continuously during work. Blood for lactate determination was sampled at rest and during the last 30 seconds of each exercise period from a catheter in an antecubital vein.

For methodological details, see Chapter I

Results

The results are summarized in Table V

The average heart rate at rest in the sitting position on the cycle ergometer was 7 beats/min lower during oxygen than air breathing ($P>0.05$) Throughout the exercise periods, both at the lower and the higher work load the heart rate was in all subjects lower during oxygen than air

TABLE V Heart rate and blood lactate during submaximal leg work breathing oxygen at 3 ata and air at 1 ata.

	air 1 ata mean ± S D			O_2, 3 ata mean ± S D			Difference 3 ata — 1 ata, mean ± standard error of the mean.		
Load, kpm/min range	rest	load I 590 500—750	load II 1140 1000—1400	rest	load I 640 500—750	load II 1140 1000—1400	rest	load I 590 550—750	load II 1140 1000—1400
Heart rate, beats/min	80 ±12	122 ±8	163 ±13	75 ±9	106 ±7	147 ±13	—7 ±3 1	—16 ** ±1.5	—16*** ±2.4
lactate, mmoles/l	0.98 ±0.13	1.54 ±0.51	4 14 ±1.37	0.92 ±0.17	1 40 ±0.56	3.28 ±1.50	—0.06 ±0.11	—0.14 ±0.20	—0.86 ±0.36

breathing The average difference was 16 beats/min after 5 minutes at the lower work intensity and 16 beats/min after 5 minutes at the higher work intensity Both differences are highly significant ($P<0.001$)

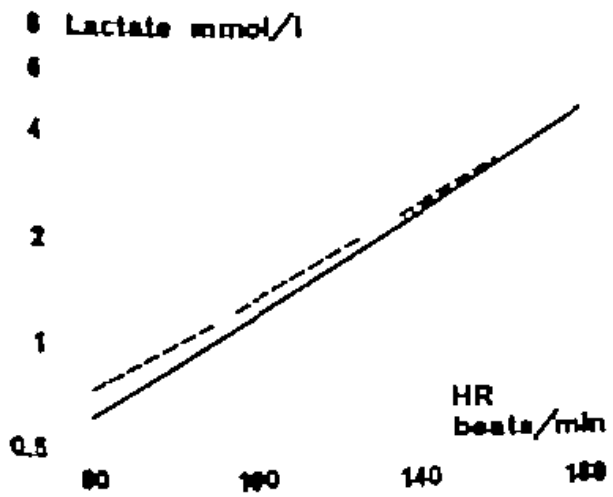


Fig 9 Relation between log lactat and heart rate t submaximal work loads. ——— regression line, air breathing t 1 ata. log lactate = 0.0073 HR — 0 65 —0,87 SD = 0.154
regression line, oxygen breathing t 3 ata. log lactate = 0 0066 HR — 0,53 —0.82, SD = 0 152.

At the lower work load the lactate concentration was not significantly different from that during air breathing ($P>0.05$) At the higher work load it was 0 86 mmoles/l lower during oxygen breathing ($P<0.05$) The relation between heart rate and lactate concentration followed almost the same course during oxygen and air breathing (Fig 9)

Discussion

The difference in heart rate between oxygen breathing at 3 ata and air breathing was of the same order of magnitude at the higher and the lower

work load It was larger than the difference in heart rate between oxygen breathing at 1 ata and air breathing reported by Asmussen and Nielsen (1955)

Although there does not seem to be any significant difference between mixed venous and arterial lactate concentrations during exercise (Holmgren 1959) the concentration in an arm vein may differ from that in arterial blood because of an uptake in the forearm muscles (Carlson and Pernow 1959) However the relation between log lactate concentration and heart rate during air breathing was the same as earlier described for arterialized capillary blood by Strandell (1964) in healthy young men The relation between log lactate and heart rate was the same during oxygen as during air breathing It may be tempting to interprete the correlation as an indication that the difference in production of acid metabolites between oxygen and air breathing is one factor determining the difference in heart rate However the concentration differences are very small in relation to the heart rate differences, and in the individual subjects no correlation between the magnitude of heart rate difference and lactate difference between oxygen and air breathing can be found.

Cardiac output intravascular pressures and blood gases studied by means of heart catheterization

Subjects

Eight male volunteers, aged 19-26 years, were studied. Seven of them were students, one was a teacher Their average working capacity determined as W_{170} was 1280 kpm/min (range 1150-1650 kpm/min) the highest value was recorded for a subject who takes part in competitive cross-country skiing With the latter exception, the subjects were considered of average physical fitness.

Procedure

A Gensini catheter was introduced through an antecubital vein and advanced to the pulmonary artery A teflon catheter was introduced about 15 cm in the proximal direction in the brachial artery Exercise was performed during oxygen breathing at 3 ata and for comparison during air breathing at 1 ata. Each subject exercised for 7-8 minutes at a work load yielding a heart rate of about 120 beats/min rested for 10 minutes and then exercised at a work load yielding a heart rate of about 160 beats/min The resting measurements during oxygen breathing were made after 4 minutes of oxygen breathing Between the different measurement periods

(rest, load I load II) the subject was disconnected from the oxygen breathing system to keep the continuous oxygen exposure times short Each oxygen breathing exercise period was preceded by 4 minutes of oxygen breathing

Measurements

Pressure measurements in the brachial and pulmonary artery were performed at rest and after 3-4 minutes of exercise. Samples of arterial and mixed venous blood were taken for measurement of Po_2 oxygen saturation, Pco_2 and pH, expired gas was collected for determination of total lung ventilation, CO_2 elimination and O_2 uptake (during air breathing) and cardiac output was determined between 4½ and about 7 minutes of work. During oxygen breathing cardiac output was determined with the dye dilution technique, during air breathing as well with the Fick method.

For methodological details, see Chapter I

Results

The results are summarized in Table VI A and B and Fig 10

Air breathing at 1 ata

The *stroke volume* was significantly larger at the lower work load than at rest in the sitting position ($P<0.001$) It did not increase significantly with increased work intensity ($P>0.05$)

The *arterio-venous O difference* increased substantially from an average value at rest of 60.5 ml/l to 111.4 ml/l at the lower load ($P<0.001$) It increased slightly further with increased work intensity in all subjects to an average value at the higher work load of 134.7 ml/l ($P<0.05$)

The *arterial Pco* was 38.3 mm Hg at rest and remained on a constant level at both work loads. The *arterial pH* decreased from 7.413 at rest to 7.348 at the highest work load ($P<0.05$)

The mixed venous Pco_2 increased moderately from 39.6 mm Hg at rest to 54.5 mm Hg at the highest work load ($P<0.01$) The *mixed venous pH* decreased from 7.384 at rest to 7.252 at the highest work load ($P<0.01$)

The integrated mean pressure in the *brachial artery* increased approximately linearly with increased heart rate. The *pulmonary artery* mean pressure increased moderately with increased work load and heart rate

Oxygen breathing at 3 ata. At rest

The arterial Po_2 was 1808 mm Hg The *heart rate* was the same during

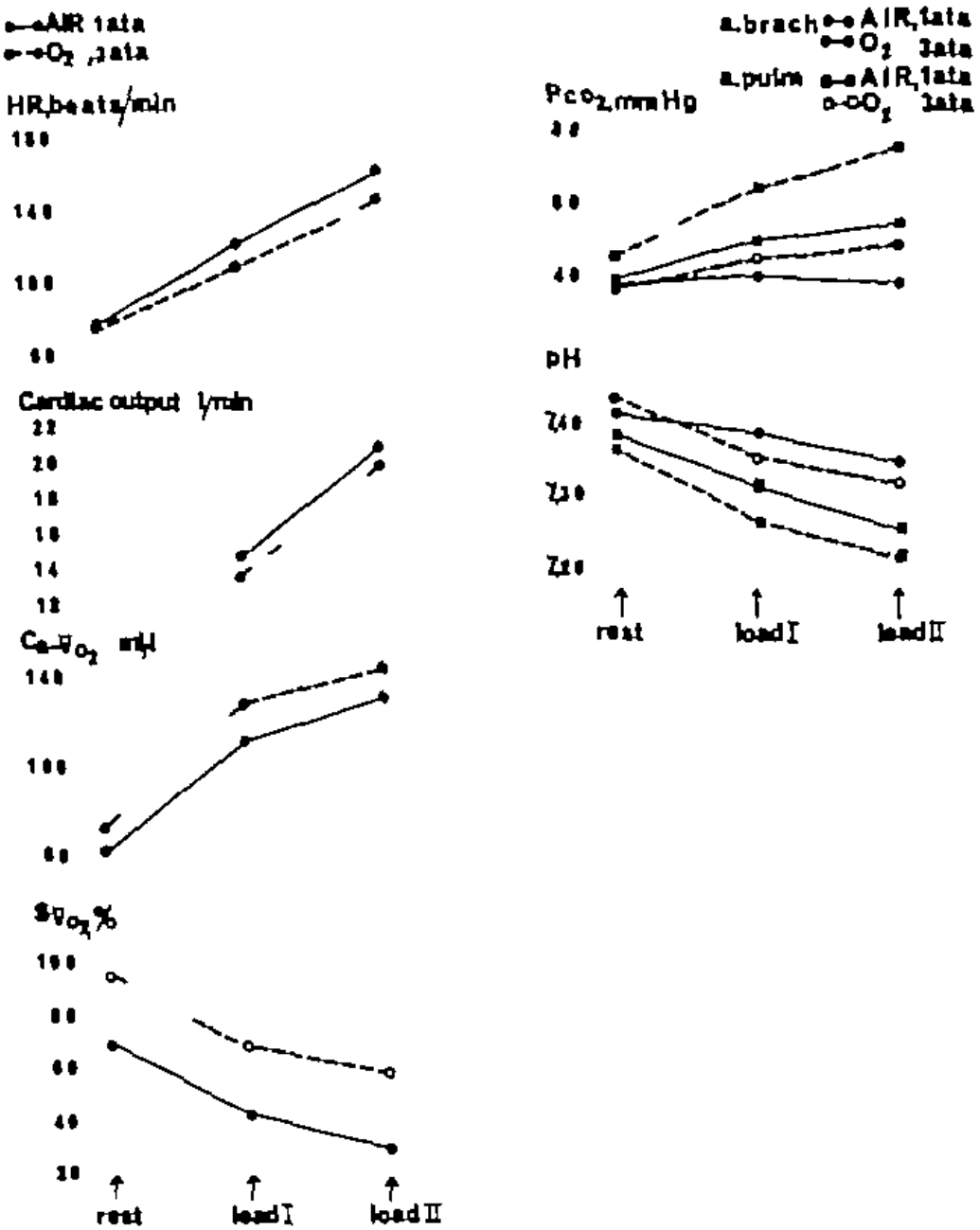
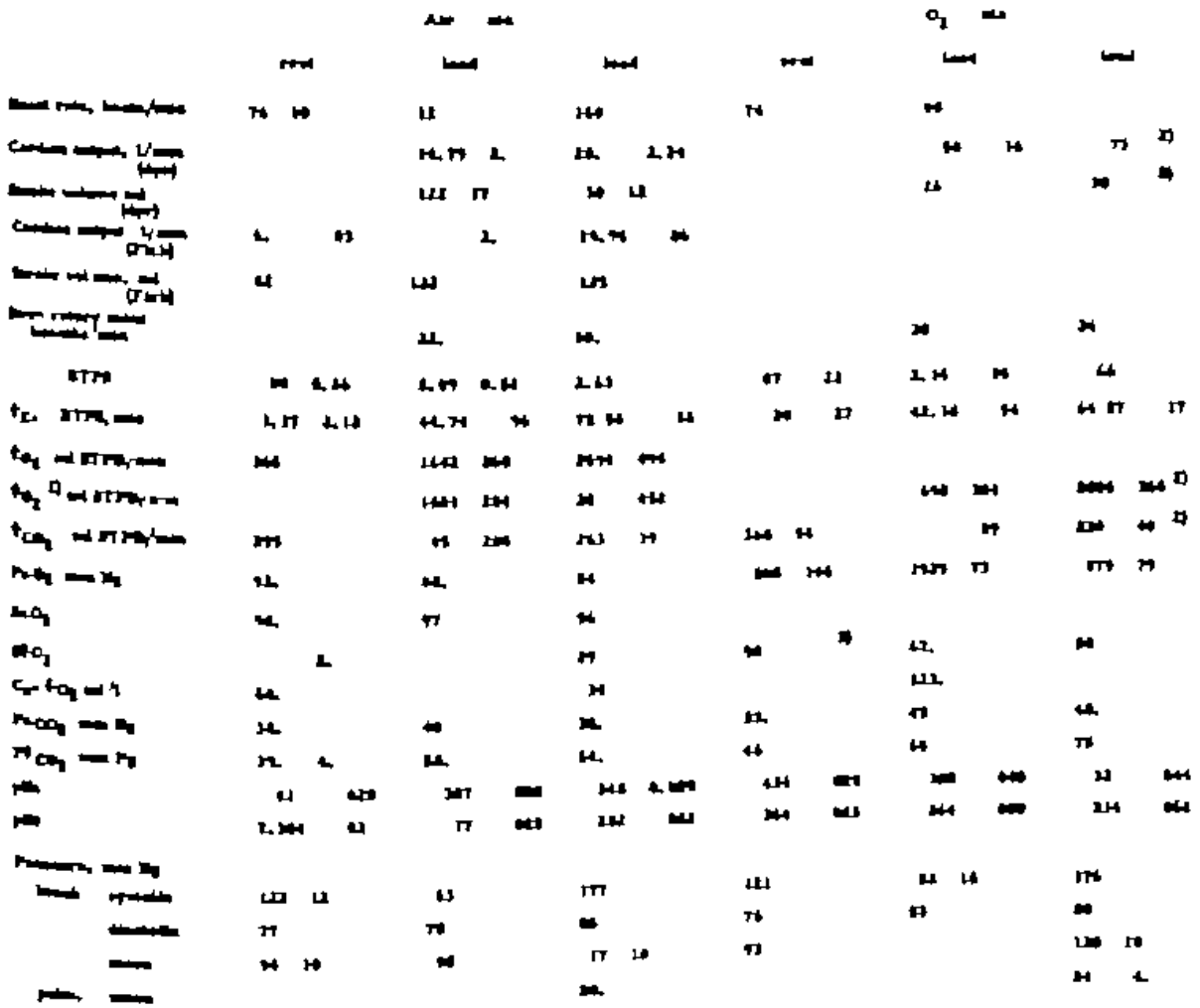


Fig 10 Heart rate cardiac output, arterio-venous O_2 difference and mixed venous (= pulmonary artery) O_2 saturation. P_{CO_2} and pH in the brabcial artery and pulmonary artery A erage values t rest and after about 6 min at the lower and the higher load *during air breathing* t 1 ta *and oxygen breathing at 3 ata.* = 8 *except for* cardiac output, load II (n = 5)

oxygen as during air breathing The *arterio-venous O difference* was 71 0 ml/L 11 ml larger than during air breathing ($P<0.01$) The *CO_2 elimination* and the *lung ventilation* were significantly larger during oxygen than air breathing ($P<0.01$) whereas the *arterial Pco* was unchanged. The *mixed venous O saturation* was 92 4 per cent, that is substantially higher than during air breathing ($P<0.001$) No significant differences in *arterial*

Table VI A. Heart catheterization data, obtained at rest in the sitting position and at light (500—850 kpm/min) and a moderately heavy (1000—1400 kpm/min) work load during air breathing at 1 ata and oxygen breating t 3 ata. Mean ± SD n = 8.



1) Calculated from cardiac output and arterio-venous O_2 difference
2) = 5. In 3 subjects the dye curves at 3 ata were not technically satisfactory
3) n = 7

or *mixed venous pH* were found compared to air breathing ($P>0.05$) The *systolic diastolic* and *mean pressures* in the *brachial* and the *pul monary artery* were the same during oxygen as during air breathing ($P>0.05$)

Oxygen breathing at 3 ata. During exercise

Pao was 1929 mm Hg at the lowest load. At the highest load it was slightly lower 1879 mm Hg ($P<0.05$)

At the lower work load no significant difference in *lung ventilation* was found between oxygen and air breathing At the higher work load, how ever the ventilation was on the average 18 per cent lower during oxygen than air breathing ($P<0.01$) The difference was caused by a difference

Table VI B. Difference between oxygen breathing at 3 ata and air breathing. Mean ± standard error of the mean. n = 6.

	O_2 3 at	air 1 ata	
	rest	load I	load II
Heart rate beat /min	2 ± 2	13 ± 2***	17 ± 2***
Cardiac output l/min		1 30 ± 0 29**	1 55 ± 0 67
Stroke volume ml		3 ± 4	5 ± 9
Respiratory rate breaths/min	1 8 ± 0 9	2 0 ± 1 1	5 6 ± 1 1**
$\dot{V}_T$ l BTPS	0 07 ± 0 06	0 05 ± 0 16	0 03 ± 0 13
$\dot{V}_E$ l BTPS/min	3 78 ± 0 99	2 36 ± 2 92	13 99 3
$\dot{V}_{O_2}$ [1] ml STPD/min		47 ± 53	115 ± 121
$\dot{V}CO_2$ ml STPD/ml	76 ± 18	140 ± 46	332 ± 48***
$Pa O_2$ mm Hg			
$Sa O_2$ %			
$S O_2$ %	4 6 ± 0 6	25 9 ± 1 2***	28 5 ± 1 3**
Ca O ml/l	11 0 ± 2 6**	12 4 ± 2 3***	6 9 ± 4 0
P CO_2 mm Hg	0 8 ± 0 9	4 6 ± 1 6	10 5 ± 1 2***
P CO_2 mm Hg	7 4 ± 2 3	14 7 2 5***	20 6 ± 4 0**
pHa	0 021 ± 0 014	0 029 ± 0 014	0 027 ± 0 011
pH_v	0 018 ± 0 012	0 053 ± 0 017	0 038 ± 0 016
P sur mm Hg			
b h yst li	1 ± 2	0 ± 3	1 ± 5
diastolic	1 ± 2	5 ± 2	4 ± 2
mean	1 ± 2	3 ± 1***	3 ± 2
a pulm. mean	0 6 ± 0 3	0 4 ± 0 6	3 6 ± 1 0

1) Calculated from cardiac output and arterio-venous O_2 difference.

in respiratory frequency the tidal volume being the same during oxygen as air breathing

The calculated *oxygen uptake* at corresponding work intensities was the same during oxygen as air breathing ($P > 0.05$)

Compared to air breathing the *heart rate* and the *cardiac output* were

about 10 per cent lower whereas the *stroke volume* was not significantly changed by oxygen breathing

The *O_2 saturation* of mixed venous blood at corresponding work intensities was substantially higher during oxygen than air breathing ($P<0.001$)

The *arterio-venous O difference* at the lower load was 12 4 ml/l larger during oxygen than air breathing ($P<0\,001$) whereas at the higher load no statistically significant difference was found the interindividual variations were large.

The *arterial Pco_2* was at the lower load 4 6 mm Hg higher during oxygen than air breathing ($P<0\,05$) and at the higher work load 10 5 mm Hg higher ($P<0\,001$)

The magnitude of the difference in $Paco_2$ between oxygen and air breathing was fairly well correlated to the difference in lung ventilation.

At the lower work load no significant difference in *arterial pH* was found ($P>0\,05$) whereas at the higher load the arterial pH was slightly lower during oxygen than air breathing ($P<0\,05$) The *mixed venous pH* was lower during oxygen breathing at both work loads ($P<0\,05$) reaching 7.214 at the higher load. The difference was caused by a higher *mixed venous Pco_2* during oxygen breathing both at the lower ($P<0\,001$) and at the higher work load ($P<0\,01$)

At the lower work load a slightly higher *diastolic* ($P<0\,05$) as well as integrated *mean pressure* ($P<0\,001$) in the *brachial artery* was found during oxygen compared to air breathing The difference was small, 5 0 mm Hg. At the higher work load no significant difference was found ($P>0\,05$) however The *systemic vascular resistance* calculated from the arterial pressure and the cardiac output was significantly higher during oxygen breathing at both work loads ($P<0\,001$) At the higher load a linear correlation between the increase in vascular resistance and the increase in arterial Pco_2 induced by oxygen breathing was found (Fig 11) At the lower load no corresponding correlation could be demonstrated.

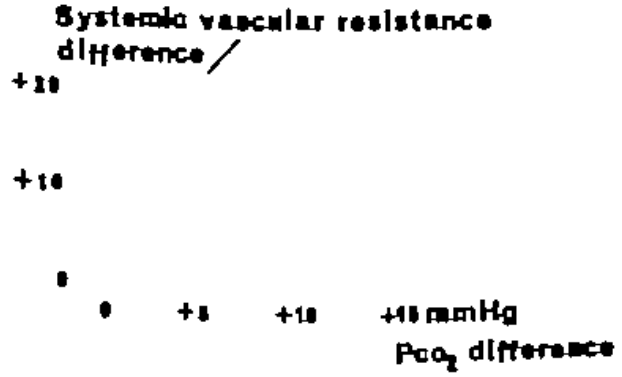


Fig 11 Relationship between the difference in systemic vascular resistance and the difference in arterial Pco_2 between oxygen breathing at 3 ata and air breathing at 1 ata at the highest load. The systemic vascular resistance is calculated as brach. artery mean pressure / cardiac output

At the lower work load the *mean pressure* in the *pulmonary artery* was the same during oxygen as during air breathing At the higher work load the pressure was significantly higher during oxygen than air breathing in spite of the smaller cardiac output. The pressure difference was not correlated to either of the factors, P_{CO_2} and pH in the pulmonary or brachial artery

Discussion

Stroke volume cardiac output and arterio-venous O_2 difference

The cardiovascular adaptation to exercise during air breathing in the present study agrees with earlier reports from the same laboratory concerning exercise in the sitting position (Bevegård, Holmgren and Jonsson 1960 Holmgren Jonsson and Sjöstrand 1960 Ekelund and Holmgren 1967)

The stroke volume was the same during oxygen as air breathing Consequently changes in exercise heart rate, induced by exposure to oxygen at 3 ata, reflect changes in cardiac output. This is important to establish if conclusions concerning the maximal aerobic work capacity are to be drawn from data obtained during hyperbaric oxygen breathing The possibility of toxic manifestations of oxygen on the myocardium has been proposed both during oxygen breathing at 3 ata (Kioschos *et al* 1969) and during oxygen breathing at normal atmospheric conditions (Daniell and Bagwell 1968) Both investigations were performed in resting dogs. In the light of the present study it seems much more likely that the decrease in stroke volume during oxygen breathing was caused by the labile stroke volume of dogs under differing experimental conditions (Rushmer and Smith 1959)

During oxygen breathing the arterial oxygen content was increased by about 30 per cent. With the moderate decrease in cardiac output and increase in arterio-venous O difference, less than 20 per cent of the increase in arterial oxygen content was utilized Consequently the mixed venous blood was comparatively highly saturated also during rather heavy work.

Gas exchange lung ventilation and blood gases

No difference in oxygen uptake at corresponding work loads between oxygen and air breathing were found This is in agreement with earlier reports concerning oxygen breathing at 1 ata (Asmussen and Nielsen 1955) The possibility that the oxygen uptake rate at a given mechanical work intensity can be decreased by prolonged exposure to high oxygen pressure has been proposed by Banister (1969) The sparse data in Banister's preliminary communication makes it impossible at present fully

to interpret them. Salzano *et al.* (1967) on the other hand found a larger oxygen uptake during oxygen breathing at 2 ata than during air breathing at 1 ata at corresponding work loads. They considered it most probably caused by the increased air density. The present data concerning exercise at 3 ata during air breathing apparently contradict this explanation (Chapter II).

Although the oxygen uptake did not differ during oxygen as compared to air breathing the CO_2 elimination was decreased during oxygen breathing. As both arterial and mixed venous P_{CO_2} was increased during oxygen breathing this is probably due mainly to an accumulation of CO_2 in the body. The compartmentalization for CO_2 in the body is not so well known, that it is possible to calculate whether a decreased tissue respiratory quotient also contributes to the decreased CO elimination. The higher CO elimination at rest during oxygen as compared to air breathing may be explained by an increased metabolic rate or at least partly by hyper ventilation. Oxygen is earlier reported to stimulate ventilation at rest (Asmussen and Nielsen 1946, Lambertsen *et al.* 1953).

In agreement with earlier investigations at 1 ata (Asmussen and Niel sen 1946, 1958, Lambertsen *et al.* 1953, Åstrand 1954, Dejours 1959) a decrease in lung ventilation during oxygen compared to air breathing was found at the heavier load, whereas at the lower load no differences were recorded. The decrease in ventilation during oxygen breathing at the higher load, 18 per cent, is of the same order of magnitude as that found by Telfer and Jennett (1965) during oxygen breathing at 3 ata, Lambertsen *et al.* (1959) at 2 ata and by Åstrand (1954) and Asmussen and Nielsen (1958) during oxygen breathing at 1 ata. The mechanisms behind the decrease in ventilation are less well known (Asmussen and Nielsen 1946, 1958, Bannister and Cunningham 1954, Dejours *et al.* 1959). As proposed by Dejours (1959) a reduced or ceased impulsive activity from the chemoreceptors may be the most probable explanation. In that case it seems likely that the activity from the chemoreceptors ceases at a P_{O_2} not higher than 1 atmosphere, as the ventilation decrease by oxygen is the same at 3, 2 and 1 ata.

Intravascular pressures

The difference in mean brachial artery pressure between oxygen and air breathing at corresponding work loads was small. Only at the lower load was a significant although small, increase in pressure found during oxygen breathing. On the other hand, in relation to the cardiac output and the heart rate the pressure was higher during oxygen breathing, the calculated

vascular resistance being therefore higher Partly this seems to be the effect of the higher arterial P_{CO_2} as the difference in resistance between oxygen and air breathing at the highest load is correlated to the difference in Pa_{CO_2} (Fig 11) Other factors, such as the direct effect of oxygen on vascular tone, must contribute, however Thus also at the lower load, where Pa_{CO_2} during oxygen breathing was almost within the normal range, the vascular resistance was higher during oxygen breathing

Very small differences in pulmonary artery pressure were found between oxygen and air breathing at corresponding work loads, slightly higher pressures being found during oxygen breathing only at the higher work load. The reason for the pressure difference is not clear A decreased inspiratory oxygen pressure increases the pulmonary vascular resistance (von Euler and Liljestrand 1946) The effect of an increase seems to be more complicated. If the oxygen pressure in the pulmonary artery is increased, an increased vascular resistance will result (Moulder *et al.* 1964 Bain, Lancaster and Adams 1965) This factor does not seem to contribute to the pressure increase during oxygen breathing in the present study however as the oxygen saturation in the pulmonary artery was higher during oxygen breathing at rest and at the lower load when no pressure difference was found.

Conclusions

1 At submaximal work loads the cardiac output was lower and the arteno-venous O difference higher during oxygen breathing at 3 ata than during air breathing The differences were much smaller however than the difference in arterial oxygen content.

2 The stroke volume was found to be the same during oxygen as during air breathing

3 The pressures in the brachial and pulmonary artery were almost equal during oxygen and air breathing at corresponding loads. However in relation to the cardiac output they were higher during oxygen breathing

4 The lung ventilation during heavy exercise was decreased during oxygen breathing at 3 ata. The difference was of the same order of magnitude as that reported by other authors during oxygen breathing at 1 ata.

3. Adaptation to maximal leg exercise

Subjects

Twelve male volunteers, aged 19-25 years, were studied. Eleven of them

were students, one was a teacher Their average physical working capacity, determined as W_{170}, was 1240 kpm/min (range 1150-1350 kpm/min) In six subjects arterial and femoralvenous catheterization was performed five of them had taken part in the heart catheterization study earlier described. In the remaining six subjects only heart rate and performance time during exhaustive work were measured.

Procedure

In six subjects, percutaneous catheters were introduced in an artery (brachial artery in five, radial artery in one subject) and the right femoral vein. In the remaining six subjects only heart rate and performance time were measured.

Exercise was performed during oxygen breathing at 3 ata and for comparison during air breathing at 1 ata. The subjects first exercised for 5 minutes at a submaximal load selected to give a heart rate of about 120 beats/min (warm-up) After that they rested for 10 minutes and then worked until exhaustion at a work load which they could sustain for 5-6 minutes. The resting measurements during oxygen breathing were preceded by 4 minutes of oxygen breathing Also the exercise periods were preceded by about 4 minutes of oxygen breathing

Measurements

The heart rate was recorded continuously Arterial and venous blood (six subjects) for the determination of O saturation, P_{CO_2} pH, lactate and pyruvate concentrations, and arterial blood for P_{O_2} determination was sampled at rest, after 1 and 2 minutes and during the last 30 seconds of the exhaustive exercise period. P_{O_2} in the femoral vein was measured only at exhaustion.

Results

Heart rate and performance time

At rest the heart rate was 74 beats/min (SD 8) during air breathing and 70 beats/min (SD 9) during oxygen breathing at 3 ata. The difference is not significant ($P>0.05$)

During exercise breathing air the heart rate at the exhaustive load increased to 156 beats/min (SD 9) after 1 min. It increased further to 183 beats/min (SD 9) at exhaustion. During oxygen breathing it was throughout the work period in all subjects lower than during air breathing. The difference was largest in the early phase of work being 18 beats/min

after 1 minute and significantly lower 13 beats/min ($P<0.05$) after 4 minutes. The heart rate at exhaustion was 171 beats/min (SD 7)

The *performance time* was 6 15 min (SD 1 4) during air and 6 47 min (SD 2 1) during oxygen breathing The values are not significantly different ($P>0.05$) Of the individual subjects eight worked longer during oxygen and four during air breathing

Arterio-femoralvenous difference in blood gases and lactate
The results are summarized in Table VII and Fig 12

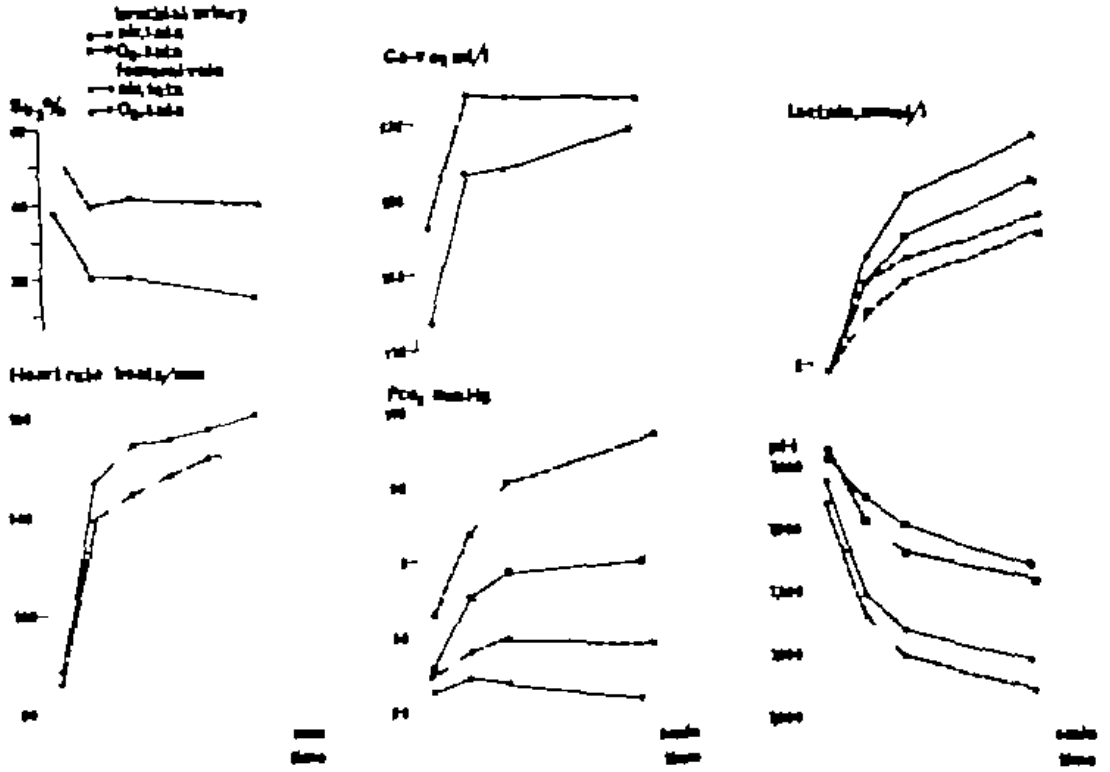


Fig 12 Femoralvenous O_2 saturation heart rate, arterial-femoralvenous O_2 difference arterial and femoral venous Pco_2, lactate concentration and pH. Average values (n = 6) at rest after 1 and 2 min, and immediately before the end of exhaustive work during air breathing at 1 ata and O_2 breathing at 3 ata. Continuous lines denote air at 1 ata, dashed lines oxygen at 3 ata. Pco_2, lactate and pH from the brachial artery squares, from the femoral vein circles.

The six subjects studied by femoralvenous catheterization did not differ from the rest of the group with respect to influence of oxygen on performance time or heart rate at rest and during work.

At rest
During air breathing the *O saturation* in the femoral vein was 37.3 per cent the *arterio-venous O difference* being 117 3 ml/l.

Table VII A. Data obtained at rest in the sitting position, after 1 and 2 min and at exhaustion at work loads ranging between 1450—1800 kpm/min during 1 breathing at 1 ata and oxygen breathing at 3 ata. Mean±SD n = 6.

B. Difference between oxygen breathing at 3 ata and air breathing at 1 ata. Mean± standard error of the mean. n = 6.

Time after start of work, min	1 ata rest	1 ata work 1	1 ata work 2	1 ata exhaustion (av. 3.77)	O_2, 3 ata rest	O_2, 3 ata work		O_2, 3 ata exhaustion (av. 3.75)
Heart rate, beats/min	76 7	154 8	168	83	73 9	135 12	149 17	178 9
O_2 mm Hg	82.3 5.	91.5 5.5	3.8	86.9 5.3	983 8	1950 08	1853 11	821 96
O_2 %	98	2 6	94.6 1.2	93.9				
O_2 mm Hg				9				5.8
O_2 %	37	28 5.8	26.5	14	54	39.8 5		
$C_{a-v}O_2$ ml/l	3	57.5 3.2	58 3.	149 16	3 .7	78. 8	77	77
CO_2 mm Hg	34 5	38	37	34.5	38	46	49.3	44. 5
CO_2 mm Hg	1.0 5	40	6 7.0	39 7	55 9	77 5.2		54.5
lactate mmol/l	1 +0.22	27 .89	8.86 .43	76 5	+0.	73 .32	60 5	8.96 .06
lactate$_{art}$ mmol/l	1.43 +0.80	84 8.44	1.06 .52	1 74	.61 +0.75	6.42	59	73 .76
pyruvate mmol/l	0.034 +0.003	0.43 +0.052	0.47 +0.036	0.1 +0.045	0.066 +0.002	0.50 +0.069	0.66 +0.054	46 +0.064
pyruvate$_{art}$ mmol/l	0.032 0.017	0.26 +0.009	228 +0.032	0.231 +0.8	064 +0.029	0.156 +0.048	0.54 +0.034	0.176 +0.049
pH	7.3 +0.017	7.348 +0.01	306 +0.008	7.241 +0.036	36 +0.017	309 +0.026	7.263 +0.07	.213 +0.036
pH	7.375 +0.014	7.193 +0.0 8	2.1 +0.027	7.092 +0.030	7.341 +0.017	7.161 +0.0 4	7.099 +0.010	044 +0. 39

O_2, 3 ata rest	1 ata work 1	1 ata work 2	1 ata exhaustion
3	1	1***	11
3		2	3
			48.0***
			1
**	.2***	20.9***	23.1**
8	1.8	1.3	1.8
26	3.5***	.0**	9.3
	6	7	9
		3	3
3	.2	3	5.6
	14	23.3	33
3.9	7.3	9.8	8.3
-0.03	39	2.49	2.80**
0.36	8.5	0.32	0.33
0.6	42	3.3***	-4.25***
58	8.54	8.68	52
-0.01	0.8	-0.00	.026
+0.01	+0.039	+0.042	+0.036
-0.006	-0.163	-0.064	+0.033
+0.015	+0.034	+0.03	+0.027
0.023	-0.139	+0.045	+0.028
+0.01	+0. 18	+0.03	+0.009
-0.034	-0.132	-0.042	-0.048**
+0.012	+0.011	+0.009	+0.012

During oxygen breathing the *arterial Po* was 1883 mm Hg The *venous O_2 saturation* was 54.5 per cent, which is significantly higher than during air breathing ($P<0.01$) The *arteno-venous O_2 difference* was 143 8 ml/l 26.5 ml/l higher than during air breathing ($P<0.05$)

Exhaustive work

Air breathing After 1 minute of exercise the *O saturation* in the femoral vein was 20 6 per cent. It decreased further during the exercise period and was at exhaustion 16 0 per cent ($P<0.05$) The corresponding *Po_2* was 17 8 mm Hg

The *arterial Pco* decreased slightly throughout the work period. The *femoralvenous Pco* increased from 60.5 mm Hg after 1 minute to 70.8 mm Hg at exhaustion

Both the *arterial* and *venous lactate* concentrations increased during exercise the veno-arterial difference not changing significantly from the first minute to exhaustion.

The *arterial pH* decreased to 7 241 and the *venous pH* to 7 092 at exhaustion

Oxygen breathing The *arterial Po* was 1892 mm Hg after 1 minute of work and remained unchanged. The *venous O_2 saturation* was 39 8 per cent after 1 minute It remained on the same level during the whole work period, being 41 1 per cent at exhaustion The *venous Po_2* at exhaustion was 65 8 mm Hg The *arteno-venous O difference* after 1 minute of work was 21 5 per cent higher than that during air breathing ($P<0.001$) No significant difference was found at exhaustion

The *art rial Pco* was higher during work than at rest ($P<0.01$) It did not change significantly from the first to the last minute of work. At exhaustion it was 48 8 mm Hg, *i.e* 14.5 mm Hg higher than during air breathing ($P<0.05$) *Pco_2* in the *femoral vein* increased from 77 3 mm Hg after 1 minute to 104.5 mm Hg at exhaustion ($P<0.01$) The value at exhaustion is 33 7 mm Hg higher than corresponding value during air breathing ($P<0.01$)

The *arterial lactate concentration* increased during work to 8.86 mmoles/l at exhaustion. Compared to air breathing this is significantly lower ($P<0.001$) However the value after 1 minute did not differ significantly from that during air breathing The *femoralvenous lactate concentration* increased to 9.93 mmoles/l at exhaustion, 4 25 mmoles/l less than during air breathing ($P<0.001$) The *veno-arterial lactate difference* was at exhaustion ($P<0.05$) but not after 1 and 2 minutes higher during oxygen than air breathing.

The *arterial pH* was at exhaustion slightly lower during oxygen than air breathing ($P<0.05$). Also the *venous pH* was at exhaustion lower than during air breathing ($P<0.01$).

Discussion

The heart catheterization study showed that the exercise stroke volume was the same during oxygen as during air breathing. Consequently the lower heart rate at exhaustion during oxygen breathing means that the cardiac output is lower than during air breathing. Thus the subjects were exhausted in spite of a lower calculated cardiac output and a higher femoralvenous O saturation than during air breathing. This gives evidence for that the O_2 transport is not limiting the oxygen uptake.

The intramitochondrial Po_2 is supposed to be about 1 mm Hg (Chance *et al.* 1962). In that case the O diffusion gradient from capillary blood to the mitochondrion was 3-4 times larger during oxygen than air breathing as judged from the venous Po_2. As, in spite of this, no sign of a significant increase in maximal oxygen uptake was found as judged from performance time arteriovenous O difference and maximal heart rate, not either diffusion from blood to muscle tissue seem to be limiting for aerobic work capacity in normal subjects.

The statement above is valid for the maximal muscular oxygen uptake rate. However even if it is not limiting for the maximal oxygen uptake an initial delay in cardiac output increase may limit oxygen uptake in the beginning of work (Margaria *et al.* 1965, Hughes *et al.* 1968). The higher femoralvenous O saturation after the end of the first minute of work than at exhaustion during air breathing may be taken as an indication that the oxygen transport by blood circulation does not either limit oxygen uptake in this quite early phase of work (*cf.* Hartley and Saltin 1969). However conclusions regarding the muscle oxygen supply at the onset of work premises still earlier measurements than in the present study.

In spite of the very high femoralvenous Po_2 during oxygen breathing the femoralvenous lactate concentration was only slightly lower than during air breathing. Consequently a high lactate production cannot be taken as evidence of a lack of molecular oxygen in the active muscle.

Both the arterial and the femoralvenous pH was slightly lower during oxygen than air breathing. It is not possible from these values to draw any definite conclusions regarding tissue pH. However as a high venous lactate concentration contributed relatively less to the low femoralvenous pH during oxygen than air breathing the pH difference between tissue and

blood is probably less during oxygen breathing (Diamant, Karlsson and Saltin, 1968) and the tissue pH may be the same under the two conditions.

Conclusions

1 The maximal aerobic work performance was not significantly increased by oxygen breathing at 3 ata.

2. As judged from the heart rate and the stroke volume, determined during submaximal work, the maximal cardiac output was not reached at exhaustion during oxygen breathing at 3 ata.

3 Po_2 in venous blood from the active muscles was 3-4 times higher during oxygen than air breathing

4 It was concluded that neither circulation nor diffusion in the active muscle are limiting for the muscle oxygen uptake.

5 The venous lactate concentration was lower during oxygen than air breathing although it was rather high in relation to the venous Po_2. The high lactate concentration cannot be taken therefore as evidence of a lack of molecular oxygen in the active muscles.

6 The femoralvenous pH at exhaustion was slightly lower during oxygen than air breathing

CHAPTER V

Adaptation to exercise at decreased inspiratory oxygen pressure

Introductory remarks

The data obtained during oxygen breathing at 3 ata demonstrated that the aerobic work performance is not significantly improved by an increase in arterial oxygen content. This seems to indicate that the oxygen transport capacity of the cardiovascular system is not the main limiting factor for aerobic work performance in healthy young subjects. To elucidate the problem further it was considered of interest to investigate to what extent a decreased arterial oxygen content could be compensated for by an increased O_2 desaturation of the venous blood.

The influence of a decreased arterial oxygen saturation on work performance in normal subjects has been described by numerous authors during sojourns at high altitude, during acute exposure to simulated altitude in low pressure chambers and during hypoxic gas mixture breathing (*e. g.* Dill 1938, Stenberg, Ekblom and Messin 1966). For a correct interpretation of results reported in the literature it is important to state under what conditions hypoxia is studied. Thus, during a prolonged sojourn at altitude, adaptation to hypoxia of the cardiovascular system as well as cell metabolism (see Hurtado 1965) take place. Some of these changes are very rapid indeed. Asmussen and Nielsen (1945) showed that the hemoglobin concentration was increased already after 24 hours exposure to simulated altitude because of a decrease in plasma volume. In unpublished experiments it was found that a major part of this concentration increase is completed within 30 minutes (Eklund and Kaijser). For the present discussion effects of short-time exposure to hypoxia are of dominating interest.

From investigations concerning acute exposure to a lowered inspiratory oxygen pressure during work, the general conclusion can be drawn that the maximal O_2 uptake is decreased by approximately the same amount (taken as a percentage) as the arterial oxygen content (Stenberg, *et al.*

1966 Hansen *et al* 1967 Ceretelli *et al.* 1967) However it is not well known if there exists a margin, by which the oxygen content may be reduced before the maximal oxygen uptake decreases, although it is discussed by Hughes *et al* (1968)

The cardiovascular adaptation to decreased inspiratory oxygen pressure is characterized by an increased heart rate at corresponding submaximal work intensities, a moderate increase in cardiac output and probably a somewhat decreased stroke volume. However the maximal heart rate, cardiac output and stroke volume seem not to be changed compared to normoxaemia, at least at simulated altitudes below 4 000 meters (Stenberg *et al* 1966 Hughes *et al* 1968)

1 Adaptation to dynamic forearm work

Subjects

Five male volunteers and one female, aged 22 24 years, were studied. Their average forearm work capacity determined as W_{max} was 13 6 kpm/min (range 9-18 kpm/min)

Procedure

Dynamic forearm work was performed at stepwise increasing loads 4 minutes at 50 per cent of W_{max} 4 minutes at 100 per cent of W_{max} and until exhaustion at 150 per cent of W_{max} *e g* the mode of exercise was the same as that used in the study of forearm work during hyperbaric oxygen breathing

The subjects were studied during 1) air breathing, 2) breathing 15 per cent O in N and 3) 10 per cent O in N_2.

A brachial artery and the deep vein of the active forearm were catheterized as described in Chapter I

Measurements

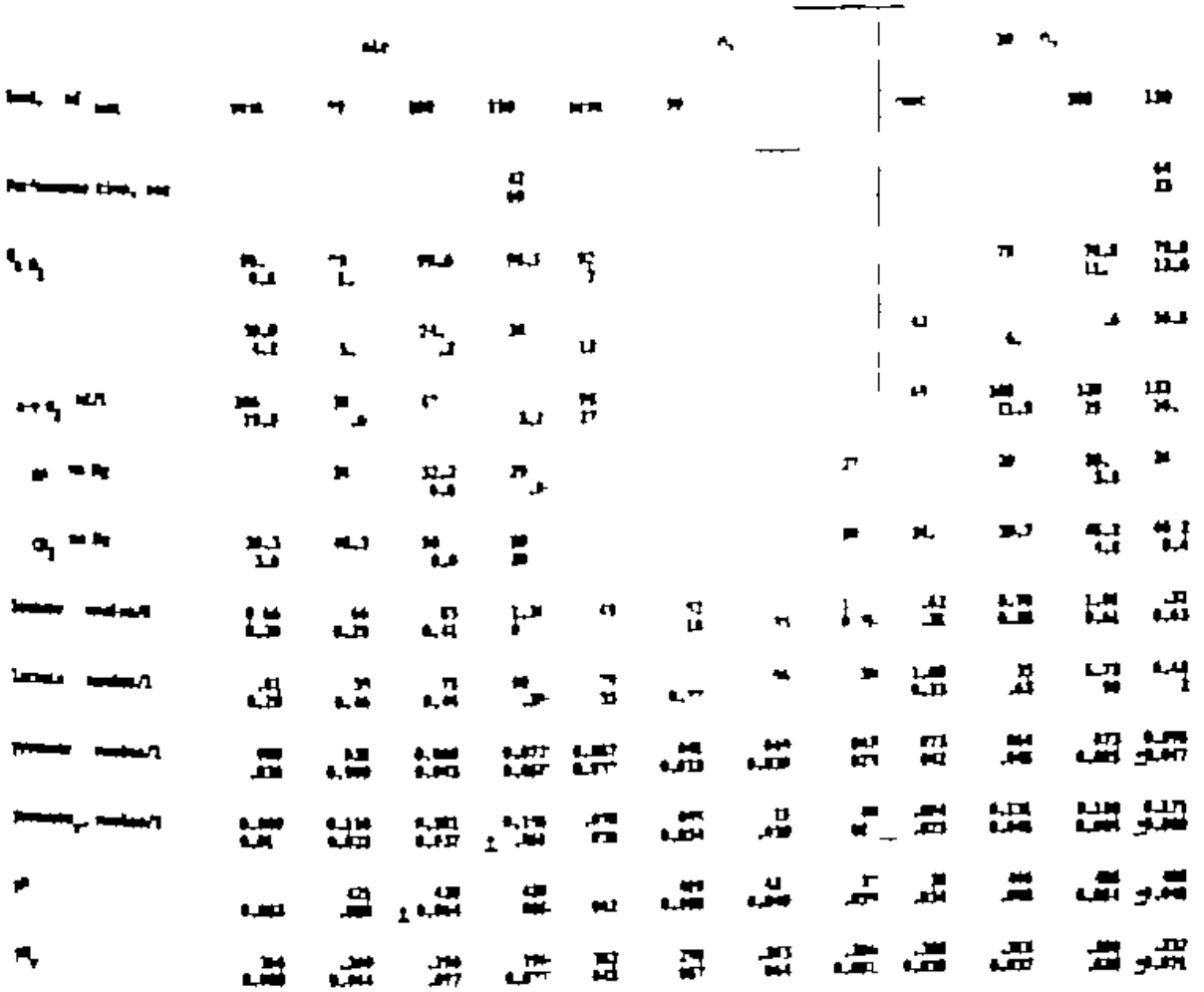
Sampling of arterial and deep venous blood was made at rest and at the end of each work period for the determination of oxygen saturation, P_{CO_2} pH, lactate and pyruvate concentrations. Also the maximal performance time at the highest load was measured.

For methodological details see Chapter I

Results

The results are summarized in table VIII A and B and Fig 13

Table VIII A. Data obtained during forearm work [illegible] stepwise in[illegible]d loads d[illegible]ring air 15 per cent and 10 per cent oxygen breathing [illegible]. Sample [illegible]ken at rest, after 4 min at the two lower loads and at exhaustion. Mean ± SD



Air breathing

Arterial blood The *O saturation* was 98 3 per cent at the lowest load No significant changes in O saturation, P_{CO_2}, *pH* or *pyruvate concentration* occurred during work as compared to rest.

The *lactate concentration* was 0 66 mmoles/l at rest and increased to 1.26 at exhaustion ($P<0.05$)

Venous blood The *O saturation* decreased from 39.9 per cent at rest to 31.8 per cent at the lowest work load ($P<0.05$) It decreased further to 24.9 per cent at the second load ($P<0.05$) and remained on the same level, 28 6 per cent at the highest load. The *arterio-venous O difference* increased from 106.5 ml/l at rest to 130 6 ml/l at the lowest load ($P<0.05$) and increased further to 140.9 ml/l at the highest work load ($P<0.05$)

Table VIII B. Difference between 1) 15 per cent oxygen and air 2) 10 per cent oxygen and al breathing. Mean ± standard error of the mean. n = 6.

	15 % O_2 air				10 % O_2 air			
Load % of ^{VO_2max}	rest	50	100	120	rest	80	100	120
Performance time sec.				17 26				78 22
O_2 %	4 8 .1	6.6 ***	6.3 *** .2	4.1 *** .8	10.3 *** 3.6	10.9 *** 3.7	20.1 *** 4.5	20.3 9 4
Sv O_2 %	2. 5	1	1.8	0 3	7.5 2.9	7.4 2.8	.3 2.4	12.1 *** 2.8
a-v O_2 ml/l	0 2			2.4 5	38.4 *** 9.8	24.9 *** 4.3	27.1 .8	18.5 7.1
P_{CO_2} mm Hg	0		2.5 2.	1.8	5.7 1 3	5.7 2.7	.5 2.6	4.5 2.6
P_{O_2} mm Hg			6.3	8.3	3.6 1 1	.6 2.1	13.3 4.3	13.1 7 7
Lactate$_v$ mmoles/l	0.57 4	87 15	0.71 1	0 58 .08	0.78 .20	0.86 .17	1.57 1.08	.66 0.79
pH_v mmoles/l	00.4 01	7.24 02	0.07 0 031	.21 032	.074 .024	0 049 .014	0.054 0.038	0 043 0.042

Pco₂ increased from 38 3 mm Hg at rest to 48 3 mm Hg at the lowest load ($P<0.05$). It increased further to 58.5 mm Hg at the second load ($P<0.05$). It remained on the same level at exhaustion.

The *lactate concentration* increased with increasing work intensity from 0 81 mmoles/l at rest to 4 80 mmoles/l at the highest load ($P<0.001$)

The *pyruvate concentration* increased from 0 089 mmoles/l at rest to 0 156 mmoles/l at exhaustion ($P<0.05$)

pH decreased from 7 366 at rest to 7.269 at the lowest work load ($P<0.05$) and further to 7 196 at the highest load ($P<0.01$)

The *performance time* to exhaustion was 142 sec.

15 per cent oxygen breathing

Arterial blood The *O saturation* was 92 1 per cent at rest. It increased significantly to 94 4 per cent at the highest work load ($P<0.05$)

Pco₂ was 31 7 mm Hg at rest, and decreased slightly to 27 2 mm Hg at exhaustion ($P<0.05$)

The *lactate concentration* was 0 48 mmoles/l at rest, and increased to 1 11 mmoles at exhaustion ($P<0.05$)

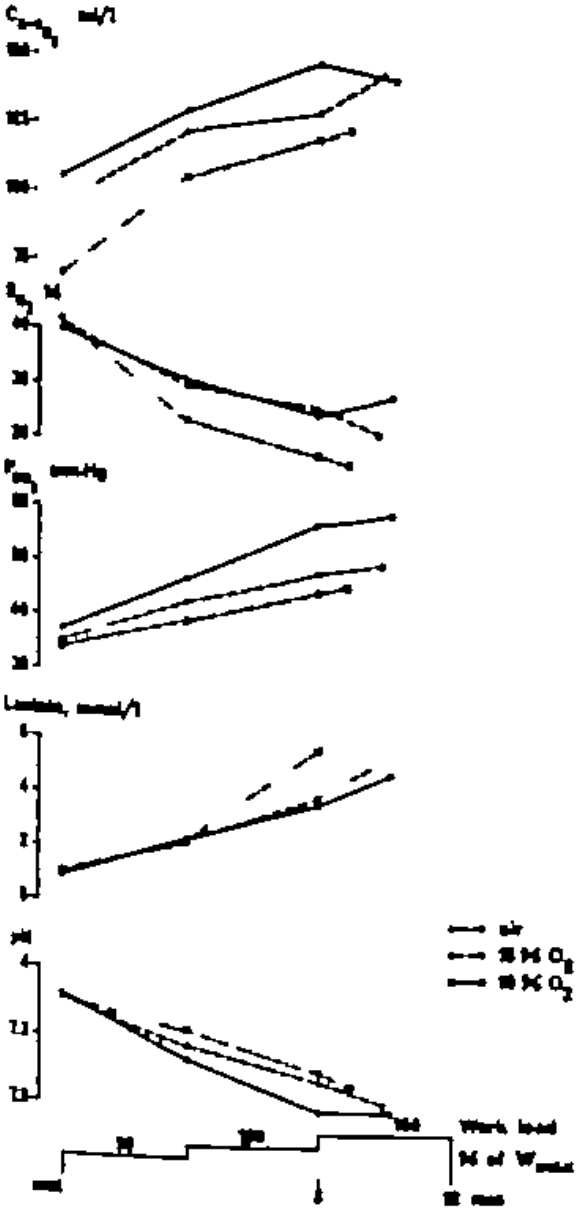


Fig 13 Arterial-deep venous O_2 difference and O_2 saturation, P_{CO_2}, lactate concentration and pH in the deep vein of the active forearm during work at stepwise increased loads. Average values ($n = 6$) at rest after 4 min at the first and the second load, and at exhaustion during air breathing, 15 per cent and 10 per cent oxygen breathing

The *pyruvate concentration* was 0.057 mmoles/l at rest, and did not change significantly during work.

pH was 7.418 at rest and did not change significantly during work ($P > 0.05$)

Venous blood The *O_2 saturation* decreased from 42.8 per cent at rest to 30.5 per cent at the lowest load ($P < 0.05$). It decreased further and was 21.9 per cent at exhaustion. The *O_2 saturation* was not significantly different from that during air breathing at the lower loads ($P > 0.05$) whereas at exhaustion it was 6.7 per cent lower than during air breathing ($P < 0.05$). The *arterio-venous O difference* was 95.7 ml/l at rest. It had increased to 125.6 ml/l at the second load ($P < 0.05$) and increased further to 143.7 ml/l at exhaustion ($P < 0.05$). It was slightly lower than during

air breathing in most subjects at the two lower work loads but at exhaustion it did not differ significantly from that during air breathing ($P>0.05$)

P_{CO_2} increased from 35.2 mm Hg at rest to 42.8 mm Hg at the lowest load ($P<0.05$) and increased further to 50.0 mm Hg at exhaustion. In most subjects it was lower than during air breathing on all work levels. If only the value at exhaustion is regarded, however it did not differ significantly from that during air breathing ($P>0.05$)

The *lactate concentration* increased from 0.79 mmoles/l at rest to 5.30 mmoles/l at exhaustion ($P<0.01$) Neither the *lactate* nor the *pyruvate concentration* differed from that during air breathing at corresponding loads.

The *pH* was 7.362 at rest. At the lowest load it was 7.295 or in most subjects higher than during air breathing The value at exhaustion, 7.207 did not differ from that during air breathing ($P>0.05$)

The *performance time* to exhaustion at the highest work load was 125 sec. It did not differ significantly from the performance time during air breathing ($P>0.05$)

10 per cent oxygen breathing

Arterial blood The *O saturation* was 78.7 per cent at rest. It did not change significantly during work ($P>0.05$)

P_{CO_2} was 31.0 mm Hg at rest and decreased to 24.5 mm Hg at exhaustion ($P<0.05$)

The *lactate concentration* was 0.62 mmoles/l at rest and increased to 1.31 mmoles/l at exhaustion ($P<0.05$)

The *pyruvate concentration* was 0.073 mmoles/l at rest and did not change during work.

pH was slightly high at rest, and did not change during work. At exhaustion it was 7.455

Venous blood The *O saturation* at rest was 42.4 per cent. It did not differ from that during air or 15 per cent O breathing It decreased to 24.2 per cent at the lowest work load ($P<0.05$) and decreased further to 16.5 per cent at the highest work load ($P<0.05$) It was at all work intensities lower than during air breathing ($P<0.05$) and at the two highest intensities lower than during 15 per cent O_2 breathing ($P<0.05$)

The *arterio-venous O difference* increased from 66.9 ml/l at rest to 105.7 ml/l at the first work load ($P<0.05$) and further to 120.2 ml/l at the second load ($P<0.05$) It remained on the same level at the highest load. It was lower than during air and 15 per cent O breathing at rest as well as at all work intensities.

P_{CO_2} was 34.5 mm Hg at rest and increased to 39 7 mm Hg at the first work load ($P<0.05$) It increased further to 45 2 mm Hg at the second load ($P<0.05$) and remained on the same level at exhaustion

The *lactate concentration* was 1 00 mmoles/l at rest and increased to 6 48 mmoles/l at exhaustion. It did not differ from that during air or 15 per cent O_2 breathing at rest or at the first two work loads. At exhaustion, how ever it was higher than during air as well as 15 per cent O breathing ($P<0.05$)

The *pyruvate concentration* was 0 094 mmoles/l at rest. It increased to 0.182 mmoles/l at the second work load ($P<0.05$) and 0 171 mmoles/l at exhaustion. It did not at any level of work differ from that during air or 15 per cent O_2 breathing

The *pH* was 7.358 at rest. It decreased to 7.318 at the lowest load, which is significantly higher than during air breathing ($P<0.05$) It de creased further to 7.237 at exhaustion. The value at exhaustion was not significantly different from that during air breathing ($P>0.05$)

The *performance time* to exhaustion at the highest load was 64 sec. It was significantly lower than corresponding values during 15 per cent O_2 and air breathing ($P<0.05$)

Discussion

Earlier investigations regarding the effect of low oxygen pressure in the inspiratory air on aerobic work performance concern heavy work with large muscle groups. The maximal oxygen uptake is generally found to decrease by an amount approximately corresponding to the decrease in arterial O_2 content (*cf* Stenberg *et al* 1966 Hansen *et al* 1967 Cerretelli *et al.* 1968)

The present study indicates that when work is performed with a small muscle group it is possible to compensate for a decrease in arterial O_2 saturation of about 5 per cent by a more complete oxygen desaturation of the blood perfusing the active muscles. This further underlines the con clusions drawn from the experiments with forearm work at hyperbaric oxygen pressure that the oxygen transport by the local blood circulation does not limit aerobic work capacity under normal conditions.

With a 20 per cent decrease in arterial oxygen saturation, the deep vein saturation can be reduced slightly further but not to a magnitude which makes possible an unchanged arteno-venous O difference and an unchanged performance time for exhaustive work.

The deep vein lactate concentration at exhaustion was higher during pronounced arterial hypoxaemia than under conditions of ordinary arterial

O saturation. Partly because of hyperventilation, partly because of the smaller O_2 extraction per unit blood during 10 per cent O breathing the deep vein P_{CO_2} is lower and the deep vein pH at exhaustion does not differ from that during air breathing

Conclusions

1 A decrease in arterial oxygen saturation of about 5 per cent did not decrease the arterio-venous O difference or the maximal performance time for exhaustive forearm work significantly
2 A decrease of about 20 per cent reduced the performance time and the arterio-venous O_2 difference significantly
3 The conclusion was drawn that there is a margin of at least 5 per cent in arterial O saturation before the maximal O_2 uptake is reduced.
4 At exhaustion the deep vein pH was the same during air 10 per cent and 15 per cent oxygen breathing

2 Adaptation to maximal leg work

In a first set of experiments work performance at an ambient pressure of 560 mm Hg was compared to that at 1 ata. In a second set of experiments four of the subjects were studied at 630 mm Hg as compared to 1 ata. The ambient pressure was selected with the results from the first set of experiments as guidance in an attempt to find the margin for decrease in arterial O content without a decrease in aerobic work performance

Subjects

Six male volunteers, aged 23-26 years, were studied. Their average work capacity determined as W_{170} was 1220 kpm/min (range 1150-1350 kpm/min)

Procedure

Percutaneous catheters were introduced in a brachial artery and the right femoral vein.

Exercise was performed in the first set of experiments at 1 ata and 560 mm Hg in the second set at 1 ata and 630 mm Hg ambient pressure. The subjects first worked for 5 minutes at a warm-up load, selected to give a heart rate of about 120 beats/min. The work intensity was then increased instantaneously to a load which the subjects were assumed to sustain for about 6 minutes.

Measurements

The *heart rate* was recorded continuously during the work periods. Arterial pressure was recorded at rest and after about 4 minutes at the submaximal and the maximal work load. Arterial and femoralvenous blood for the determination of Po_2, O_2 saturation Pco_2, pH, lactate and pyruvate concentrations was sampled at rest, after 4½ minutes at the submaximal and after 1 and 2 minutes as well as during the last 30 seconds at the maximal work load. Oxygen uptake was measured at rest and during repeated periods of about 1½ minute during maximal work.

For methodological details, see Chapter I

Results

1 Comparison between air breathing at 560 mm Hg and 1 ata

The results are summarized in Table IX and Fig. 14.

At rest

The *arterial O_2 saturation* was 98.5 per cent at 1 ata and 91.8 per cent at 560 mm Hg ($P<0.01$). No other significant differences between 560 mm Hg and 1 ata were recorded.

During exercise at 1 ata

The *heart rate* increased from 76 beats/min at rest to 118 beats/min at the submaximal work load and 182 beats/min at exhaustion at the maximal load.

The *arterial O_2 saturation* was 98.5 per cent at rest and decreased slightly to 95.0 per cent at exhaustion ($P<0.05$). The *femoralvenous O_2 saturation* was 46.9 per cent at rest and 30.3 per cent at the submaximal work load. It decreased to 21.5 per cent after 1 minute at the maximal load ($P<0.05$) and decreased further to 14.9 per cent at exhaustion ($P<0.05$). The *femoralvenous Po* decreased from 23.8 mm Hg at rest to 18.8 mm Hg at the submaximal work load ($P<0.05$). It remained on the same level in the beginning of the maximal work but decreased to 15.5 mm Hg at exhaustion ($P<0.05$). The *arterio-venous O_2 difference* increased from 98.1 ml/l at rest to 131.0 at the submaximal work load ($P<0.05$). It increased further to 148.7 ml/l after 1 minute at the maximal load ($P<0.05$) and was still higher 163.4 ml/l, at exhaustion ($P<0.05$).

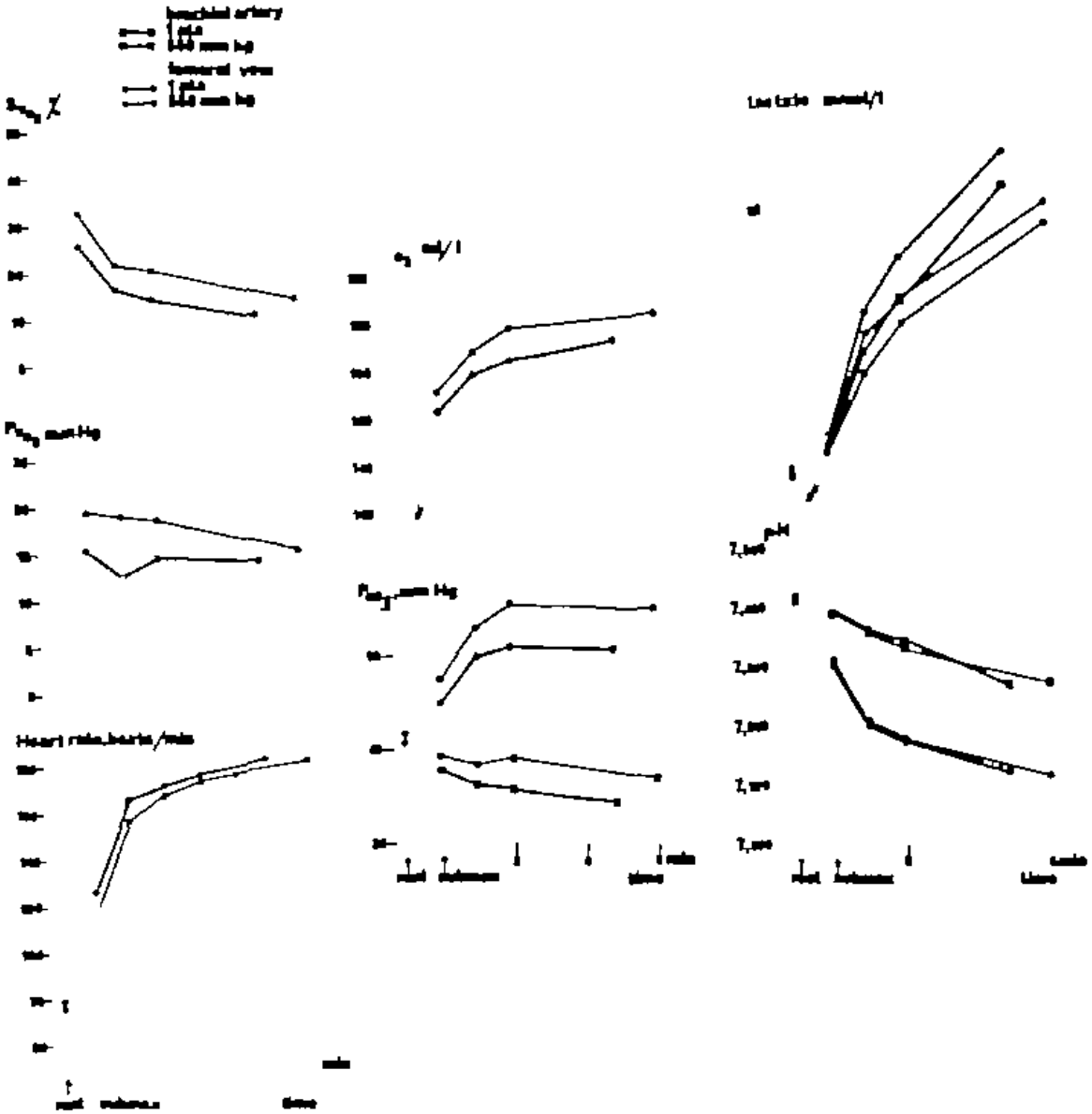


Fig. 14 Femoralvenous O_2 saturation, P_{O_2}, P_{CO_2}, lactate concentration and pH arterial-femoralvenous O_2 difference and heart rate at rest, during submaximal work, and after 1 and 2 min, and immediately before the end of exhaustive work. Average values ($n = 6$) at 1 ata and 560 mm Hg ambient pressure.

The *arterial Pco* was 36.7 mm Hg at rest. It remained on the same level during submaximal work and during the first two minutes at the maximal load but decreased significantly to 33.5 mm Hg at exhaustion ($P<0.05$). The *femoralvenous Pco* was 43.5 mm Hg at rest, increased to 54.3 mm Hg at the submaximal work load ($P<0.05$) and to 65.7 mm Hg after 1 minute at the maximal load ($P<0.05$). It increased further to 70.5 after 2 minutes ($P<0.05$) and remained at the same level until exhaustion.

The *arterial lactate concentration* increased from 1.04 mmoles/l at rest to 1.81 mmoles/l at the submaximal load ($P<0.05$) and increased con

Table IX A. Data obtained rest in the sitting position after 5 min a the submaximal work load and after 1 and 2 min and exhaustion t the maximal load during air breathing at 1 ta nd 560 mm Hg ambient pressure. Maximal loads anging between 1550—1700 kpm/min. Mean ± SD. n=6.

	1 ata					560 mm Hg				
	rest	submax.	max work			rest	submax.	max.work		
Time at the work load, l		5	1	2	exhaustion		5	1	2	exhaustion
Performance time, l					5 97					4 83
Heart rate beats/ml	76 ±10	116 ±7	157 ±13	168 ±9	182 ±9	78 ±12	126 ±8	166 ±8	172 ±8	183 ±9
P_{aO_2} mm Hg	88.2 ±6.8	92.5 ±10.2	82.7 ±10.0	80.3 ±10.9	78.3 ±8.1	55.6 ±4.8	50.8 ±3.5	44.8 ±3.5	46.3 ±4.7	46.8 ±5.4
S_{aO_2} %	96.5 ±1.1	97.5 ±0.5	96.1 ±1.4	96.2 ±0.8	95.0 ±1.1	91.8 ±3.0	88.5 ±2.4	86.0 ±2.5	85.2 ±3.0	84.3 ±5.5
P_{vO_2} mm Hg	23.6 ±6.9	18.8 ±4.7	17.8 ±3.3	17.2 ±3.1	15.5 ±2.9	21.5 ±5.6	19.4 ±2.4	12.8 ±2.5	14.0 ±1.8	13.8 ±2.1
S_{vO_2} %	46.9 ±11.7	30.3 ±7.8	21.5 ±6.5	20.3 ±5.8	14.9 ±4.1	41.2 ±12.2	25.1 ±5.1	16.3 ±4.1	14.4 ±3.0	10.8 ±2.8
C_{a-vO_2} l/l	98.1 ±21.8	131.0 ±19.2	148.7 ±19.3	158.7 ±17.0	163.4 ±9.1	96.0 ±19.3	124.5 ±17.1	139.3 ±13.3	144.6 ±13.2	152.8 ±10.7
P_{aCO_2} mm Hg	36.7 ±4.0	38.7 ±6.7	36.8 ±6.3	36.8 ±7.3	33.5 ±8.6	33.7 ±2.7	35.5 ±3.0	32.5 ±3.5	31.7 ±3.5	28.7 ±2.5
P_{vCO_2} mm Hg	43.5 ±2.7	54.3 ±11.2	65.7 ±14.2	70.5 ±14.2	69.5 ±12.8	41.7 ±3.7	49.8 ±4.5	59.3 ±5.5	61.5 ±6.2	61.0 ±8.0
$Lactate_a$ moles/l	1.04 ±0.32	1.81 ±1.02	3.04 ±0.83	7.18 ±1.32	11.37 ±1.39	0.85 ±0.12	2.11 [1] ±1.14	6.78 ±2.81	8.05 ±1.19	12.94 ±1.59
$Lactate_v$ moles/l	1.02 ±0.39	1.73 ±0.67	5.92 ±1.44	8.29 ±1.84	12.26 ±1.59	0.86 ±0.20	2.46 [1] ±1.40	7.39 ±2.11	9.93 ±1.97	14.34 ±2.61
Pyruvate moles/l	0.072 ±0.035	0.108 ±0.032	0.138 ±0.042	0.171 ±0.033	0.196 ±0.058	0.067 ±0.019	0.111 ±0.017	0.179 ±0.041	0.194 ±0.093	0.223 ±0.039
$Citrate_v$ moles/l	0.049 ±0.024	0.097 ±0.030	0.199 ±0.060	0.217 ±0.046	0.226 ±0.048	0.054 ±0.014	0.118 ±0.026	0.240 ±0.028	0.232 ±0.022	0.244 ±0.024
pH_a	7.429 ±0.020	7.384 ±0.014	7.348 ±0.014	7.336 ±0.035	7.273 ±0.054	7.423 ±0.030	7.396 ±0.010	7.367 ±0.010	7.342 ±0.020	7.275 ±0.037
pH_v	7.386 ±0.018	7.304 ±0.022	7.202 ±0.045	7.162 ±0.047	7.122 ±0.042	7.378 ±0.810	7.312 ±0.024	7.209 ±0.030	7.179 ±0.030	7.115 ±0.050

1) n=5

B. Differences between ues obtained at 560 mm Hg and 1 ta. M and standard error of the mean. n=6.

	560 mm Hg 1 ta			
	rest	submax.	max.work	
		5	1	2	exhaustion
Heart rate beats/ml	2 ±4	8 ±5	9 ±4	4 ±1	1 ±2
P_{aO_2} mm Hg	32.4 ±3.5	41.7 ±4.6	37.9 ±3.0	33.8 ±3.0	31.5 ±2.2
S_{aO_2} %	-4.7 ±1.1	-8.0 ** ±0.9	10.1 *** ±1.2	11.0 ** ±0.9	10.5 ±2.0
P_{vO_2} mm Hg	2.3 ±2.1	3.4 ±1.8	5.0 ±1.0	3.2 ** ±0.7	2.0 ±1.6
S_{vO_2} %	5.3 ±3.8	5.2 ±1.3	5.2 ±1.5	6.0 ±1.5	4.1 ±1.7
C_{a-vO_2} l/l	2.1 ±5.5	-6.5 ±2.3	-9.4 ±3.5	-9.6 ±4.3	10.7 ±2.3
P_{aCO_2} mm Hg	3.0 ±1.7	3.2 ±2.1	-4.3 ±2.2	7.1 ±2.0	-4.8 ±2.6
P_{vCO_2} mm Hg	1.8 ±0.9	-4.5 ±3.0	-6.4 ±4.0	-9.0 ±4.0	-8.5 ±3.3
$Lactate_a$ moles/l	0.39 ±0.20	0.13 [1] ±0.37	1.74 ±1.19	6.87 ±2.37	1.57 ±0.85
$Lactate_v$ moles/l	-0.16 ±0.14	0.44 [1] ±0.37	1.67 ±1.10	1.64 ±0.43	2.08 ±1.22
Pyruvate moles/l	-0.005 ±0.017	0.003 ±0.013	0.021 ±0.024	0.023 ±0.027	0.027 ±0.017
$Citrate_v$ moles/l	-0.015 ±0.010	0.021 ±0.013	0.041 ±0.018	0.015 ±0.016	0.002 ±0.014
pH_a	-0.002 ±0.009	0.002 ±0.007	0.019 ±0.006	0.016 ±0.008	0.002 ±0.014
pH_v	-0.008 ±0.004	0.008 ±0.004	0.007 ±0.013	0.007 ±0.009	-0.007 ±0.020

tinuously during work to 11 37 mmoles/l at exhaustion. No significant veno-arterial lactate difference was recorded at rest or at the submaximal work load. At the maximal load, however the venous concentration was higher than the arterial, reaching 12.26 mmoles/l at exhaustion. The *arterial pyruvate concentration* was 0 072 mmoles/l at rest, increased to 0 108 mmoles/l at the submaximal work load ($P<0.05$) It increased further to 0 196 mmoles/l at exhaustion ($P<0.01$) The *femoralvenous pyruvate concentration* did not differ from the arterial at rest or at the submaximal load but during maximal work it increased more than the arterial, reaching 0 226 mmoles/l at exhaustion. At the maximal work load the veno-arterial differences for lactate and pyruvate were significant, both after 1 minute ($P<0.05$) and at exhaustion ($P<0.01$) The veno-arterial differences were of the same order of magnitude at exhaustion as after 1 minute.

The *arterial pH* was 7 425 at rest, 7.394 at the submaximal work ($P<0.05$) and decreased continuously during the maximal work to 7.271 at exhaustion. The differences between the first and the second minute as well as between the second minute and exhaustion were significant ($P<0.05$)

The *mean pressure* in the *brachial artery* was 87 mm Hg at rest and 95 mm Hg at the ubmaximal work load. It increased significantly to 116 mm Hg shortly before exhaustion ($P<0.01$)

During exercis at 560 mm Hg ambient pressure

The heart rate at rest was the same at 560 mm Hg as at 1 ata. At the submaximal work it was almost unchanged compared to 1 ata in three subjects and increased in three, the average difference compared to 1 ata not statistically significant ($P>0.05$) During maximal work the heart rate increased faster at 560 mm Hg, the difference after 2 minutes being 4 beats/min ($P<0.05$) The maximal heart rate was the same under the two conditions.

The arterial O saturation was 91 8 per cent at rest, decreased slightly to 88 5 per cent at the submaximal load ($P<0.05$) and decreased further to 84 5 per cent at exhaustion ($P<0.05$) The *femoralvenous O saturation* at rest did not differ significantly from that at 1 ata. At the submaximal work as well as after 1 and 2 minutes and at exhaustion at the maximal work load it was lower than at 1 ata. The difference at exhaustion was 4 1 per cent ($P<0.05$) The *femoralvenous* Po_2 was not significantly lower than that at 1 ata at rest or at the submaximal work load but both after 1 and 2 minutes at the maximal work ($P<0.01$) At exhaustion no significant difference was found ($P>0.05$) The *arterio-venous O dif*

ference at rest and at the submaximal work load did not differ significantly from that at 1 ata ($P<0.05$). At the maximal work it was 9.4 ml/l smaller after 1 minute ($P<0.05$) and 10.7 ml/l smaller at exhaustion ($P<0.01$).

The *arterial* Pco_2 did not differ significantly from that at 1 ata, neither at rest nor at the submaximal nor after 1 minute at the maximal work load ($P>0.05$). After 2 minutes at the maximal work load it was significantly lower at 560 mm Hg ($P<0.05$) but at exhaustion the difference was insignificant ($P>0.05$). The *femoralvenous* Pco_2 did not differ significantly between the two conditions at rest, at the submaximal work or during the first two minutes of the maximal work. At exhaustion however it was 8.5 mm Hg lower at 560 mm Hg ambient pressure ($P<0.05$).

The *arterial lactate concentration* did not differ significantly between 560 mm Hg and 1 ata at rest, at the submaximal work or after 1 minute at the maximal work load. After 2 minutes at the maximal work load all subjects had higher arterial lactate concentrations at 560 mm Hg and at exhaustion five of the six subjects had higher lactate concentrations, whereas one did not differ between the two conditions. The *femoralvenous lactate concentration* was not significantly different between the two conditions at rest, at the submaximal work load or after 1 minute at the maximal work. After 2 minutes and at exhaustion however all subjects had higher lactate concentrations at 560 mm Hg. The arterial and femoralvenous pyruvate concentrations did neither at rest nor during submaximal or maximal work differ from that recorded at 1 ata.

The *arterial pH* was the same at the two environmental conditions at rest, at the submaximal work load and at exhaustion ($P>0.05$). *The femoralvenous pH* was at rest as well as at the submaximal and the maximal work load the same at 560 mm Hg as at 1 ata ($P>0.05$).

The *mean pressure in the brachial artery* was the same under the two conditions both at rest, at the submaximal and at the maximal work.

The *performance time* was in all subjects shorter at 560 mm Hg than at 1 ata, the average performance time being 4.83 min as compared to 5.97 min at 1 ata.

2. *Comparison between air breathing at 630 mm Hg and 1 ata*

The results are summarized in Table X and Fig. 15.

1 ata

No major differences were recorded compared to corresponding values at 1 ata in the above reported experiments.

Table X. A. Data obtained t rest in the sitting position, after 5 min at the submaximal work load and after 1 and 2 min and at exhaustion at the maximal load during a breathing at 1 ata and 630 mm Hg ambient pressure. Maximal loads ranging between 1350—1700 kpm/min. Mean and range n = 4

B. Difference between values obtained t 630 mm Hg and 1 ta. Mean±standard error of the mean. n = 4 1) denotes that all individual differences are in the same direction.

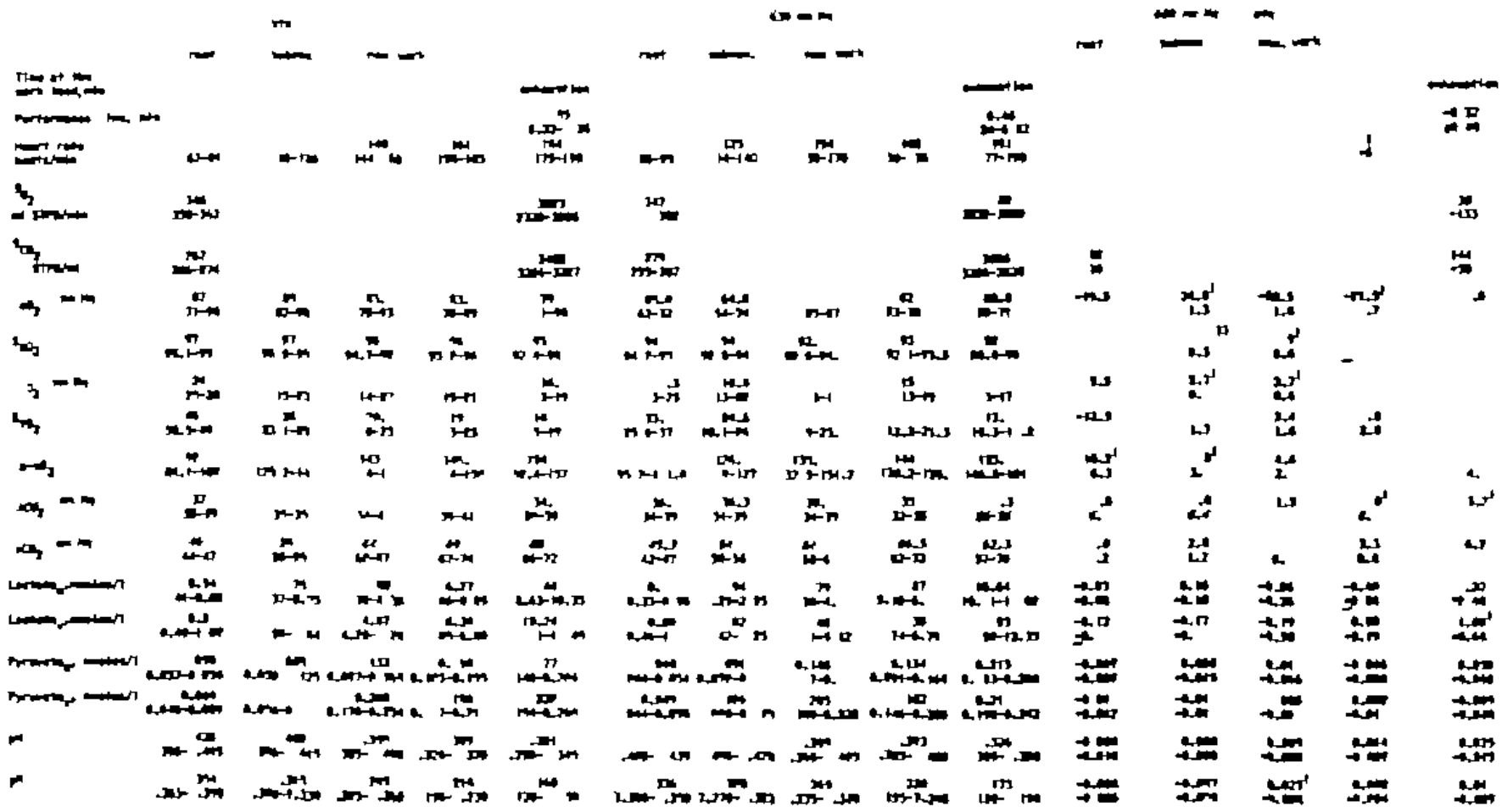
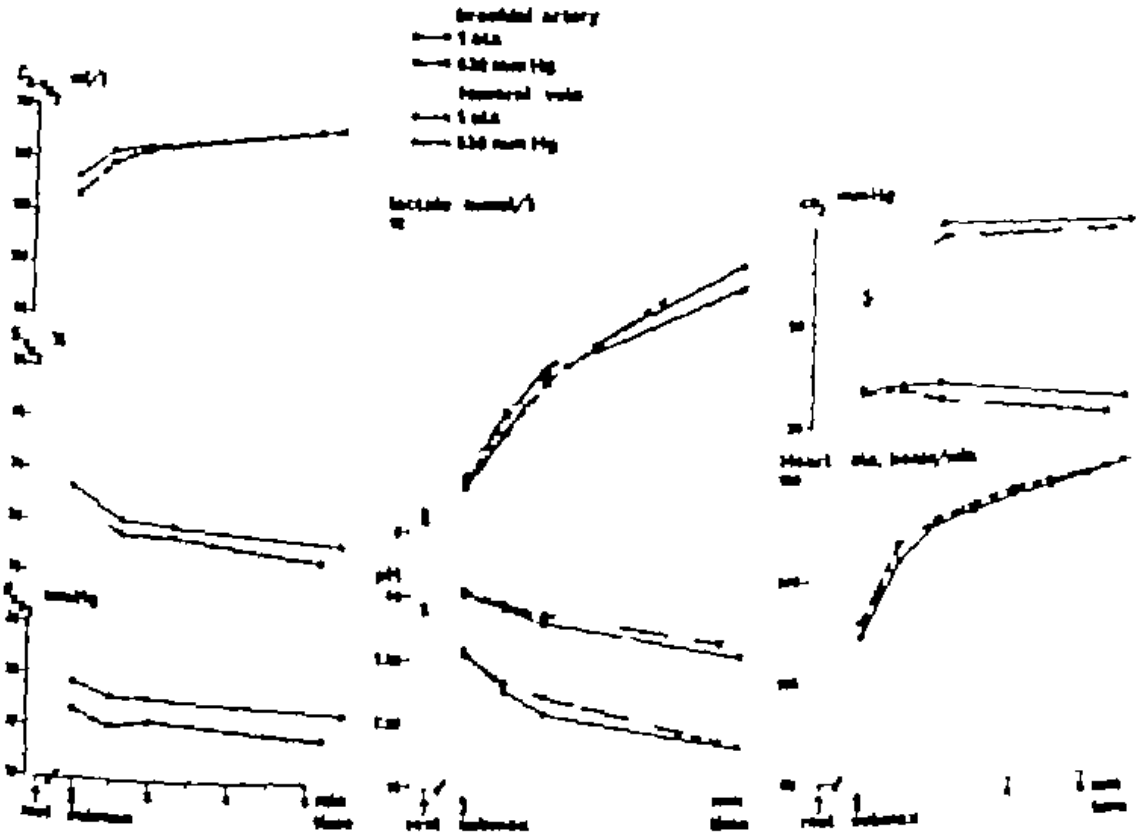


Fig. 15 Arterial-femoralvenous O_2 difference (extrac- tion, and P_{CO_2}, arterial and femoralvenous lactate concentration pH at rest, during submaximal work and after 1 and 2 min and before the end of exhaustive work. Average values (n = 4) during air breathing at 1 and 630 mm Hg ambient pressure.



Ambient pressure 630 mm Hg

The *arterial O saturation* was at rest 2.6 per cent lower than at 1 ata. Both at the submaximal work load and at exhaustion it was in all subjects lower at 630 mm Hg than at 1 ata, the average difference at exhaustion being 2.8 per cent. The *venous O saturation* was at rest lower than at 1 ata in all subjects, the average difference being 12.5 per cent. At the submaximal work no consistent difference was found, whereas at exhaustion three subjects had a lower and one an unchanged saturation at 630 mm Hg compared to at 1 ata. *The arterio-venous O_2 difference* did not differ consistently between 630 mm Hg and 1 ata neither at rest nor during submaximal work or at exhaustion.

No consistent differences in *arterial Pco* were found at rest or at the submaximal work load, whereas at the maximal work load both after 1 and 2 minutes and at exhaustion P_{CO_2} was lower at 630 mm Hg than at 1 ata in all subjects. *The femoralvenous Pco* did not either differ consistently between the two conditions at rest or at the submaximal work

load, whereas after 2 minutes and at exhaustion at the maximal work load, it was slightly lower in all subjects at 630 mm Hg than at 1 ata.

The *femoralvenous lactate concentration* did not differ between the two conditions at rest, at the submaximal work load or in the beginning of the maximal work but was at exhaustion in all subjects slightly higher at 630 mm Hg No consistent differences in *arterial* or *femoral venous pyruvate concentrations* were found.

No consistent differences in *arterial* or *femoral venous pH* were found at any time of work.

The *performance time* and the *maximal oxygen uptake* did not differ consistently between the two conditions.

Discussion

When the arterial oxygen content was decreased about 10 per cent at an ambient pressure of 560 mm Hg this was partly compensated by a more complete desaturation of the venous blood during exhaustive exercise. Hence, the O extraction from the blood was decreased to a lesser extent than the decrease in arterial O content. This is in accordance with Doll and Keul (1969).

The mentioned finding suggested that a certain decrease in arterial oxygen content should be possible without a decrease in aerobic work performance. This was confirmed in maximal exercise experiments at 630 mm Hg With a decrease in arterial O saturation of about 3 per cent the venous O saturation was decreased by the same magnitude. The arteriovenous O difference as well as the performance time to exhaustion and the maximal O uptake remained unchanged

It can be noted that under hypoxaemic conditions (560 mm Hg ambient pressure) the maximal work performance is reduced, although in no subject was the femoralvenous Po_2 as low as the so called "critical O pressure" of about 10 mm Hg (Stainsby and Otis 1964) Doll and Keul (1969) have recently reported Po_2 values in the femoral vein of about 10 mm Hg during heavy work breathing 12 7 per cent oxygen. However with 12.7 per cent oxygen in the inspiratory gas the arterial O_2 saturation was probably lower than in the present experiments. In the report by Doll and Keul nothing is stated about oxygen uptake or work performance. However the present experiments make it probable that the femoralvenous Po_2 below which the oxygen uptake is decreasing is higher than that calculated by Stainsby and Otis from experiments in dogs. The difference may however be caused by a difference in the fraction blood at the sampling site that drains active muscle tissue

Under hypoxaemic conditions the lactate concentration in the femoral-venous blood is significantly higher than under normal conditions. This is in accordance with findings reported by Keul and Doll (1969) At the same time the femoralvenous P_{CO_2} is lower during hypoxaemia and the venous pH is the same under the two conditions. This finding is valid both for the slight hypoxaemia at 630 mm Hg and the more marked hypoxaemia at 560 mm Hg This lends further support to the hypothesis that the increasing tissue acidity during exhaustive work is a limiting factor for muscle performance.

Also the arterial lactate concentration is higher during exhaustive work under hypoxaemic conditions. This is in accordance with the findings by Asmussen *et al.* (1948) although other authors have failed to show an increase in arterial lactate concentration during exhaustive work under hypoxaemic conditions (Edwards 1936 Stenberg *et al* 1966 Hansen *et al* 1967)

No difference in integrated mean pressure in the brachial artery was found between moderately hypoxaemic (at 560 mm Hg) and ordinary conditions. This is apparently not in agreement with the findings by Stenberg *et al.* (1966) However Stenberg's experiments were performed at a higher simulated altitude where probably the hyperventilation during work was more pronounced and the arterial P_{CO_2} lower (*cf* Chapter IV p 49)

Conclusions

1. A 3 per cent decrease in arterial oxygen content was found to be compensated for by a more complete oxygen extraction from the blood.
2. A more marked decrease in saturation, about 10 per cent, could only be partly compensated for and the maximal oxygen uptake decreased.
3. Thus there seems to be a margin of at least 3 per cent for lowering of the arterial oxygen content before the maximal oxygen uptake is influenced.
4. During exhaustive work under hypoxaemic conditions (Sa_{O_2}~85 per cent) the femoralvenous P_{CO_2} was lower than under normoxic conditions. The venous lactate concentration, on the other hand, was higher and the resulting venous pH at exhaustion was the same under the two conditions.
5. It was concluded that the decrease in pH could be a limiting factor for muscle performance.

CHAPTER VI

Adaptation to dynamic forearm work at decreased muscle temperature

Introductory remarks

To test the hypothesis that muscle metabolism is the limiting factor for aerobic work performance it was considered of interest to study muscle performance and oxygen extraction under conditions influencing the maximal metabolic rate In the following results of a preliminary study are reported.

The rate of enzymatic reactions usually increase with temperature up to a temperature where thermal inactivation of the enzymes occur Comparatively little is known about the optimum temperature for aerobic muscle metabolism. However Abramson *et al* (1957) found that the metabolic rate of the forearm was increased when the temperature was elevated above the normal resting level and Asmussen and Boje (1946) reported that the capacity for exhaustive leg work increased after prewarming of the active muscle Consequently it was assumed that a moderate decrease in muscle temperature of 2 3 C should reduce the maximal metabolic rate.

For the experiment to be conclusive from a metabolic point of view the temperature reduction should not interfere significantly with neuromuscular function or muscular blood flow Clarke, Hellon and Lind (1958) showed that the muscle temperature could be decreased to 27 C without a decrease in the rectified and integrated muscle action potentials at the end of an exhaustive sustained contraction. The same investigation also indicated that no major changes in muscle blood flow occurred

Hall Munoz and Fitch (1947) found the direct effect of heating of the cat gastrocnemius to be an increase in strength. Nukada and Müller (1955) reported an increased performance time for dynamic work and Nukada (1955) and Clarke *et al.* (1958) an increased performance time for sustained isometric contraction when the forearm temperature was reduced to 27 C. However Clarke *et al.* explained the findings by the assumption that during a sustained contraction, when the washout of

metabolites was impaired a decreased metabolic rate may be advantag eous.

Subjects

Four male volunteers, aged 23-27 years, were studied. Their average fore arm work capacity determined as W_{max} was 15.9 kpm/min (range 15-18 kpm/min)

Procedure

A catheter was introduced in the distal direction into a deep vein of the right forearm. The subjects performed dynamic forearm work on the hand ergometer described earlier for 5 minutes at a submaximal load correspond ing to 50 per cent of W_{max}, rested for 5 minutes, and worked until ex haustion at 150 per cent of W_{max}.

The forearm was chilled for 10-12 minutes by moist fabric packings filled with ice before the work test. For comparison exercise was performed at corresponding loads with the arm in room air. The order of the tests (warm-cold) was varied between the subjects.

Measurements

The blood temperature in the deep forearm vein was measured by a thermocouple introduced through the catheter. Blood was sampled at rest, after 5 minutes at the submaximal, and at exhaustion at the highest load for the determination of Po_2, O_2 saturation, Pco_2, pH, lactate and pyruvate concentrations.

For methodological details, see Chapter I.

Results

The results are summarized in Table XI and Fig. 16.

Room temperature

The average *temperature* in the deep forearm vein was 34.1°C at rest (range 31.6-36.0). It increased slightly to 34.8 at exhaustion (range 32.9-36.3).

The deep vein *O saturation* decreased from 44.7 per cent at rest (range 25.8-58.0) to 28.5 per cent at the lower load (range 24.3-34.3) and 28.6 per cent at exhaustion (range 22.6-35.6).

The deep vein *lactate concentration* increased from 0.88 mmoles/l (range 0.71-1.63) at rest to 2.08 mmoles/l (range 1.10-3.99) at the lower load, and 4.04 mmoles/l at exhaustion (range 3.08-4.94).

Table XI. Data obtained at rest, after 5 min at submaximal and at exhaustion at a maximal load during dynamic forearm work at room temperature and after cooling of the forearm. Mean and range. n = 4

	room temperature			cooled forearm		
	rest	load I	load II	rest	load I	load II
Temperature °C	34.1 31.6–36.0	34.7 32.6–36.0	34.8 32.9–36.3	32.7 30.2–34.6	32.8 28.9–34.5	32.4 30.0–33.6
S_{vO_2} %	44.7 25.8–58.0	28.5 24.3–34.3	28.6 22.6–35.6	59.4 43.2–75.0	32.3 24.8–42.3	37.1 31.3–45.2
P_{vO_2} mm Hg	25.4 15–39	19.3 15–21	18.8 17–20	25.4 15–39	17.3 15–21	19.8 18–24
P_{vCO_2} mm Hg	40.0 35–44	48.5 45–53	59.3 46–70	35.8 30–43	45.3 41–53	50.0 46–57
$Lactate_v$ mmoles/l	0.86 0.71–1.63	2.08 1.10–3.99	4.04 3.06–4.94	0.89 0.56–1.66	2.46 0.87–4.47	3.96 2.89–6.06
$Pyruvate_v$ mmoles/l	0.076 0.055–0.109	0.078 0.057–0.099	0.107 0.078–0.141	0.055 0.035–0.070	0.092 0.078–0.109	0.112 0.094–0.127
pH	7.408 7.37–7.50	7.348 7.310–7.385	7.250 7.200–7.330	7.453 7.425–7.485	7.381 7.375–7.390	7.321 7.285–7.340

The *pyruvate concentration* was 0.076 mmoles/l at rest (range 0.055-0.105) 0.078 mmoles/l at the lower load (range 0.057-0.098) and 0.107 mmoles/l at exhaustion (range 0.078-0.141)

The *pH* was 7.408 at rest (range 7.375-7.430) 7.349 at the lower load (range 7.310-7.385) and 7.250 at exhaustion (range 7.200-7.330)

The average *performance time* to exhaustion was 290 seconds (range 209-363



Fig. 16. Differences between chilled forearm and forearm in room temperature. Values from the individual subjects of performance time at the highest load and O_2 saturation, P_{CO_2}, lactate concentration and pH in the deep vein of the active forearm at rest, at load I and at load II. n = 4

After cooling

After 10-12 minutes in ice packings, the average resting temperature in the deep forearm vein was 1·4 C lower than before cooling (range 0·7 3·0). The temperature increased moderately during exercise. The forearm vein temperature at exhaustion was 2·4 C lower than in the experiments conducted in room air (range 1·0-3·6).

At rest or at the lower work intensity no consistent difference in deep vein O_2 *saturation* was found between the cooled forearm and the forearm with ordinary temperature. However during work until exhaustion the O_2 saturation in the deep vein in all subjects decreased less after cooling than under ordinary conditions.

The *lactate concentration* did not differ consistently between the two conditions.

The *pyruvate concentration* was not consistently different after cooling compared to normal conditions at rest or at exhaustion. At the low work load however it was in all subjects slightly higher after cooling.

pH was higher after cooling in all subjects at rest in 3 subjects at the lower load and in all subjects at exhaustion.

The *performance time* to exhaustion was decreased in all subjects by cooling, the average difference being 95 seconds which corresponds to per cent.

Discussion

The experiment was not intended to be a comprehensive study of the relationship between muscle performance and muscle temperature but rather a qualitative confirmation that a decrease in maximal muscle metabolic rate directly influences maximal muscle performance.

A moderate decrease in muscle temperature, yielding a 2-3 C decrease in deep vein blood during exhaustive work, caused a 33 per cent reduction in performance time to exhaustion. At exhaustion the O saturation in the deep venous blood was higher after cooling than under normal conditions whereas the lactate concentration did not differ consistently suggesting that the most likely explanation of the decreased performance was a decreased aerobic metabolic rate.

Conclusions

1. A moderate decrease 2-3 C in muscle temperature was found to reduce the performance time for exhaustive dynamic forearm work.

2 The O_2 saturation in the deep vein at exhaustion was higher after cooling than under ordinary conditions.
3 The deep vein lactate concentration at exhaustion was the same after cooling as under ordinary conditions.
4 pH in the deep vein was higher after cooling than under ordinary condition.
5 The conclusion was drawn that the performance time was dependent on the muscle temperature and that this is due to an effect of temperature on the aerobic metabolic rate.

Part C. GENERAL DISCUSSION

1 The local blood perfusion as a limiting factor for aerobic work capacity

If the local blood flow limits the maximal oxygen uptake, variations in arterial oxygen content would directly induce changes in maximal oxygen uptake. Furthermore the altered oxygen uptake would be seen as a change in arteno-venous O difference During exhaustive forearm work the arte rio-venous O difference was not influenced by variations in arterial oxygen content between about 5 per cent below and 30 per cent above normal Since the maximal performance time was not significantly altered either it seems reasonable to conclude that the maximal oxygen uptake was not influenced Hence, the *local circulation* seems not to be a directly limiting factor for oxygen uptake (Fig 17)

The calculated Po_2 in the deep vein was at least twice as high when the arterial oxygen content was increased by oxygen breathing at 3 ata as when it was normal. This speaks against the assumption that the *oxygen diffusion* from the capillary blood to tissue is a limiting factor for oxygen uptake

If the arterial oxygen content is gradually decreased a level will be reached below which the oxygen supply to the active muscle tissue will limit aerobic work performance The magnitude of the decrease in arterial oxygen content which can be fully compensated for by a more complete oxygen extraction, seems to be somewhere between 5 and 10 per cent.

The muscle blood perfusion was not directly measured in the present study However from the arteno-venous O difference it can be calculated that at submaximal work loads a substantial increase (30 per cent) in arterial oxygen content decreased the blood flow although to a moderate degree A 20 per cent decrease in arterial O saturation from the normal

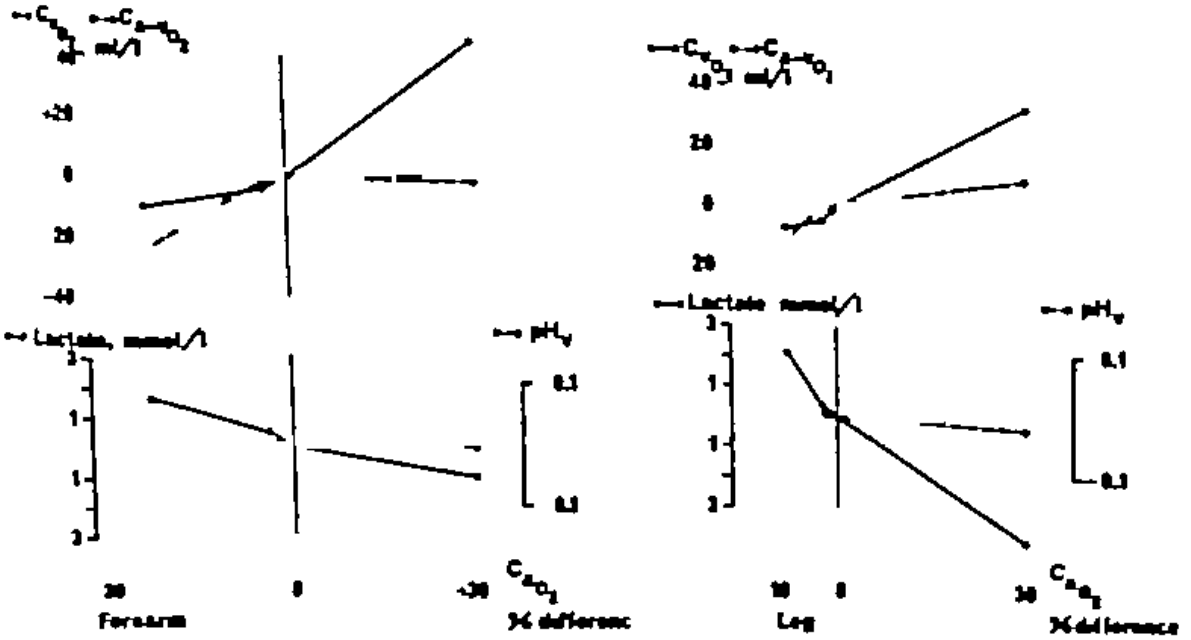


Fig 17 Left. Deviations in arterial-deep vein O_2 difference, deep vein O_2 content, lactate concentration and pH at exhaustion during dynamic forearm work from the values recorded under normoxaemic conditions as a function of changes in arterial O_2 content from normoxaemia.

Right. Deviations in arterial-femoral venous O_2 difference, femeral venous O_2 content, lactate concentration and pH at exhaustion during leg work from the values recorded under normoxaemic conditions as a function of changes in arterial O_2 content from normoxaemia.

level increased the blood flow somewhat. The direct effect of oxygen on vascular tone may partly explain the differences in blood flow (Carrier *et al* 1964) Nevertheless, factors associated with the muscle metabolism are generally supposed to be more important for the blood flow regulation. The hyperbaric oxygen experiments lend support to the hypothesis that the pyruvate concentration in muscle blood is at least one factor of importance.

During maximal exhaustive work the calculated blood flow was not influenced by changes in arterial oxygen content as evidenced by similar arterio-venous oxygen differences and similar performance times within a wide range of arterial oxygen pressures. Hence, blood flow seems not to be regulated by the local P_{O_2} under these conditions.

2. Central circulation as a limiting factor for aerobic work capacity

Work performed with large muscle groups, *e g* leg work on a cycle ergometer places a large demand on the central circulation. Under such condi-

tions central circulation could be limiting for aerobic work capacity although the local vessels permit an increased blood perfusion. In that case an increased oxygen utilizing muscle mass would not increase the maximal oxygen uptake. Findings in the literature are conflicting on this point, however Christensen and Högberg (1950) found a slightly higher maximal oxygen uptake during skiing and Åstrand and Saltin (1961) during treadmill running compared to cycling Taylor *et al* (1955) recorded a slightly larger maximal oxygen uptake during work with arms + legs than with only legs in contrast to Stenberg *et al* (1967)

When in the present study the arterial oxygen content was increased by oxygen breathing at 3 ata, subjects performing maximal work were exhausted when the cardiac output was calculated to be smaller than their maximal. Furthermore, at exhaustion, the femoralvenous oxygen saturation was much higher than during air breathing and the maximal performance time was not increased. Consequently the cardiac output seems not to limit the maximal oxygen uptake (Fig 17)

This is further supported by the observation that a small decrease in arterial oxygen content was possible without decreasing aerobic performance The margin was found to be above 3 and below 10 per cent. Probably it was not much larger than 3 per cent as the performance time was substantially reduced by a 10 per cent decrease in oxygen content. The decrease in femoralvenous saturation, 4 per cent may be regarded as the possible maximum for the margin

Regarding the cardiac output as a limiting factor for muscle oxygen uptake also the conditions at the start of heavy work must be discussed. It has been proposed by Margaria *et al* (1968) that the cardiac output will increase slower than the maximal possible oxygen utilization rate. The present findings do not support such a hypothesis, as the O_2 saturation in the femoral vein is higher after 1 minute of work than at exhaustion during maximal work with ordinary arterial oxygen content. However the present findings are not conclusive concerning the earliest phase of work, since this should have premised still earlier blood sampling

3 Muscle metabolism as a limiting factor for aerobic work capacity

The investigations concerning variations in arterial O content gave by elimination rise to the assumption that the maximal metabolic rate of the muscle under ordinary conditions in healthy young subjects is the limiting factor for aerobic work capacity In an attempt, by more direct means to find support for this conception, the effect of cooling the active muscles

during dynamic forearm work was studied A moderate decrease in forearm temperature reduced the performance time for exhaustive work substantially As, at the same time, the O_2 saturation in the deep vein was significantly higher than at ordinary temperature condition, whereas the lactate concentration in the venous blood was fairly unchanged it was concluded that the decreased performance was caused by a decrease in aerobic metabolism

4 The point of exhaustion

The point of exhaustion is settled by the contribution of several factors, among them *psychological factors* such as motivation tolerance of discomfort and pains. In the present study psychological factors may have influenced the performance time during exhaustive work to a larger or lesser degree in the individual subjects. In the group however they are probably at random. Furthermore, most volunteers took part in several experiments, and were well aquainted with the experimental situation, so minimizing the subjective influence on the point of exhaustion.

Physiological mechanisms determining the point of exhaustion may interfere with excitation, excitation-contraction coupling, the energy yielding processes or the function of the contractile elements. The effect may be due to factors such as an insufficient supply of substrate or oxygen, or it may be due to a change in the internal environment of the cell caused by metabolite accumulation.

The oxygen supply of the active muscle by blood perfusion and diffusion from blood to the muscle cell does not set the point of exhaustion This is obvious since wide variations in blood oxygen content and pressure did not influence the performance time. An exhausted substrate supply may be limiting for prolonged work but not for work of the durations in the present study (Bergström and Hultman 1966)

For many years the accumulation of lactic acid in the muscle cells has been looked upon as a main cause for muscle fatigue (Hill 1927) The present study does not lend support to the hypothesis that lactate accumulation *per se* determines the point of exhaustion, as the lactate concentration of the muscle vein blood varied under different conditions of arterial oxygenation. It is true that the lactate concentration in blood differs from that in the muscle cell during heavy exercise (Diamant *et al.* 1968) but as the conclusion in the present study rests on paired observations in the same subjects at corresponding work loads, differences in blood lactate probably reflect differences in tissue lactate.

In the experiments with varying arterial oxygen content the venous P_{CO_2} varied inversely with venous lactate concentration. As a result, the venous pH reached the same value at exhaustion within a wide range of conditions regarding arterial oxygen content during forearm work and with one exception also during leg work (Fig 17)

During leg work, breathing oxygen at 3 ata, the femoralvenous pH at exhaustion was slightly lower than under the other conditions studied. During oxygen breathing also the arterial pH was lower Furthermore, compared to air breathing the femoralvenous P_{CO_2} was substantially higher and the lactate concentration lower However during heavy work the lactate concentration in muscle tissue is higher than in blood (Diamant *et al.* 1968) whereas CO can be assumed to diffuse more readily from tissue to blood. Consequently the difference in femoralvenous pH may be explained without supposing a difference in tissue pH. Thus, although more definite conclusions should premise direct measurement in the tissue, the present study lends support to the hypothesis that the increased acidity in the muscle cell may set the point of exhaustion.

Of mechanisms by which acidity may exert its effects, the interference with the oxidative phosphorylation (Myers and Slater 1957) or processes more directly related to the contraction (Merton 1954 Bendall 1960) can be suggested from available reports in the literature.

In the cooled muscle, the point of exhaustion was reached at a higher venous pH than under ordinary conditions. This was due to a lower venous P_{CO_2} interpreted as the result of a retarded maximal aerobic metabolic rate However in contrast to the conditions during arterial hypoxaemia, an increase in lactate production did not occur Consequently a certain inhibition also of the anaerobic metabolism may have occurred. This is supported by determinations of the glycolytic flux at varying temperatures by Kohen, Kohen and Thorell (pers. comm.) On the other hand, the lower pH at exhaustion may be due to an altered pH sensitivity of any of the mechanisms underlying muscle contraction, induced by the decreased temperature. Our present knowledge of the processes leading to muscle contraction seem to be too incomplete to allow a more comprehensive explanation

5. The adaptation of oxygen transport capacity to metabolic capacity

The experiments with oxygen breathing at hyperbaric pressure indicated that the maximal oxygen uptake for the modes of exercise studied was achieved already under normal atmospheric conditions. The hypoxaemic

experiments showed that a small decrease in arterial oxygen content is possible without decreasing the oxygen uptake From the results it has been concluded that the oxygen transport *per se* does not determine the maximal oxygen uptake. However the conclusion must also be drawn that the margin between the maximal respiratory metabolic rate of the muscles and the maximal oxygen supply by local blood circulation (fore arm work experiments) as well as central circulation (leg work experiments) is small under ordinary conditions in ordinary young subjects.

It can be concluded that the capacity of the circulatory system in the ordinary subject is well adapted to maximal demands by the musculature. Findings by other authors have indicated that not either the lungs have an oxygen transport capacity which much exceeds that of the cardiovascular system (Blomqvist Jonson Jr and Saltin 1969 Freedman 1970) Thus significant correlations are found between measures of aerobic work capacity and dimensional and functional measures of the cardiovascular system and the lungs (Sjöstrand, 1953 1954 1962 Holmgren and Åstrand 1966)

Within the scope of the mentioned correlations large variations may exist, however The correlation between arterio-venous oxygen difference and performance time for dynamic forearm work (Fig 8) may indicate individual differences in relative capacity of the oxygen transport and utilization system. To judge from the experiency of this laboratory (*e g* Holmgren *et al.* 1960) as well as others (Saltin *et al.* 1968 Ekblom 1969) the earliest effect of physical training is an increased aerobic work capacity without an increase in cardiovascular dimensions. This is accomplished by a more complete oxygen extraction from the blood The phenomenon has often been ascribed to a more efficient vasoregulation but it might as well be caused by an increased capacity of the oxygen utilization system in the muscles. As a consequence, the margin for the capacity of the circulatory system in relation to the oxygen utilization system in the muscle cell should be smaller in the well-trained subject. Data are also reported in the literature, which indicate that the margin for oxygen transport by the lungs in relation to the overall oxygen transport capacity is decreased after training (Anderson and Shephard 1968)

Part D SUMMARY and CONCLUSIONS

The local circulation as a limiting factor for aerobic work performance was tested by varying the arterial oxygen content in forearm work experiments. Variations between 5 per cent below and 30 per cent above the ordinary arterial oxygen content did neither significantly influence the maximal

arteno-venous oxygen difference nor the performance time during exhaustive work. The oxygen saturation and the calculated oxygen pressure in the deep vein of the active forearm was 2 3 times higher during oxygen breathing at 3 ata than during air breathing It was concluded that neither the local blood perfusion nor the oxygen diffusion from blood to muscle cell limit the local maximum oxygen uptake.

To test if the central circulation could be a limiting factor for oxygen uptake when large muscle groups are utilized leg work was performed with varying arterial oxygen content. An increased arterial oxygen content was achieved by oxygen breathing at 3 ata. An analysis of the cardiovascular adaptation to steady state exercise at submaximal work loads during oxygen breathing at 3 ata revealed that the cardiac output is moderately reduced during oxygen breathing This was caused by a heart rate decrease, the stroke volume being unchanged. Only minor differences in pulmonary artery and brachial artery pressures were found between oxygen and air breathing Exhaustive work was performed with ordinary increased or decreased arterial oxygen content. With an increased arterial oxygen content by oxygen breathing at 3 ata, the subjects stopped because of exhaustion with a lower calculated cardiac output and a far higher femoral-venous oxygen saturation than during air breathing It was concluded that the cardiac output did not limit the maximal oxygen uptake during leg work. It was also found that it was possible to decrease the arterial oxygen content slightly without decreasing the aerobic work performance. The margin amounted to between 3 and 10 per cent, probably not much larger than 3 per cent, however

In spite of the 2-4 times higher venous oxygen pressure during oxygen breathing at 3 ata compared to air breathing, only a moderate reduction in venous lactate concentration was seen during forearm as well as during leg work. It was concluded that a high lactate production should not be taken as evidence for lack of molecular oxygen in the muscle tissue. It may however be taken as further evidence, that the capacity of the aerobic metabolism limits the oxygen uptake of a muscle.

In spite of varying venous P_{CO_2} and lactate concentration, the point of exhaustion seems to be reached at approximately the same venous pH under the different conditions. The finding was considered to support the hypothesis that the increased acidity in the muscle cell in healthy young subjects at work periodes of up to 10 minutes, sets the limit for its function.

The maximal rate of muscle metabolism as the limiting factor for aerobic work performance was supported by experiments with dynamic forearm work after cooling A ? 3 C decrease in muscle temperature, as evidenced

from measurement in the deep vein blood decreased the performance time for exhaustive work 33 per cent. The deep vein blood was at exhaustion less desaturated in the cooled arm than under ordinary conditions, whereas no difference in deep vein lactate concentration was found It was concluded that the decreased performance was caused mainly by a decrease of the aerobic metabolic rate

The final conclusion from work experiments with varying arterial oxygen content and muscle temperature is that oxygen transport by the blood circulation does not under ordinary conditions limit the aerobic work capacity However the different links in the transport and utilization systems for oxygen seem to be adapted to each other with rather small margins under ordinary conditions in healthy young subjects.

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SUPPLEMENTUM 347

From the Department of Clinical Physiology
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EFFECT OF POSTURE ON CIRCULATION AND RESPIRATION AT REST AND DURING EXERCISE IN HEART DISEASE

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Contents

		Page
I	List of symbols and abbreviations	5
II	Acknowledgements	9
III	Introduction	11
IV	Methods	13
V	Patients	16
VI	Procedures	23
VII	Postural effects on the central circulation and respiration at rest and during exercise in patients with *mitral stenosis*	25
VIII	Postural effects on the central circulation and respiration at rest and during exercise in patients with *pulmonary stenosis*	41
IX	Postural effects on the central circulation in patients with *atrial septal defect*	52
X	Ventilation, alveolar gas exchange and $D_{L_{CO}}$ during exercise in patients with *atrial septal defect* with special reference to body posture	59
XI	Ventilation, alveolar gas exchange and $D_{L_{CO}}$ in patients after corrective surgery of *atrial septal defect*	65
XII	General discussion	71
XIII	Summary	77
XIV	References	80

List of symbols and abbreviations

These are based on the recommendations of Pappenheimer et al. (1950) and Gandevia and Hugh-Jones (1957)

1 Gas measurements.

a. Gas phase

I	= inspired gas
E	= expired gas
A	= alveolar gas
T	= tidal gas
D	= dead space gas
STPD	= standard temperature, pressure, dry (0 C., 760 mm Hg)
BTPS	= body temperature, pressure, saturated with water vapor
ATPS	= ambient temperature, pressure, saturated with water vapor
V	= volume, ml or l
V	= flow ml or l/min.
P	= pressure, mm Hg
F	= fractional concentration
R	= respiratory exchange ratio

b. Blood phase

a	= arterial
v	= venous
v	= mixed venous
S	= saturation, %

2. Other symbols used.

VC	= vital capacity l BTPS
FRC	= functional residual capacity l BTPS
RV	= residual volume, l BTPS
TLC	= total lung capacity l BTPS
$FEV_{1.0}$	= forced expiratory volume in 1 sec., l BTPS
$FEV_{\%}$	= $FEV_{1.0}$ in per cent of VC
MVV_{40}	= maximal voluntary ventilation at 40 breaths/min. l BTPS/min.
MVV_{free}	= maximal voluntary ventilation at a free respiratory rate, l BTPS/min.
$D_{L_{CO}}$	= pulmonary diffusing capacity for carbon monoxide, ml min.$^{-1}$ mm Hg^{-1}

V_E/V_{O_2} = ventilatory equivalent for oxygen $\frac{\text{l, BTPS/min.}}{\text{l, STPD/min.}}$

StB = standard bicarbonate, mEq/l

Sup = the patient in the supine position

Sitt = the patient sitting on a bicycle ergometer

Heart rhythm S = sinus

F = atrial fibrillation

BSA = body surface area according to DuBois formula, m^2

W_{150} = physical working capacity at heart rate 150 cpm, kpm/min.

W_{max} = "maximal working intensity kpm/min.

THb = total amount of hemoglobin, g

BV = blood volume, l

HV = heart volume, ml

3 Hemodynamic symbols

$\dot{Q}$ = cardiac output, l/min.

$\dot{Q}_s$ = cardiac output in systemic circulation, l/min.

$\dot{Q}$ = cardiac output in pulmonary circulation, l/min.

SV = stroke volume, ml

ΔSV = the relative change in stroke volume =

$$= \frac{SV_{sup} - SV_{sitt}}{SV_{sup}} \times 100 \ \%$$

HR = heart rate, cpm

AVD = arterio-venous oxygen difference, ml/l

AVD_P = AVD of the pulmonary circulation, ml/l

P = pressure, mm Hg

S = systolic

D = diastolic

M = mean

Ao = aorta

LV = left ventricle

PCV = pulmonary capillary venous

PA = pulmonary artery

RV = right ventricle

ed = end diastolic

RA = right atrium

SVC = superior vena cava

SVR = systemic vascular resistance = $\frac{P_{Ao}}{\dot{Q}_s}$ mm Hg/(l/min.)

PVR = pulmonary vascular resistance = $\frac{\bar{P}_{PA} - \bar{P}_{PCV}}{\dot{Q}_p}$ mm Hg /(l/min.)

4. Statistical symbols

p	=	probability
$\bar{x}$	=	mean value
d	=	difference
r	=	coefficient of correlation
SD	=	standard deviation
SD_d	=	standard deviation of the differences
C	=	coefficient of variation $= \frac{SD_d \cdot 100}{\sqrt{2} \cdot \bar{x}}$ %

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Introduction

The ability to perform exercise of short duration (5—10 min.) with large muscle groups is mainly determined by the oxygen transport capacity which is normally limited by circulatory factors.

In young and old individuals cardiac output increases as a linear function of oxygen uptake during submaximal work loads in both the supine and sitting positions (for references see Bevegård and Shephard 1967) The slope for the regression line of cardiac output on oxygen uptake varies little between normal individuals, while the intercept may vary markedly The average intercept for normal young subjects in the supine position is defined in the following to mean normokinetic circulation. If the level of the regression line is lower than normal, the circulation is said to be hypokinetic. In this state the oxygen transport is delivered with a smaller cardiac output than in the normokinetic state. In the presence of a hypokinetic circulation the peripheral oxygen utilization increases. In old subjects (above 60 years) the circulation has been described to be more hypokinetic than in young subjects (Strandell 1964) although the slope of the line is the same as in young individuals.

The level of the regression line is correlated with the size of the stroke volume in relation to the volume of the cardiovascular system. The circulation becomes hypokinetic on changing from the supine to the sitting position in normal young and old subjects. The slope of the regression line is, however unchanged. The cause of a lower stroke volume and cardiac output in the upright position is the lower filling energy available for the ventricles because of the gravitational shift of blood distribution (Sjöstrand 1941 Lagerlöf et al. 1951 Bevegård et al. 1960 Mc Gregor et al. 1961 Strandell 1964)

Patients with heart disease usually have a lower stroke volume in the supine position in relation to circulatory dimensions, such as heart volume and blood volume, than normal subjects (Sjöstrand 1953 Wade and Bishop 1962, Marshall and Shephard 1968) leading to a hypokinetic circulation. Several authors have reported a low cardiac output in patients with mitral stenosis (Eliasch et al. 1952, Holmgren et al. 1958 Bishop and Wade 1963, Granath 1965) Ikkos et al. (1966) described patients with tight pulmonary stenosis with a hypokinetic circulation at rest and during work.

Clinical experience suggests that patients with heart disease are less orthostatic than normal subjects. Several studies have dealt with the orthostatic response to change from the supine to the head up position at rest (McMichel and Sharpey Schafer 1944, Eliasch et al. 1961 Abelmann and Fareeduddin 1967 Lorentsen et al. 1967) Donald et al (1953) found a slight fall in cardiac output in patients

with heart disease, but there were marked interindividual variations. Lorentsen et al. (1967) reported a fall in cardiac output and stroke volume in most cases without, or with only a slight degree of heart failure. Patients in left ventricular failure showed the same response. Two patients with tricuspid valvular insufficiency even displayed an increase in cardiac output and stroke volume after changing from the supine to the sitting position.

Abelmann et al. (1969) found in 33 patients with heart disease that hemodynamic abnormalities characteristic of heart failure were associated with increased orthostatic tolerance, measured as the response of heart rate and diastolic blood pressure to change in posture. There are very few circulatory studies reported in cardiac patients during exercise in the upright position.

Epstein et al. (1969) pointed out that exhausting levels of exercise could be achieved in the upright position by patients with cardiac disease, with no evidence of pulmonary oedema appearing. They reported further that in patients with impaired cardiac function bicycle exercise in the supine position imposed a greater circulatory stress than when the same exercise was performed in the sitting position. This was manifested by a faster heart rate and higher systemic arterial pressures in the supine position. Details of the individual patients were not, however, given. Most clinical investigations on cardiac patients are performed in the supine position and these do not necessarily reflect the situation of the central circulation during ordinary daily activity.

The principal aim of this investigation was to study how changes in body position affect the central circulation, ventilation, alveolar gas exchange and pulmonary diffusing capacity at rest and during exercise in three different groups of patients. The *first group* was selected to represent low pulmonary blood flow (i.e. hypokinetic circulation) and high pulmonary vascular pressures, with an almost normal resistance in the pulmonary vascular bed, as found in patients with moderately tight mitral stenosis. The *second group* was selected to represent low blood flow and low pressure levels in the pulmonary circulation, as in patients with tight pulmonary stenosis. The *third group* represented high blood flow in the pulmonary circulation with almost normal pulmonary pressures, as found in patients with atrial septal defect.

Some of the patients in this last group were also investigated about 1 year after operation to study how decrease in pulmonary blood flow would affect ventilation, alveolar gas exchange and pulmonary diffusing capacity.

Methods

The week before heart catheterization the patients were thoroughly investigated with physical and x-ray examination, and determination of working capacity in the sitting and supine positions with simultaneous recording of a six lead electrocardiogram (Holmgren and Strandell 1961)

Exercise tests were performed on an electrically braked bicycle ergometer (Holmgren and Mattson 1954) with the work load increasing stepwise at the end of every six minutes. Using the approximately linear relationship between heart rate and work load working capacity at a fixed heart rate (150 beats/min) was obtained by inter or extra polation. The exercise test was usually stopped before the pulse rate had reached 150—170 beats/min. because of symptoms. This was particularly so in the patients with mitral stenosis, and in some patients with pulmonary stenosis and ASD Therefore, the concept "maximal work intensity introduced by Strandell (1964) has been used to express the working capacity

Maximal work intensity (W_{max}) was taken to be the highest work load that the patient could sustain for six minutes, with an increase proportional to the completed part of the period at the next higher load when symptoms prevented the patients from continuing the work for six minutes. An interval of 1—2 days elapsed between work tests in the sitting and supine positions

Total amount of hemoglobin (THb) was determined with the alveolar CO method of Sjöstrand (Sjöstrand 1948) with a reproducibility of 3 7 % (Normal values see Holmgren 1969)

Blood volume (BV) was calculated from THb and hemoglobin concentration of finger blood, and correction was made for the difference between body hematocrit and peripheral vessel hematocrit by a factor 0 91 (Ekelund 1965)

Heart volume (HV) was determined in the prone position according to the method described by Larsson and Kjellberg (1948) except that the angulation of the x ray tube was omitted.

Heart catheterization was performed as described earlier from this laboratory (Bevegård et al. 1960) A no 9 double lumen catheter was introduced percutaneously into an ante-cubital vein and passed into the pulmonary artery A teflon catheter was introduced in the right brachial artery by the percutaneous technique and advanced into the left ventricle through the aortic valve with the aid of a metal guide wire. In patients with mitral stenosis another teflon catheter was introduced percutaneously so that its tip was in the right atrium. In patients with atrial septal defect such a teflon catheter was placed with its tip in the superior vena cava. Blood sampled from this catheter was taken to represent approximately mixed venous blood from the systemic circulation (Dexter 1956)

Blood pressures were recorded with electrical transducers* the zero pressure reference level in the supine position being the midthoracic level and in the sitting position at the level of the sternal insertion of the fourth rib The tip of the double lumen catheter was moved to a wedge position in the lower part of the right lung and the tip of the teflon catheter in the right atrium was placed to be at approximately the same level, i.e. the sternal insertion of the fourth rib in supine position. During exercise in the sitting position the patient breathed through a fixed mouthpiece to maintain an approximately constant horizontal reference level.

Cardiac output (Q) was measured by the direct Fick method. Expired gas was collected in Douglas bags and was analysed for oxygen and carbon dioxide by the Scholander method (Scholander 1947) Gas volumes were measured with a spirometer and converted to BTPS *Blood samples* were drawn simultaneously from the pulmonary artery and the aorta for one minute in the middle of the gas collection period, which was 6 minutes at rest and 2—3 minutes during work.

Blood gas analysis Oxygen saturation and hemoglobin concentration were determined spectrophotometrically (Holmgren and Pernow 1959)

Partial pressure of carbon dioxide (P_{aCO_2}) was measured with a Severinghaus P_{CO_2} electrode (Severinghaus and Bradley 1958) and of oxygen (P_{aO_2}) with a Clark microcathode electrode in a stainless steel cuvette kept at 37 ° C. The reproducibility of measurement of P_{aCO_2} and P_{aO_2} was 0 5 and 1.2 % respectively

Standard bicarbonate base excess and *pH* were determined according to Siggaard Andersen et al. (1960)

Diffusing capacity of the lungs for CO (D_{LCO})

The methodology techniques and errors involved have been reported earlier (Holmgren 1965 Freyschuss and Holmgren 1965) therefore only brief information on methodology is included F_{O_2} and F_{CO_2} were analysed with the Scholander technique Gas volumes were measured with a spirometer *Carbon monoxide in the gas phase* was analysed with a hopcalite CO-meter as described by Linderholm and Sjöstrand (1956) *Carbon monoxide content* in blood was determined by the method described by Linderholm, Sjöstrand and Söderström (1956) The carbon monoxide capacity of blood was taken to equal 1 34 times the hemoglobin concentration. The reproducibility for measuring D_{LCO} during exercise in this laboratory is 7 2 %

Static spirometry with determination of total lung capacity and its subdivisions, was performed with helium dilution method in a closed system (Holmgren 1954) Normal values were those given by Grimby and Söderholm (1963)

Dynamic spirometry including forced expiratory volume in 1 sec., $FEV_{1.0}$ this volume related to the vital capacity FEV% maximal voluntary ventilation at a fixed respiratory rate of 40 breaths/min. and at free rate, MVV_{40} and MVV_{free} were

) Model EMT 34 and 35 Elema Schönander, Stockholm, Sweden.

determined with a Bernstein spirometer* Predicted normal values were those given by Berglund et al. (1963)

To study the reproducibility of the spirometric data over a longer period, the investigation was repeated with 2—120 days interval in the mitral stenosis group The coefficient of variation of the difference between the two measurements was for vital capacity 1.8 % for functional residual capacity 11 4 % for total lung capacity 4 3 % and for maximal voluntary ventilation 7 3 %

Calculations

The physiological dead space was calculated using the Bohr formula and assuming the alveolar carbon dioxide tension to be equal to the arterial carbon dioxide tension (Enghoff 1938, Riley and Cournand 1949)

Alveolar oxygen tension and alveolar ventilation were obtained from the alveolar gas equation (Riley et al. 1946) The *diffusing capacity* was measured by means of the steady state CO-method, calculated as the quotient between the carbon monoxide uptake (V_{CO}) and the driving pressure alveolar CO-tension minus mean capillary CO-tension ($P_{ACO}-\bar{P}_{cCO}$) The V_{CO} was calculated according to Filley et al. (1954) Assuming the same dead space for CO and CO_2, the P_{ACO} was calculated by means of the Bohr equation. The $\bar{P}_{CO}$ was calculated according to Lindeholm (1957)

All calculations concerning THb, alveolar gas exchange and D_{LCO} were performed by an IBM 7044 computer

Statistical methods

Statistical calculations were mainly performed according to Dahlberg (1940) and Snedecor (1959) Significance of differences between mean values were tested by Student s t-test. Differences between regression lines were tested with covariance analyses according to Hald (1960) The following probability (p) levels of significance were used.

$p<0.001$	=	highly significant	(***)
$0.001<p<0.01$	=	significant	(**)
$0.01<p<0.05$	=	probably significant	(*)

) Kifa, Stockholm, Sweden

V

Patients

Anthropometric and functional data are given in tables I—VI

The patients selected for this study were referred to the Thoracic Clinics for preoperative evaluation and/or surgical treatment. There were 38 patients in all, with ages ranging from 16 to 60 According to diagnosis they were placed into three groups.

MITRAL STENOSIS (MS) (Tables I—II)

This group consisted of 9 patients with mitral stenosis. There were 7 women and 2 men with a mean age of 50 years(range 42—59) The diagnosis was made on basis of typical physical findings, x ray examination, heart catheterization and later confirmed by surgery One patient (no 1) had an additional aortic stenosis of mild degree (peak systolic left ventricular aortic pressure difference of 20 mm Hg) With this exception and another patient (no 2) with very mild mitral regurgitation seen on angiocardiography all had pure mitral stenosis. Four of the patients had a history of rheumatic fever and two had been operated upon with commissurotomy 10 and 2 years respectively before the investigation, but had signs of restenosis (nos. 2 and 4)

According to the classification of the New York Heart Association (1955) (N Y H.A.) the patients belonged to functional group II—III and none had clinical signs of right heart failure. Four patients were in atrial fibrillation and the others in sinus rhythm. All except one (no 6) were digitalized and case no. 7 also had quinidine.

Heart volume (HV) in the prone position averaged 1108 ml (range 915—1320) All cases had moderately enlarged heart volumes in relation to the total amount of hemoglobin.

Total amount of hemoglobin (THb) was on an average 588 g SD ± 38 or 9 1 g/ kg body weight SD ± 0 6 These mean values do not deviate significantly from the normal range in the laboratory nor do they differ from those by Granath (1963)

Blood volume (BV) averaged 5.8 l SD ± 0 7 or 77.9 ml/ kg body weight SD ± 12 0 which is within the normal range.

Exercise test All patients stopped working because of dyspnea and general fatigue and these symptoms were more marked in the supine exercise test. The mean value of maximal working capacity (W_{max}) in the whole group was 468 kpm/min. (range 300—667) in the sitting and 383 kpm/min. (range 250—667) in the supine position. This mean postural difference of 85 kpm/min. was significant ($p < 0.01$) The mean value for the women was 414 kpm/min. in the sitting and

TABLE I Anthropometric data in 9 patients with mitral stenosis

Case no	Sex	Age years	Height cm	Weight kg	B.S.A. m^2	Heart volume supine ml	Total hemoglobin g	Blood volume l
1	F	59	151	68	1.62	1025	506	4.2
2	F	53	167	61	1.69	1255	571	5.2
3	F	55	160	66	1.68	1160	446	4.3
4	F	52	160	60	1.62	1060	439	4.0
5	F	42	158	71	1.72	1035	612	5.3
6	F	49	162	54	1.57	915	617	5.6
7	M	47	170	73	1.85	1140	639	5.2
8	F	47	164	59	1.65	1060	653	5.3
9	M	50	173	75	1.89	13 0	808	6.1

TABLE II Exercise tolerance tests in 9 patients with mitral stenosis See list of symbols and abbreviations

		Highest load, kpm/min. and highest heart rate, beats/min.						W max kpm/min.	
		Sitting			Supine				
Case no	Heart rhythm	Load	HR	Time min.	Load	HR	Time min.	Sitting	Supine
1	F	400	142	4	300	132	6	333	300
2	F	500	142	6	300	144	6	300	300
3	F	400	162	6	400	160	6	400	400
4	S	600	130	1	400	102	2	433	267
5	S	600	155	1.5	300	138	4	450	250
6	S	600	158	4	400	142	6	533	400
7	S	900	140	1	600	140	6	650	600
8	S	600	156	1 5	400	148	2	450	267
9	F	800	160	2	800	166	2	667	667

312 kpm/min. in the supine position. These values are low compared to those of normal subjects (Astrand 1956, Holmgren et al. 1957)

Stat spirometry The vital capacity and functional residual capacity were significantly decreased on an average to 84 % and 86 % respectively of the predicted normal value. The total lung capacity was decreased to 90 % of the normal value. The residual volume was not significantly changed from the normal value. The functional residual capacity and total lung capacity were diminished by the same degree and therefore the quotient between them was normal. Due to the diminished TLC the RV/TLC was slightly higher than normal (112 % $p<0.05$)

Dynamic spirometry The forced expiratory volume in 1 sec. ($FEV_{1.0}$) was significantly decreased to 82 % but the forced expiratory volume in relation to the

vital capacity (FEV %) was normal. The maximal voluntary ventilation at a fixed respiratory rate of 40 breaths/min (MVV_{40}) and at free rate (MVV_{free}) were decreased to 73 % and 69 % respectively of normal mean value

Comment None of the patients had a history of chronic bronchitis, and the spirometric data agree with what has been described earlier in mitral stenosis, i.e. a decreased vital capacity but a normal residual volume.

There were no signs of obstructive lung disease and the low maximal breathing capacity correlates with the decreased vital capacity following restrictive lung changes.

PULMONARY STENOSIS (PS) (Tables III—IV)

Five patients with congenital valvular stenosis were included in this group There were two men and three women with ages ranging from 36 to 60 years with a mean of 47 years. The diagnosis was based on physical findings, x ray examination with cineangiography of the right ventricle and heart catheterization. In 4 patients the diagnosis was verified at operation. None of the patients had other valvular disease or septal defect. The patients belonged to functional group I to II according to N.Y.H.A., and none had clinical signs of right ventricular failure. All were in sinus rhythm.

Heart volume (HV) varied between 590 and 1140 ml and was in all within the normal range in relation to total hemoglobin.

Total amount of hemoglobin (THb) varied between 390 g and 1011 g. In relation to body weight it was somewhat small in the three females, ranging from 6.3 to 6 9 g/kg body weight. For the males it was normal in one (10.0 g/kg body weight) and a little increased in one (12 6 g/kg)

Blood volume (BV) was as an average 3 7 l (range 3.0—4 6) or 55 0 ml/kg body weight for the women. This value is small compared to the normal range For the two men it was somewhat small in one (66 ml/kg body weight) and normal in the other

TABLE III Earlier power data 5 patients with pulmonary stenosis

Case no	Sex	Age years	Height cm	Weight kg	B.S.A. m^2	Heart volume supine ml	Total hemoglobin g	Blood volume l
1	F	43	154	59	1 56	590	390	3.0
2	F	36	162	80	1.84	760	432	3 4
3	F	60	162	74	1 78	730	512	4 6
4	M	57	181	101	2.22	1140	1011	6.6
5	M	37	177	66	1.83	1030	811	5 4

) Heart volume in the standing position

TABLE IV Exercise tolerance tests in 5 patients with pulmonary stenosis
See list of symbols and abbreviations

Case no	Heart rhythm	Highest load, kpm/min. and highest heart rate, beats/min.						W_{max} kpm/min.		W_{150} kpm/min.	
		Sitting			Supine						
		Load	HR	Time min.	Load	HR	Time min.	Sitting	Supine	Sitting	Supine
1	S	450	170	2	450	180		350	350	267	256
2	S	400	152	6	—	—	—	400	—	390	—
3	S	400	153	6	400	148	3	400	300	376	—
4	S	900	140	1.5	600	124	6	675	600	982	990
5	S	600	160	6	600	160	6	600	600	530	530

Exercise test All patients stopped the exercise test because of general fatigue and tiredness in the legs. Two patients (nos. 1 and 4) also complained of dyspnea. There was no main difference in symptoms between sitting and supine exercise. W_{150} was the same in both body positions in the three patients in whom it was possible to calculate. For the whole group W_{max} averaged 490 kpm/min. in the sitting and 460 kpm/min. in the supine position. The mean maximal heart rate observed in each individual was 155 beats/min. (range 140—170) and 153 beats/min. (range 124—180) in the sitting and supine positions respectively

Static spirometry The vital capacity functional residual capacity residual volume and total lung capacity were in all within the normal range.

Dynamic spirometry One patient (no 5) had a low $FEV_{1.0}$ and FEV % (73 and 78 % of predicted normal value) that could indicate an obstructive lung disease although he had no history of bronchitis or asthma. He had a normal MVV_{40}, however The other four patients were within the normal range.

ATRIAL SEPTAL DEFECT (ASD) (Tables V—VI)

The group with increased pulmonary blood flow was selected only to include patients with atrial septal defects and/or abnormal venous return. The group consisted of 24 patients, 14 women and 10 men with a mean age of 40 years (range 16—57) The diagnosis was based on physical findings, x ray examination, heart catheterization and in most patients the diagnosis was verified at operation. Two patients had an atrial septal defect of primum type, but none of these had signs of mitral insufficiency All the others had a defect of the secundum type and some of these also had partial anomalous pulmonary venous drainage.

The patients all belonged to functional group I—II according to the N.Y.H.A., i.e none had any symptoms at rest, and during ordinary physical activity some had symptoms dominated by dyspnea. None had clinical signs of heart failure. Two patients were in atrial fibrillation and the rest were in sinus rhythm.

TABLE I Anthropometric data in 24 patients with atrial septal defect

Case no	Time to op.	Sex	Age years	Height cm	Weight kg	B.S.A. m^2	Heart volume ml	Total hemoglobin g	Blood volume l
1	pre	F	39	165	72	1.77	1160	725	5.8
	post		40	165	76	1.83	810	616	5.0
2	pre	F	48	162	67	1.71	905	—	—
	post		49	162	66	1.70	650	489	4.4
3	pre	M	52	178	87	2.06	1290	864	6.0
	post		53	178	95	2.13	1130	734	5.3
4	pre	M	53	183	79	2.03	1600	711	5.5
	post		55	184	77	2.01	1200	707	5.9
5	pre	F	26	168	57	1.66	1140	610	5.8
6	pre	M	25	170	64	1.75	1150	665	4.7
7	pre	F	57	156	71	1.70	1390	610	4.8
8	pre	F	52	156	59	1.58	980	556	4.8
	post		53	157	60	1.58	710	—	—
9	pre	M	44	180	59	1.78	1840	49	6.2
	post		46	183	63	1.85	1285	649	5.2
10	pre	F	37	161	48	1.48	790	—	—
	post		39	161	47	1.46	620	343	5.1
11	pre	M	41	170	55	1.65	—	698	5.8
12	pre	F	57	169	64	1.74	1340	677	5.9
	post		58	169	64	1.75	1555	621	5.0
13	pre	F	37	155	63	1.60	740	455	4.2
14	pre	F	50	162	68	1.73	910	525	4.6
15	pre	M	46	182	86	2.09	1650	942	7.5
16	pr	F	21	171	60	1.72	640	452	4.1
	post		23	170	63	1.74	650	400	3.7
17	pre	F	36	163	53	1.57	630	459	4.1
18	pre	M	16	167	66	1.69	830	582	4.7
19	pre	M	41	173	72	1.86	1300	631	5.1
	post		42	173	80	1.94	1100	641	5.1
20	pre	M	49	190	86	2.14	1415	817	6.4
	post		50	190	90	2.21	890	686	5.6
21	pre	F	38	161	93	1.94	1540	678	5.6
22	pre	F	22	173	56	1.69	1080	510	4.6
23	pre	M	38	166	52	1.58	1100	433	4.6
24	pre	F	45	162	70	1.75	915	547	4.8

) Heart volume in standing position.

Heart volume (HV) averaged 1222 ml (range 630—1840). In all patients, except one (no 17) it was abnormally increased in relation to total hemoglobin.

Total amount of hemoglobin (THb) was determined in 21 patients, 9 men and 12 women. The mean value for both sexes was within the variation of normal individuals. In female patients it was 567 g SD±93 corresponding to 8.7 g/kg body weight and in males it amounted to 715 g SD±111 or 10.5 g/kg body weight.

TABLE VI Exercise tolerance tests in 24 patients with atrial septal defect
See list of symbols and abbreviations

Case no	Time to op.	Heart rhythm	Highest load, kpm/min. and highest heart rate, beats/min. Sitting Load	HR	Time min.	Supine Load	HR	Time min.	W_{max} kpm/min. Sitting	S pine	W_{150} kpm/min. Sitting	Supine
1	pre	S	600	142	6	600	134	6	600	433	677	391
	post	S	800	150	6	600	128	6	667	467	776	740
2	pre	S	—	—	—	—	—	—	—	—	—	—
	post	S	600	160	6	—	—	—	600	—	509	—
3	pre	S	600	164	1.5	400	150	1.5	450	250	—	—
	post	S	900	152	6	600	124	6	900	600	900	—
4	pre	S	900	172	6	900	168	6	900	800	748	800
	post	F	900	146	2	600	156	6	700	600	—	—
5	pre	S	600	178	6	—	—	—	600	—	433	—
6	pre	S	900	148	6	900	135		700	625	751	800
7	pre	S	600	158	6	600	134	6	600	467	575	850
8	pre	S	400	138	2	500	114	6	267	300	—	—
	post	S	400	158	6	400	149	2	400	500	338	—
9	pre	F	450	146	6	450	146	6	450	450	470	462
	post	F	900	195	2	600	102	6	700	600	529	—
10	pre	S	600	144	2	—	—	—	467	—	529	—
	post	S	400	126	6	400	120	6	400	400	550	—
11	pre	S	600	150	2	600	142	1	467	433	535	547
12	pre	F	400	146	6	400	146	6	400	400	438	413
	post	F	600	190	2	—	—	—	467	400	429	—
13	pre	S	600	170	6	—	—	—	600	—	463	—
14	pre	S	600	152	6	600	162	3	600	500	397	486
15	pre	S	900	153	1.5	—	—	—	675	—	975	—
	post	S	600	175	2	—	—	—	467	—	360	—
16	pre	S	600	180	4	600	138	1	553	467	400	—
17	pre	S	600	142	6	400	126	6	600	400	663	600
18	pre	S	900	175	6	900	168	4	900	800	611	675
19	pre	S	900	166	6	900	168	4	900	800	806	851
	post	S	1200	170	3	—	—	—	1050	—	926	—
20	pre	S	600	94	2	400	98	6	467	400	—	—
	post	S	1000	132	1	900	122	2	833	750	—	—
21	pre	S	400	150	6	400	136	6	400	400	400	493
22	pre	S	600	164	6	600	164	6	600	600	497	496
23	pre	S	900	185	1.5	600	158	6	675	600	509	543
24	pre	S	800	170	2	600	150	4	667	534	598	655

Blood volume (BV) was on an average 4.9 1 SD±0 7 corresponding to 75 ml/kg body weight for women and 5 6 1 SD±1.0 or 85 ml/kg body weight for men, and not significantly changed from the normal values.

Exercise tests in the sitting position was performed in 23 patients. Three of these

patients had no symptom or discomfort during the test and these three all worked to a pulse rate of 160 beats/min. One of these patients was 16 years old and the other two were over 40 years old.

The dominating symptom during the test was dyspnea in 12 patients (55 %) Tiredness of the legs was noticed in 6 (27 %) and general fatigue in 7 patients (32 %)

In 15 patients W_{150} could be calculated, according to the method described, in both the sitting and supine positions. The mean W_{150} in the sitting position was 592 kpm/min. (range 400—806) and in the supine position 617 kpm/min (range 413—850) The postural difference of 25 kpm/min. was not significant.

Static spirometry The vital capacity was below 2 SD of the mean normal value in 45 % of the patients. On an average the vital capacity was significantly reduced, being 85 % of the predicted normal mean value. Functional residual capacity and residual volume were normal both in absolute values and in relation to the total lung capacity

Dynamic spirometry The forced expiratory volume in 1 sec. ($FEV_{1.0}$) was significantly decreased to 85 % but the FEV % was normal and none of the patients fell outside 2 SD of the normal mean value The maximal voluntary ventilation at a fixed respiratory rate of 40 breaths/min. and at a free rate were significantly decreased to 76 % and 79 % respectively of the predicted normal values.

Comments No patients had signs of obstructive lung disease. The slight decrease of the vital capacity with 15 % fits well with the large material of Siltanen (1968) who found a 18 % decrease of the VC in patients with ASD The decrease in VC was not related to the pulmonary blood flow or to the age. Siltanen found in a postoperative follow-up study that significant improvement of VC correlated best with changes in heart volume, but there was generally no relation to the flow and pressure in the lung circulation. In the present study the group with abnormally small VC had a mean heart volume in the supine position that was 205 ml larger than that in the group with a normal VC, although the difference was not statistically significant.

VI

Procedures

MITRAL STENOSIS (MS)

All catheterizations were performed in the morning and the patients had only a light meal 2 hours before the investigation. Approximately 15 minutes after the catheters had been introduced, blood pressures (including the pressure difference across the mitral valve) and cardiac output were measured at rest in the supine position. Five of the patients were then studied during exercise in the supine position (nos 1 and 3 on two loads, the others on one load only). The highest work load during heart catheterization was on an average 85 % of W_{max} performed some days earlier in the supine position. After 30—45 minutes rest the patients were investigated on the bicycle in the sitting position. Pressures and flow were measured after 3—4 min. rest, and during the 4th—6th min. at work on the same loads as in the supine position. The other four patients were studied in the same way in the sitting position before the exercise in the supine position. Also in these cases the time interval between exercise periods in the two positions was 30—45 min. and at the end of a similar period all patients were studied once more supine at rest. There were no complications of exercise either in the supine or sitting postures and no patient complained of dizziness or fainted while sitting at rest on the bicycle. The total amount of blood that was withdrawn from the patient during the heart catheterization varied between 110 and 150 ml.

In all patients the diffusing capacity was measured 2 or 3 days after the heart catheterization also in the morning. The same work loads were chosen in both body positions as during the heart catheterization.

PULMONARY STENOSIS (PS)

The heart catheterizations were all done in the morning and performed as described earlier. In all patients the central pressures and the cardiac output were first measured at rest in the supine position. Two patients were then moved over to a bicycle and were studied at rest and during exercise in the sitting position first and then during work in the supine position. The other three patients exercised in the supine position first and then in the sitting position (case no. 4 and 5 on two loads, the others on one load). The time interval between the two investigations was 30 to 45 min. In three patients the diffusing capacity was determined some days after the heart catheterization, and the same work loads being used for the two investigations

ATRIAL SEPTAL DEFECT (ASD)

In seventeen patients the heart catheterizations were performed at the Thoracic Clinics as described. Cardiac output and central pressures were measured first at rest and then during work in the supine position. Seven of these patients were also investigated in the sitting position at the same work load as that used in the supine position. In two patients exercise in the sitting position was performed before the work in the supine position, in the other five the order was reversed. Seven patients had been investigated preoperatively in other hospitals. In these only diffusing capacity and alveolar gas exchange were studied in the supine and sitting positions.

VII

Postural effects on the central circulation and respiration at rest and during exercise in patients with MITRAL STENOSIS

Nine patients with MS, described in Chapter V were selected to represent low blood flow and high pressure in the pulmonary vascular bed but with normal vascular resistance to flow. The patients had moderately tight stenosis with a valve area of 1.2—2.3 cm^2 calculated according to Gorlin and Gorlin (1951).

RESULTS

Hemodynamic data

Mean values during exercise are given in Table VII.

Oxygen uptake ($\dot{V}_{O_2}$) in the supine position during the first determination was 23 % higher than that found during basal conditions prior to the investigation. The basal metabolic rate as an average was 6 % higher than the predicted normal value (Harris and Benedict 1919). High oxygen consumption during catheterization is a normal finding and this increase corresponds well with that found in other investigations (Bevegård et al. 1960). There was no difference between the first and second determination at rest in the supine position. In the sitting position the oxygen uptake at rest was significantly 10 % higher than in the supine position. During exercise the oxygen uptake averaged 3.5 times the resting value and there was no postural difference.

Mechanical efficiency averaged 20 % in both positions. This is within the normal variation for the work loads studied (Åstrand 1960).

Heart rate (HR) at rest in the supine position was 73 beats/min. (range 52—95) and there was no difference between duplicate determinations in the supine position. In the sitting position the heart rate was 17 beats/min. greater ($p<0.001$) than when supine. Patients with atrial fibrillation responded in the same way as patients in sinus rhythm. During work the heart rate averaged 120 beats/min. (range 76—150) in the supine position and there was no significant postural difference (Fig. 1).

Arterial oxygen saturation ($S_{a_{O_2}}$) was normal at rest averaging 97.0 % (range 94—100) *and 97.2 % (range 95—100) in the supine and sitting positions* respectively. $S_{a_{O_2}}$ was mainly unchanged during exercise, although it decreased to 90 % in one patient. The mean $S_{a_{O_2}}$ was 1.9 % higher in the sitting than in the supine position although the difference was not significant.

Mixed venous oxygen saturation ($S\bar{v}_{O_2}$) averaged 69.3 % (range 66—75) at rest in the supine position and decreased in the upright position to 59.3 % (range

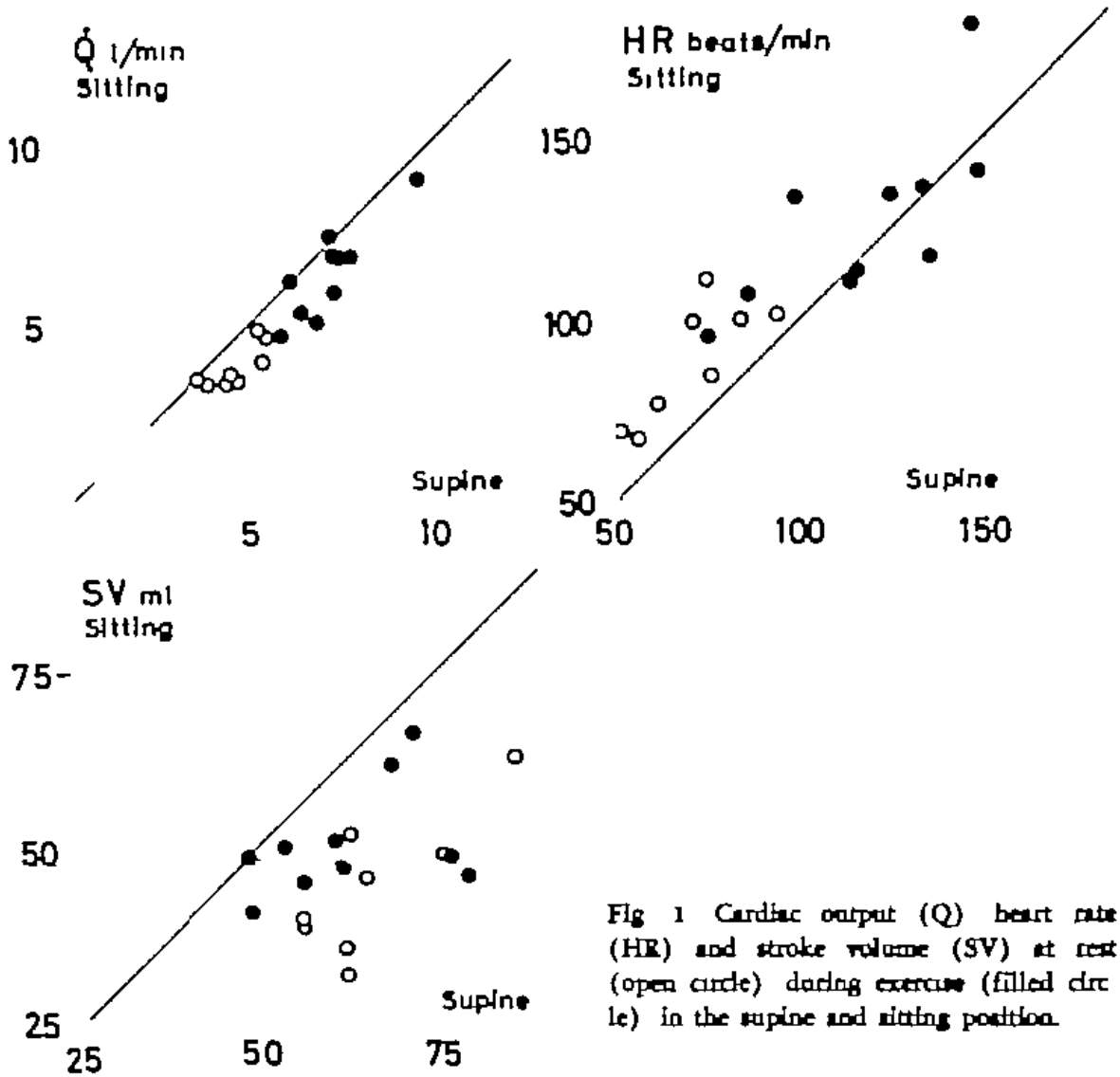
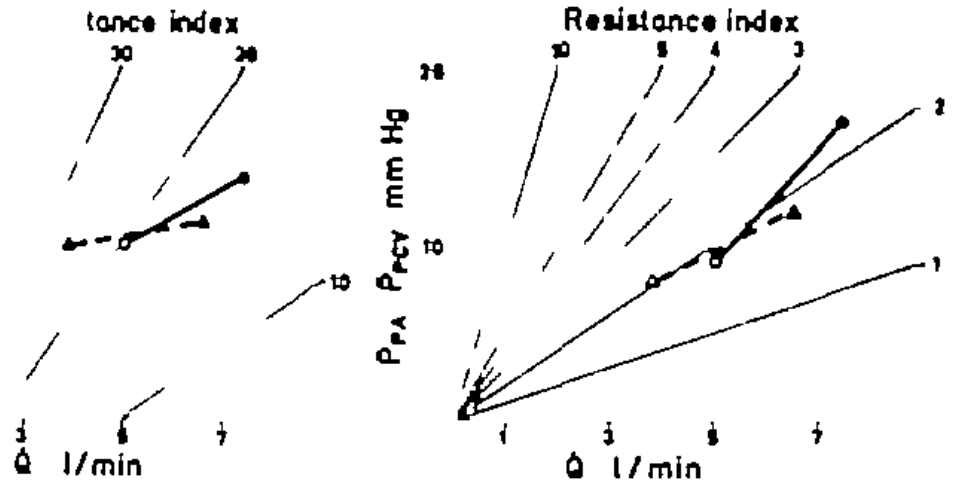


Fig 1 Cardiac output (Q) heart rate (HR) and stroke volume (SV) at rest (open circle) during exercise (filled circle) in the supine and sitting position.

52—65) During exercise the $S\bar{v}_{O_2}$ decreased markedly in both positions. The mean values were 33 3 % (range 24—46) and 29 1 % (range 19—42) in the supine and sitting positions respectively This difference of 4.2 % was not significant.

The oxygen capacity of the blood from the aorta and the pulmonary artery increased from rest to exercise in both body positions. There was also a significantly higher value in the sitting position than in the supine position both at rest and during exercise. The largest difference in capacity was thus between supine rest (mean 178 ml/l) and sitting exercise (mean 194 ml/l)

Arterio venous oxygen difference (AVD) at rest, supine, averaged 51 5 ml/l (range 43—61) and was unchanged at the second determination at rest. All patients in atrial fibrillation were two standard deviations above the mean normal AVD when plotted against $\dot{V}_{O_2}$ All except one of the patients with sinus rhythm were within the normal range (Fig. 2) At rest in the sitting position the AVD was significantly higher than in the supine position in all cases and averaged 73 4 ml/l (range 61—89) During work there were significantly higher values in the



ean pressure (P_{Ao}) in relation to cardiac output (Q) The mean pressure the pulmonary ascular bed (P_{PA}—P_{PCV}) in relation to cardiac output (Q) es are isoresistance lines. Symbols as in fig. 2

old individuals (Bevegård et al 1960 Strandell 1964) The regression rdiac output on oxygen uptake in this material was $Q = 3.70 + 0.0041$ ie supine position and $\dot{Q} = 2.50 + 0.0045\ \dot{V}_{O_2}$ in the sitting position ** and 0.96*** respectively)

volume (SV) at rest in the supine position was 67 ml (range 52—92) reased, not significantly to 63 ml (range 49—86) during exercise. When ition was changed at rest to sitting the stroke volume decreased to 44 ml 32—63) and this fall of 34 % from the supine resting value was highly icant. On exercise in the sitting position the stroke volume increased to 51 ange 41—66) The difference in stroke volume between supine and sitting ions was still significant and averaged 19 % less in the sitting position 1)

mean pressure (P_{Ao}) was within the normal range at rest and during n the supine position. Pressures averaged 101 mm Hg and 121 mm Hg changing to the sitting position did not alter the mean pressure at cise in the sitting position the aortic pressure did not increase to the during exercise when supine. The postural difference of 14 mm Hg ificant ($p < 0.05$)

resistance (SVR) at rest, increased significantly on transition sitting position. During exercise there was no positional l quite di jing from rest to exercise in supine is the same at rest as during y during exercise ($p < 0.001$)

levated in most of the patients mm Hg (range 13—30) This

TABLE VII *Effect of body position on some respiratory and hemodynamic variables during exercise in patients with mitral stenosis analyzed as differences between data (sitting minus supine)*

See list of symbol and abbreviations

	$\bar{X}_{sup}$	$\bar{d}$	SD_d	C	n	p
HR, cpm	120	9	17.0	9.7	11	—
Respiratory cpm	24	−2	5.7	17.6	11	—
$\dot{V}_E$ l/min	26.6	−0.2	3.3	8.9	11	—
V_T ml	1113	116	220	13.3	11	—
$\dot{V}_{O_2}$ ml/min.	905	7	62	4.8	11	—
$\dot{V}_E/\dot{V}_{O_2}$	28.4	0.6	4.2	10.3	11	—
R	0.89	0.01	0.07	5.5	11	—
V_D, ml	251	15	100.9	27.7	11	—
V_D/V_T	0.23	−0.1	0.04	15.0	11	—
$_{a}PCO_2$ mm Hg	38.5	−1.2	2.6	4.8	11	—
P_{aO_2} mm Hg	80.3	9.3	7.6	6.3	11	
P_{AO_2} mm Hg	106.4	1.9	3.8	2.5	11	—
$P_{(A-a)O_2}$ mm Hg	26.1	−7.1	9.4	29.4	11	
pH, units	7.38	0	0.02	0.2	8	—
Stb mEq/l	20.6	0.8	0.8	2.9	7	—
S_{aO_2} %	96.1	1.9	3.3	2.4	11	—
$S_{\bar{v}O_2}$ %	33.3	−4.2	7.0	15.9	11	—
AVD ml/l	121.8	16.0	11.2	6.1	11	
Q l/min.	7.4	−0.8	0.6	6.3	11	
SV ml	63	−12.0	10.2	12.5	11	
$\bar{P}_{RA}$ mm Hg	11.0	−8.7	4.8	30.6	7	
P_{PA} mm Hg	36.5	−10.4	7.5	11.5	11	**
P_{PCW} mm Hg	39.5	−15.1	7.4	16.4	11	
$\bar{P}_{Ao}$ mm Hg	121.0	−14.0	18.8	11.6	11	
PVR, units	2.4	−0.4	1.0	31.2	11	—
SVR, units	18.2	−1.0	3.6	14.5	11	—

1.2 l/min (24 %) is highly significant ($p<0.001$). During work the cardiac output increased to 7.4 l/min. (range 5.9—9.7) in the supine position and to 6.6 l/min. (range 4.7—9.0) in the sitting position. The postural difference during exercise between the cardiac outputs averaged 0.8 l/min. (11 %, $p<0.001$) (Fig. 1).

There was no significant difference between the slope of the two regression lines for cardiac output on oxygen uptake (Fig. 3). In both body positions, however, the slope was significantly less steep and the level lower than compared to normal

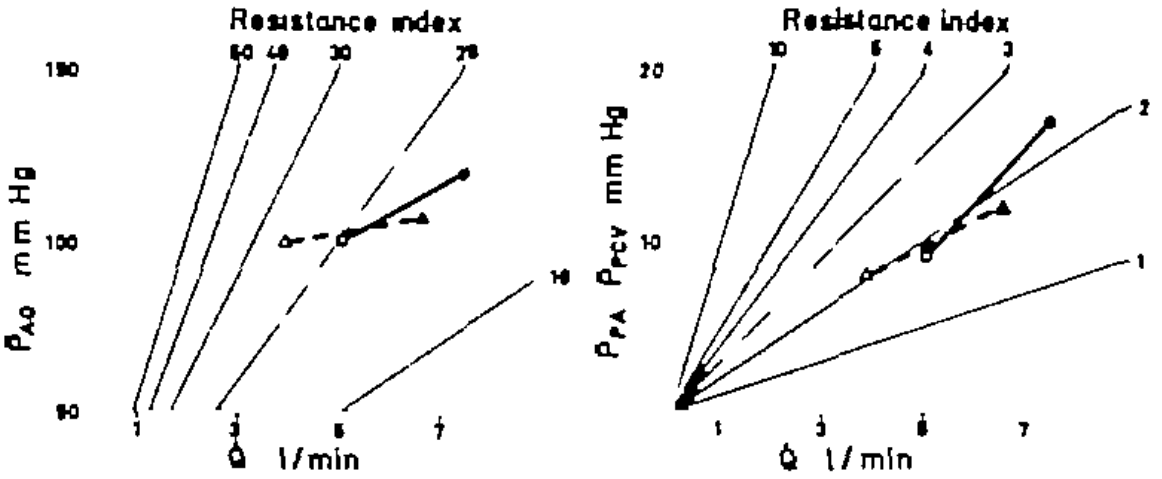


Fig. 4. Aortic mean pressure (P_{Ao}) in relation to cardiac output (Q). The mean pressure difference across the pulmonary vascular bed (P_A—P_{PCV}) in relation to cardiac output ($\dot{Q}$). The oblique lines are isoresistance lines. Symbols as in fig. 2.

young and old individuals (Bevegård et al. 1960, Strandell 1964). The regression line for cardiac output on oxygen uptake in this material was $Q = 3.70 + 0.0041\ \dot{V}_{O_2}$ in the supine position and $\dot{Q} = 2.50 + 0.0045\ \dot{V}_{O_2}$ in the sitting position ($r = 0.94^{***}$ and 0.96^{***} respectively).

Stroke volume (SV) at rest in the supine position was 67 ml (range 52—92) and decreased, not significantly to 63 ml (range 49—86) during exercise. When the position was changed at rest to sitting the stroke volume decreased to 44 ml (range 32—63) and this fall of 34 % from the supine resting value was highly significant. On exercise in the sitting position the stroke volume increased to 51 ml (range 41—66). The difference in stroke volume between supine and sitting positions was still significant and averaged 19 % less in the sitting position (Fig. 1).

Aortic mean pressure ($\bar{P}_{Ao}$) was within the normal range at rest and during exercise in the supine position. Pressures averaged 101 mm Hg and 121 mm Hg respectively; changing to the sitting position did not alter the mean pressure at rest. On exercise in the sitting position the aortic pressure did not increase to the same extent as during exercise when supine. The postural difference of 14 mm Hg was probably significant ($p < 0.05$).

Peripheral vascular resistance (SVR) at rest, increased significantly on transition from the supine to the sitting position. During exercise there was no positional difference. SVR responded quite differently on changing from rest to exercise in the two body positions. In the supine position SVR was the same at rest as during work but, when sitting, the resistance fell significantly during exercise ($p < 0.001$) (Fig. 4).

Pulmonary capillary venous pressure ($\bar{P}_{PCV}$) was elevated in most of the patients at rest in the supine position to a mean value of 20 mm Hg (range 13—30). This

TABLE VII Effect of body position on some respiratory and hemodynamic variables during exercise in 9 patients with mitral stenosis analysed as differences between data (sitting minus supine)

See list of symbols and abbreviations

	$\bar{X}_{sup}$	$\bar{d}$	SD_d	C	n	p
HR, cpm	120	9	17 0	9 7	11	—
Resp rate cpm	24	−2	5 7	17.6	11	—
V_E, l/min	6 6	−0.2	5 5	8.9	11	—
V ml	1115	116	220	13 3	11	—
V_{O_2}, ml/min	905	7	62	4.8	11	—
V_E/V_{O_2}	28 4	0.6	4.2	10.3	11	—
R	0.89	0.01	0.07	5 5	11	—
V_D, ml	251	13	100.9	27 7	11	—
V_D/V_T	0 23	−0.1	0.04	15 0	11	—
P_{aCO_2} mm Hg	38.5	−1 2	2.6	4 8	11	—
P_{aO_2} mm Hg	80 3	9 3	7 6	6.3	11	
P_{AO_2} mm Hg	106 4	1 9	3.8	2.5	11	—
$P_{(A-a)O_2}$ mm Hg	26.1	−7 1	9 4	29 4	11	
pH, units	7 38	0	0.02	0.2	8	—
St b mEq/l	20.6	0.8	0 8	2.9	7	—
S_{aO_2} %	96.1	1 9	3.3	2.4	11	—
S_{vO_2} %	33.3	−4.2	7.0	15.9	11	—
AVD ml/l	121.8	16.0	11.2	6.1	11	
Q l/min.	7 4	−0.8	0.6	6.3	11	
SV ml	63	−12.0	10.2	12.5	11	
P_{RA}, mm Hg	11.0	−8 7	4.8	50.6	7	
P_P mm Hg	56.5	−20.4	7 5	11 5	11	
P_{PCV} mm Hg	39 5	−15 1	7 4	16.4	11	
$\bar{P}_{Am}$ mm Hg	121.0	−14.0	18.8	11.6	11	
PVR, units	2.4	−0.4	1.0	51.2	11	—
SVR, units	18.2	−1.0	3.6	14.5	11	—

1.2 l/min (24) is highly significant ($p<0.001$) During work the cardiac output increased to 7 4 l/min. (range 5 9—9 7) in the supine position and to 6.6 l/min. (range 4 7—9 0) in the sitting position. The postural difference during exercise between the cardiac outputs averaged 0.8 l/min. (11 % $p<0.001$) (Fig. 1)

There was no significant difference between the slope of the two regression lines for cardiac output on oxygen uptake (Fig. 3) In both body positions, however the slope was significantly *less steep and the level lower than compared to normal*

exercise in the supine position and was 20 mm Hg lower during work with the same intensity in the sitting position (Fig. 5)

Pulmonary vascular resistance (PVR) was almost normal in all cases at rest and averaged 1.8 units (0.7—3.1). It did not change significantly during exercise. The PVR was not different in the upright compared to the supine position either at rest or during exercise (Fig. 4)

The pressure in the right atrium increased from 5.7 mm Hg (range 4—8) at rest to 11.0 mm Hg (range 8—15) during exercise in the supine position. In the sitting position the pressure also increased during exercise but to a less degree, from 0.5 mm Hg (range —2—6) at rest to 2.3 mm Hg (range —2—9) during exercise.

Effect of previous exercise on the central circulation at rest

All patients were studied twice at rest in the recumbent position, before and after the first exercise study. There were no significant differences in the hemodynamic variables between the two determinations at rest. The oxygen capacity was slightly smaller (2.3 ml/l SD±2.8, $p<0.05$) during the second resting period. Strandell (1964) also found this when studying old normal subjects. He also found a decrease in stroke volume and slightly lower filling pressures for both ventricles during the second period. The effect of previous exercise on the circulation during repeated exercise has been reported by Widimsky et al. (1963) and they found a small decrease in the pulmonary artery pressure during the second period. Burkart et al. (1967) found a significant difference between repeated exercise determinations in the same body posture. In order to diminish this effect when comparing the behaviour of the patients in different positions, four of the patients were studied first in the sitting and then in the recumbent position, in the other five the order was reversed.

Respiratory data obtained during the circulatory study

Mean values during exercise are given in Table VII

Total ventilation ($\dot{V}$) increased at rest on changing from the supine to the upright position by 1.0 l/min. ($p<0.01$) and this was due to a higher metabolic rate when sitting as the ventilatory equivalent for oxygen did not change. Also during exercise the ventilation increased linearly with increasing oxygen uptake, and there was no difference between the two body positions. The mean values in the supine position were 27.8 and 28.4 l/l oxygen uptake at rest and during exercise respectively and in the sitting position 28.8 and 29.0 l/l respectively. The regression line ($\dot{V}_E = 0.031\ \dot{V}_{O_2} - 0.87$) was slightly steeper than in normal subjects (Fig. 25 in General discussion). The higher ventilation at rest in the sitting position was achieved with a higher respiratory rate but no change of the tidal volume. During exercise the ventilation increased both by a higher respiratory rate and a

TABLE VIII Mean $\bar{d}$ and difference between means, data obtained in 9 patients with mitral stenosis during exercise at heart catheterization (cath.) and at determination of pulmonary diffusing capacity (D_L) some days later. See list of symbol and abbreviations

	SUPINE					SITTING				
	Cath	D_L	$\bar{d}$	C	p	Cath.	D_L	$\bar{d}$	C	p
HR, cpm	120	128	−8.0	9 4	—	130	140	−10	10.8	—
Resp rate, cpm	24	24	0	11 4	—	22	21	1	7 5	—
V_E, l/min.	26 6	28.2	−1.6	7.2		26.4	28 4	−2.0	9.2	—
V_T ml	1113	1160	−47	10.0	—	1229	1370	−141	10.8	
V_{O_2} ml/min	905	887	18	6.0	—	912	936	−24	8.9	—
V_E/V_{O_2}	28.4	29 8	−1 4	11.2	–	29.0	31 3	−2.3	4 9	
R	0 89	0 99	−0 10	4.9		0.91	0.95	−0 04	5.0	—
V l/min	17 7	20.1	−2 4	10.2		18 5	20 9	−2 4	13.0	—
V_D, ml	251	266	−15	25 5	—	264	272	−8	28 8	—
V_D/V_T	0.23	0 23	0	16.7	—	0.24	0.21	0.03	26 8	—
P_{CO_2} mm Hg	38 5	37 5	1.0	5.0	—	37.2	37.0	0.2	8.4	—
$P_{a_{O_2}}$ mm Hg	80 5	81 6	−1.3	5.0	—	89 3	88.7	0.6	7.2	—
$P_{A_{O_2}}$ mm Hg	106.4	109 4	−3 0	1.9		108.3	108.5	−0.2	3 6	—
$P_{(A-a)_{O_2}}$ mm Hg	26.1	27 7	−1.6	16.4	—	19.0	19.8	−0 8	30.8	—
pH, units	7 38	7 40	−0 02	0.27	—	7 38	7 40	−0.02	0.31	—
St.b mEq/l	20.6	20.6	0	3 4	—	19.8	21.2	−1 4	3 1	
Mech. eff., %	19.6	19.6	0	8 8	—	19 5	18.4	1.1	13 3	—
Hb, g/100 ml	14.1	13 1	1.0	3 5	*	14.6	13.6	1.0	4.7	

difference lower in the sitting position than in supine, as was found during catheterization.

Pulmonary diffusing capacity (D_{LCO}) was measured during exercise at the same level of $\dot{V}_{O_2}$ in both body positions. In the supine position D_{LCO} averaged 20.1 ml min.$^{-1}$ mm Hg^{-1} (range 15.2—25.1) and in the sitting position the mean value for D_{LCO} was 22.0 ml min.$^{-1}$ mm Hg^{-1} (range 12.9—28.6). The difference of 1.9 ml min.$^{-1}$ mm Hg^{-1} between the two body positions was not significant (Fig. 6). In both body positions, however, the D_{LCO} was significantly lower than the predicted normal value for the patient. The mean observed D_{LCO} of the group was 24 % lower than the predicted value (Holmgren 1965). Only 2 patients, however, fell below two standard deviations of the normal regression line. In two patients D_{LCO} was determined on two work loads in both body positions (nos. 1 and 3). The difference of D_{LCO} between the two loads ranged between 1.4 to 2.8 ml min.$^{-1}$ mm Hg^{-1} which is within the reproducibility of the method (Holmgren 1965).

COMMENTS

The aim was to determine and analyse postural effects on circulation, ventilation and gas exchange in patients with increased pressures in all parts of the pulmonary vascular bed and low pulmonary blood flow. Patients with mitral stenosis without significant increase in the resistance of the pulmonary vascular bed were selected for the study. The material, although small, corresponds rather well with that in the pre- and postoperative study by Granath (1965) concerning the severity of stenosis, without highly increased pulmonary vascular resistance or signs of right heart failure.

Patients with right heart failure tolerate the upright position well and usually do not show any drop in cardiac output and stroke volume on postural change at rest (Taquini et al. 1961, Rapaport et al. 1966 and Lorentsen et al. 1967). This increased tolerance to the effect of gravitation contrasts to the reaction in normals. The difference probably depends on several factors secondary to the abnormal circulation. Increased plasma volume in combination with increased venous tone (Follow and Mellander 1964, Wood 1965) both counteract the blood shift, and thus *the filling energy for the ventricles is maintained. Peripheral oedema seen* in patients with heart failure increases tissue pressure around the vessels and this mechanism may also decrease the venous distensibility.

In patients with heart disease without failure the reaction to gravitational stress is reported to be similar to that found in normal subjects. This has, however, only been studied at rest and usually on tilting from recumbent to 45° feet down. Cardus et al. (1958) found in patients with mitral valve disease, on tilting to an angle of 40° from the horizontal, a fall of the pressures in the pulmonary vessels, but small changes in cardiac output. Most of their cases had moderate to markedly *increase in pulmonary vascular resistance (>4 units) and thus they were at a*

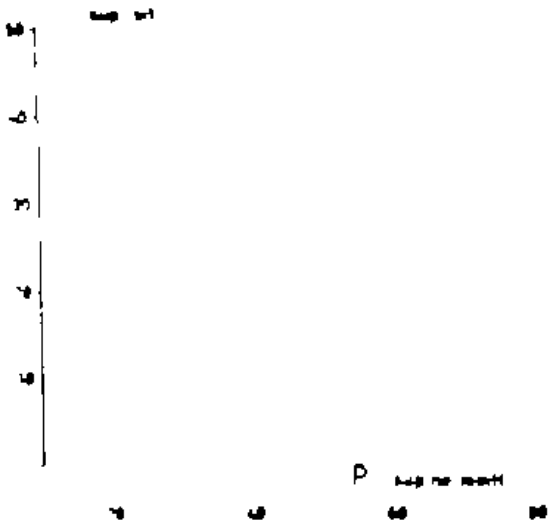


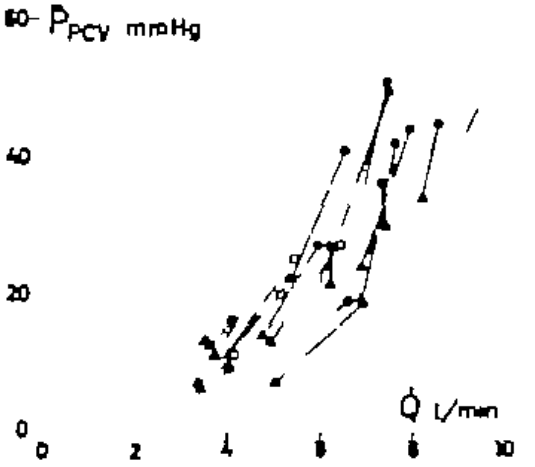
Fig. 7 Relative postural diff rence in stroke olume (ΔSV) calculated as difference be tween stroke volume in the supine and stroke volume in the sitting position in per cent f stroke olume in the supine position, in relation to the supine pulmonary artery mean pressure (P_{PA}) at rest (open circle) and during exercise (filled circle) in pa tients with mitral stenosis (r=0.85 *)

more advanced stage of the disease than the patients in this study Two patients with normal hemodynamics showed a considerable drop in pulmonary artery and PCV pressure as well as in cardiac output. Eliasch et al (1961) who compared recumbent and feet down tilted (45 —60°) patients with mitral disease found a small decrease in pulmonary vascular pressures but a more marked fall in stroke volume except in 2 patients with signs of right heart failure who did not change the pressure and flow on tilting.

The postural difference in blood flow was also demonstrated in this study On transition from supine to sitting position cardiac output fell 1 2 l/min., although the oxygen uptake was higher in the sitting position. The oxygen transport was thus maintained by an increased oxygen extraction In the sitting position the arteriovenous oxygen difference was on an average 73 ml/l corresponding to an oxygen saturation in the pulmonary artery of 59 % The stroke volume fell 34 % on an average, a value which agrees with the decrease found in normal individuals (Bevegård et al 1960) To compensate for this fall in stroke volume in an attempt to maintain cardiac output, the heart rate increases, which has special significance in patients with mitral valve obstruction Shortening of the diastolic filling time is probably one reason for the decreasing stroke volume during supine exercise seen in patients with tight mitral stenosis. If the increased heart rate alone in sitting were the main factor causing an impaired diastolic filling and the fall in stroke volume one would not expect the left atrial mean pressure (measured as PCV pressure) to drop markedly In the present study it decreased at rest from a mean of 20 mm Hg in the supine to a mean of 9 mm Hg in the sitting position.

On transition from rest to work in the sitting position the stroke volume in reased an average of 16 but was, however still significantly lower than that during exercise in the supine position. The postural difference in stroke volume during xercise varied widely between the patients. There was a correlation between the relative decrease in stroke volume on changing posture, and the left atrial and the pulmonary artery pressure levels in the supine position (Fig 7) The higher

Fig. 8. Relationship between PCV pressure ($\bar{P}_{PCV}$) and cardiac output (Q). Symbols are as in fig. 2. Lines connect values i the supine and sitting positions of indi idual patients.



the pulmonary artery and PCV pressure, the smaller was the difference in stroke volume between the sitting and supine positions. This could partly be explained on hydrodynamic grounds. The mitral valve obstruction forms a resistance to flow where the driving pressure is related to the second power of flow (Gorlin and Gorlin 1951). If it is assumed that the intra-thoracic pressure is approximately unchanged by body position, the PCV pressure may be used as an indicator of the driving pressure for the flow through the mitral valve. From the second power pressure-flow relationship it is obvious that tight and moderately tight obstructions result in large changes in driving pressure for a given change in flow (Fig. 8, 9).

When intravascular pressures are measured, and especially when comparing observations in different body positions, the zero reference level is critical, as long as absolute values are discussed, but not so when pressure differences are compared. The distending or transmural pressure also depends on the surrounding

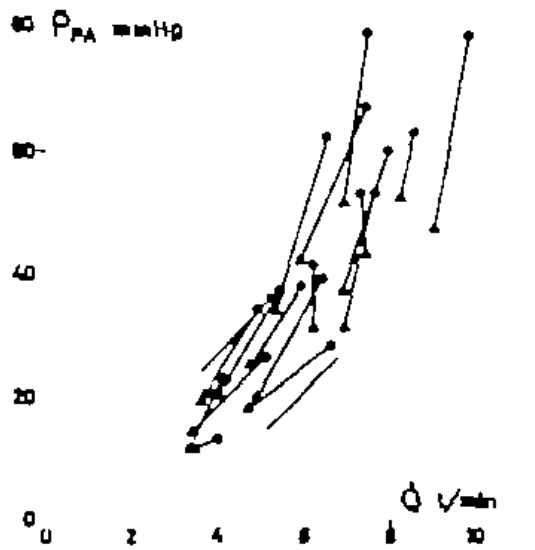


Fig. 9. Pulmonary artery mean pressure ($\bar{P}_{PA}$) in relation to cardiac output ($\dot{Q}$). Symbols as in fig. 2.

tissue pressure. In the normal lungs this latter pressure is negative when referred to atmospheric pressure and might also be changed when changing body position Such a change might introduce a bias in the pressure recordings in the sitting position but will probably only amount to a few millimeters of Hg (Cardus et al. 1958 Mead and Gaensler 1959) As such a systematic error would be relatively less at higher pressure levels it is improbable that this would explain the lower pressure when sitting observed here. Fig 5 (bottom, left) shows on the contrary that the difference between the pulmonary arterial pressure in the supine and sitting positions increases the greater the arterial pressure in the former position.

If there are no marked vessel changes in the pulmonary vascular bed the lung blood volume is to some extent correlated to the pulmonary venous pressure (Sar noff and Berglund 1952, Lindsey and Guyton 1959 Yu et al. 1967 Wilhelmsen 1968) and although the central blood volume was not measured, it could thus be presumed to be increased in the stage of the disease that all of the patients belonged to The importance of the lung blood volume and the displacement of blood from the lungs to the periphery for the filling of the left ventricle has been shown in normal subjects (Sjöstrand 1953) A change in lung blood volume also affects the vascular pressure. But if all lung vessels are perfused in the sitting position, then increasing vascular pressure will make the vessels less and less distensible and give small volume changes for given pressure changes (Borst et al. 1956) This partly explains the observation that the patients with high pulmonary artery and PCV pressures had great falls in pressure and showed less decrease in stroke volume, and thus had relatively smaller lung blood volume displacement and could keep up enough potential filling energy for the left ventricle.

Abelmann et al. (1969) who studied the tolerance to orthostatic stress at rest in normals and patients with cardio-vascular disease, found that elevation of ventri cular filling pressure was the prime determinant to increased tolerance to upright position, as they called the "heart failure response They also found that the toler ance increased with age. In this study the relative decrease in stroke volume was negatively correlated with the right atrial pressure in recumbent position (Fig. 10) Decreased distensibility of the capacitance vessels with increasing filling pressure of the right ventricle would explain this resistance to hydrostatic blood shifts.

In normal subjects and in patients with mitral stenosis with almost normal hemo-dynamics the pulmonary vascular resistance increases on tilting body position at rest (Lagerlöf et al. 1951) The patients in this study all had normal or almost normal pulmonary vascular resistance and thus had probably not developed marked structural changes in the pulmonary vessels. In the sitting position when the pul monary arteri mean pressure is above the hydrostatic pressure from top of the lung to the reference level (about 15 mm Hg) all vessels are supposed to be perfused and then pressure and flow are nearly linearly related to each other and changes in pulmonary vascular resistance are small (West 1965) This would fit well with the findings in this study where the pulmonary vascular resistance was unaltered by

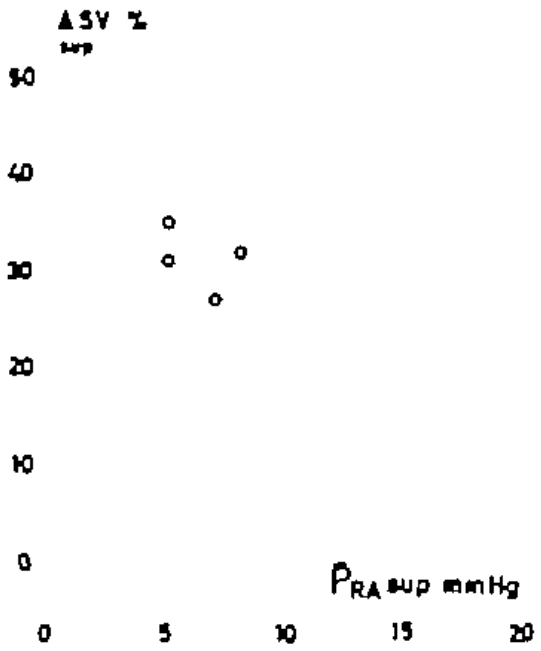


Fig. 10 Relative postural difference in stroke volume (ΔSV) in relation to supine right atrial mean pressure ($\bar{P}_{RA}$) Symbols as in fig. 1 (r=0.68)

changes in body position and the mean pressure in the pulmonary artery at rest in sitting averaged 17 mm Hg.

Topographical inequalities in the relationship between perfusion and ventilation in sitting normal individuals alter the physiological dead space (Riley et al. 1959) as a result of an increase in alveolar dead space in the sitting position because of underperfusion of pulmonary capillaries in the upper zones of the lung. Further support for this was given by observations from Mattson and Carlens (1955) and Malmberg (1966) Larson and Severinghaus (1962) however, could not confirm this The patients with mitral stenosis did not change the dead space when the position was altered either at rest or during work. This would further support the idea that the whole lung was evenly perfused also in the sitting position (Dollery and West 1960 Daly et al. 1964)

The alveolar arterial oxygen tension difference was mainly within the normal range when the age was taken into account (Vale 1967 Scherrer and Büchler 1967) and was not altered significantly by change of posture at rest. During exercise it increased significantly only in the supine position. In normal individuals $P_{(A-a)O_2}$ does not change significantly from rest to low intensity work (Hesser and Matell 1965 Vale 1967) some increase is seen at high oxygen uptakes (Holmgren and Linderholm 1958) but it increases greatly with the magnitude of the G-vector in the long axis of the body (Barr 1963) An increase in $P_{(A-a)O_2}$ could be due to three different mechanisms, either separately or in combination. These are the true right to-left shunts, the shunt component due to an unequal distribution of blood flow and ventilation within the lung and thirdly the diffusion components in the lungs. The two first components constitute about half each of the $P_{(A-a)O_2}$ in normal subjects (Mellemgaard 1966) It is not possible to separate these two factors in this study because the patients were breathing room air only Driscoll et al (1963)

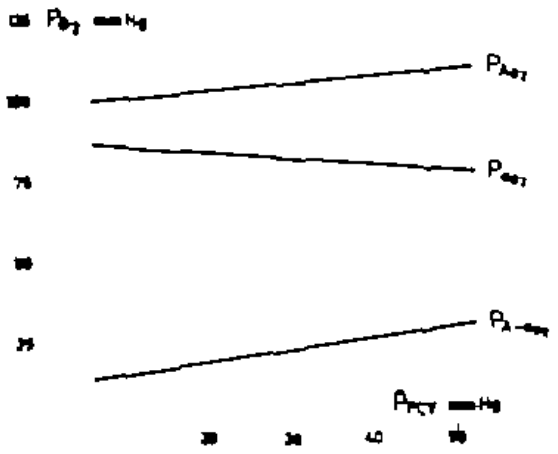


Fig. 11 Alveolar oxygen tension ($P_{A_{O_2}}$) arterial oxygen tension ($P_{a_{O_2}}$) and the alveolar-arterial oxygen tension difference ($P_{A-a_{O_2}}$) in relation to the PCV pressure (P_{PCV}) $P_{A_{O_2}}$ $r=0.46$ $P_{a_{O_2}}$ $r=0.24$, $P_{A-a_{O_2}}$ $r=0.52$ * $n=46$)

noted that the arterial oxygen desaturation at rest in mitral stenosis improved more significantly with mild exercise than with the breathing of 100 per cent oxygen *This was best explained by an improvement in ventilation perfusion balance in the* lung during mild exercise.

The mean value for the arterial oxygen pressure was the same at rest and during exercise in the supine position in this material but was greater in the sitting position. No significant correlation was found between the arterial oxygen tension and the PCV pressure in both body positions at rest and during work. The $P_{(A-a)_{O_2}}$ was, however highly correlated with the PCV pressure ($r=0.52$ $p<0.001$) (Fig. 11) This would probably best be explained by regional transudation of fluid, creating disturbances in the ventilation-perfusion balance. Guyton and Lindsey (1959) found in dogs that the ratio of wet to dry weight of the lung was propor *tional to the increase of left atrial pressure above 24 mm Hg.*

The D_{LCO} during exercise was somewhat low but mainly within the normal variation. As for normal lungs this would only give a negligible gradient for oxygen both at rest and during work (Linderholm 1959 Turino et al. 1959 Driscoll et al. 1963) Increased pulmonary capillary blood volume tends to counteract the effect of reduction in the membrane component of the D_{LCO}, so that D_{LCO} is often normal in patients with mitral valve disease (Hamer 1965) There was no positional difference in D_{LCO}, suggesting that the pulmonary capillary blood volume was unaffected by position in these patients.

VIII

Postural effects on the central circulation and respiration at rest and during exercise in patients with PULMONARY STENOSIS

This patient group was selected to represent low blood flow and low pressure levels in the pulmonary circulation. Observations were made in five subjects of whom four fulfilled these criteria and had a tight valvular stenosis with a valve area calculated according to Gorlin and Gorlin (1951) of 0.35 cm^2 (range 0.20—0.50). In one patient (no. 3) the degree of stenosis was moderate (area 1.20 cm^2).

RESULTS

Hemodynamic data

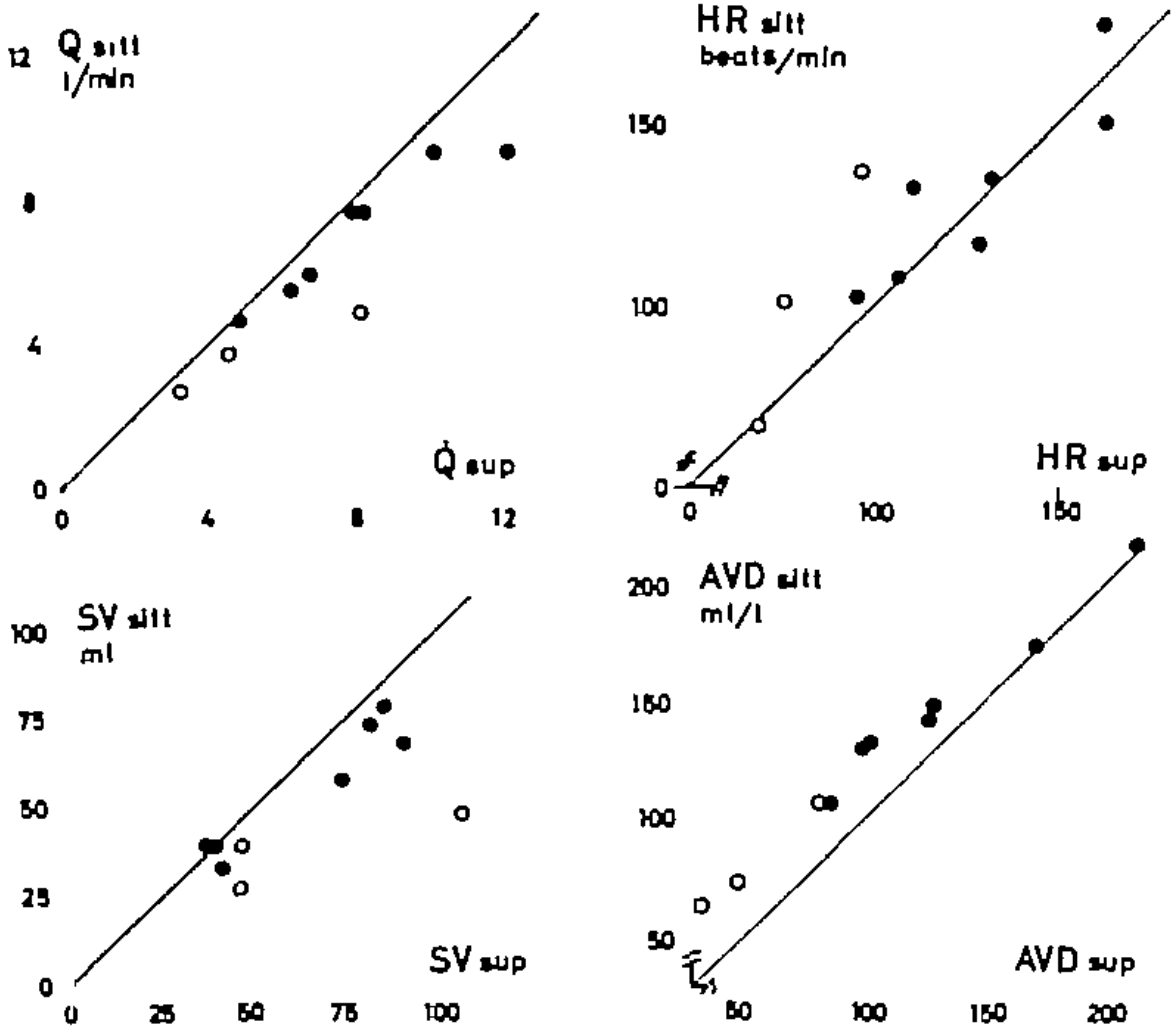
Oxygen uptake (V_{O_2}) averaged 253 ml/min. in the supine position at rest (range 218—301) and in the sitting position it was on the average 40 ml/min. higher (16 %) in the three patients studied also at rest in the sitting position. During exercise the oxygen uptake averaged 3.8 times the resting value. As a mean there was no positional difference in oxygen uptake during exercise. During supine exercise the V_{O_2} averaged 952 ml/min. (range 814—1385).

Heart rate (HR) at rest in the supine position averaged 78 beats/min. (range 69—97). At rest in the sitting position HR was in two patients (no. 1 and 3) 40 beats/min. and 25 beats/min. respectively higher than in the supine position in one patient (no. 5) there was no change.

During exercise there were no positional differences on the same load and the heart rate in the supine position averaged 130 beats/min. (range 96—162) and in sitting 133 beats/min. (range 102—177) (Fig. 12). The heart rate during the catheterization was as on average 85 per cent of the highest heart rate reached during the exercise test performed some days earlier.

Arterial oxygen saturation ($S_{a_{O_2}}$) was normal in all patients and averaged 96.8 % (range 95—99) and 98.0 % (range 97—99) respectively at rest in supine and sitting. In both body positions it increased somewhat during exercise.

Mixed venous oxygen saturation ($S\bar{v}_{O_2}$) at rest averaged 71.4 % (range 63—77) and 59.3 % (range 51—65) in the supine and sitting position respectively. During exercise it decreased markedly in both positions. In the supine position it averaged 38.6 % (range 11—56) and in the sitting position 33.7 % (range 13—43). This difference was significant ($p<0.02$). In one patient (no. 5) $S_{v_{O_2}}$ was almost equal in both positions. At the highest work load it was 11 % and 13 % in the supine and sitting positions respectively.

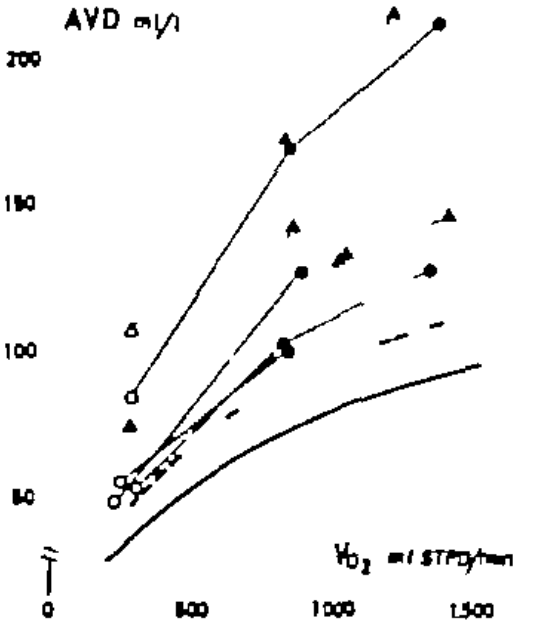


F g. 12. Cardiac output ($\dot{Q}$) heart rate (HR) stroke volume (SV) and arterio-venous oxygen difference (AVD) in the supine and sitting positions. Five patients with pulmonary stenosis studied at rest (open circle) and during exercise (filled circle)

The oxygen capacity of the blood averaged at supine rest for the three women 185 ml/l (range 177—197) and was 232 ml/l for the two men. It increased during exercise in both body positions and was as an average 8 % higher during sitting exercise than at supine rest.

Arterio-venous oxygen difference (AVD) at rest, in the supine position, averaged 54 6 ml/l blood One patient (no 3) had a normokinetic circulation with an AVD of 33 ml/l. This patient was considered to have a rather mild pulmonary stenosis with a systolic pressure in the right ventricle at rest of 72 mm Hg and a peak systolic pressure difference over the pulmonary valve of 42 mm Hg. The other four patients with tight stenosis had an increased AVD at rest compared to normals and thus displayed a hypokinetic circulation (range 48—84) (Fig. 13) The three patients studied at rest sitting all had a greater AVD compared to that when supine The AVD when sitting averaged 82.4 ml/l (range 65—108) During exercise in the supine position the AVD averaged 132.9 ml/l (range 86—

Fig. 13. Arterio-venous oxygen difference (AVD) in relation to oxygen uptake (V_{O_2}) in 4 patients with tight pulmonary stenosis. Thin full and broken lines represent the supine and sitting positions respectively. Thick lines represent corresponding regressions in normal subjects.



214). The individual maximal value for the patients with tight stenosis ranged 100—214 ml/l. In the sitting exercise the AVD was significantly ($p<0.001$) higher than in supine position. The mean value was 150 6 ml/l (range 109—217).

Cardiac output ($\dot{Q}$) at rest averaged 5 3 l/min. and 3.8 l/min. respectively in the two body positions. The patient with a normokinetic circulation (no. 3) demonstrated the most marked fall, 3 3 l/min. or 40 % of the cardiac output in the supine position. In the other two patients cardiac output fell 0 8 and 0 6 l/min. (17—18 % respectively when changing position from supine to sitting (Fig. 12). During supine exercise the cardiac output increased to 8.1 l/min. (range 4.9—12.2) and during sitting to 7.2 l/min. (range 4.7—9 3). The postural difference in cardiac output for the patients with tight pulmonary stenosis averaged 0 6 l/min. The patient with mild stenosis (no 3) had a normal cardiac output in relation to the oxygen uptake in both body positions (Fig. 14). The patients with tight stenosis

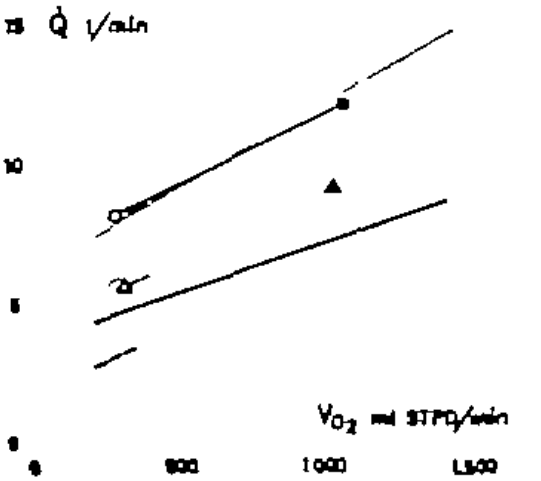


Fig. 14. Cardiac output ($\dot{Q}$) in relation to oxygen uptake (V_{O_2}). Thick full and broken lines represent 4 patients with tight pulmonary stenosis in the supine and sitting positions respectively. Patient no. 3 is indicated separately with symbols as in fig. 2. Thin lines represent normal subjects (Bevegård et al. 1960).

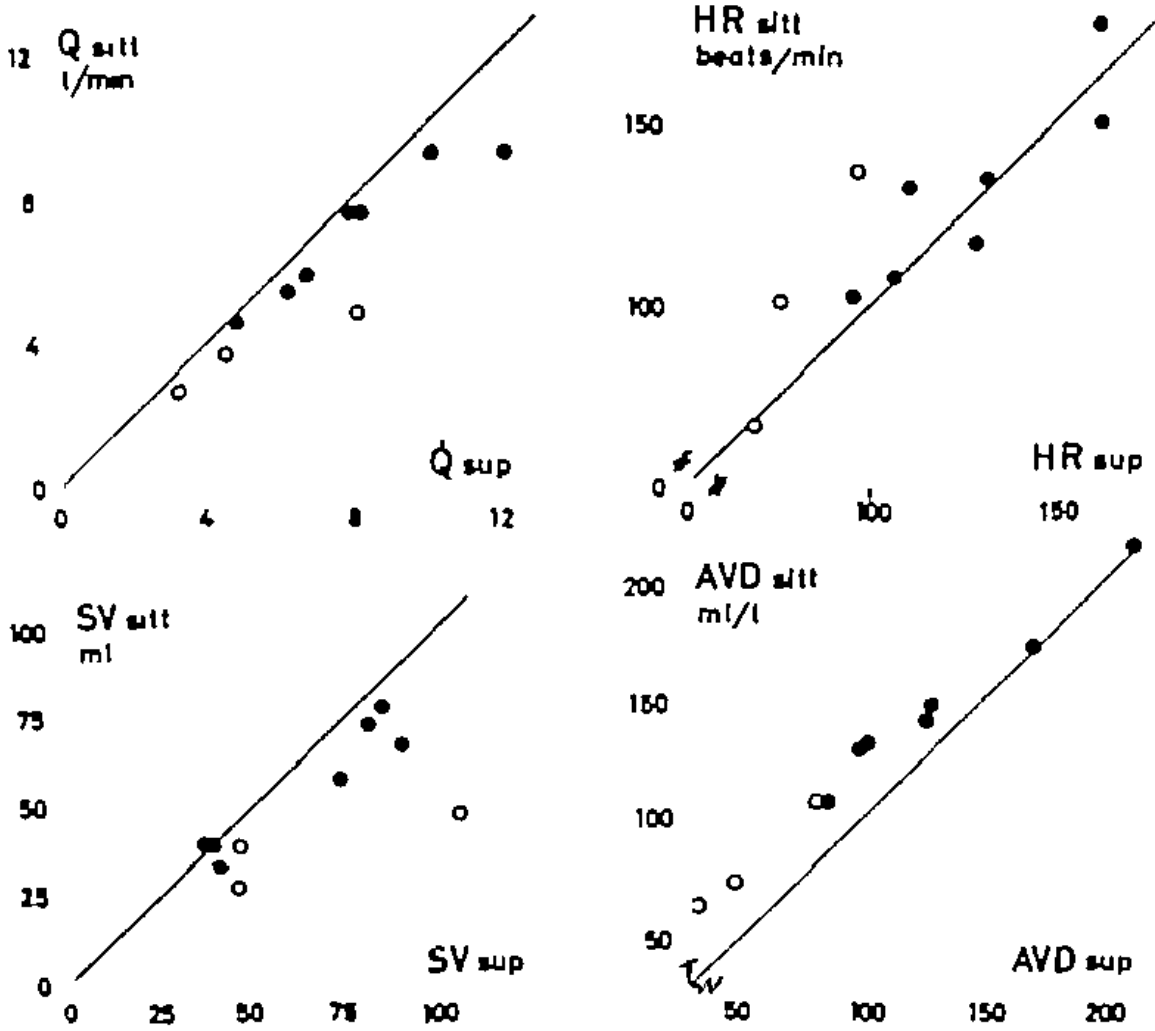


Fig. 12 Cardiac output (Q) heart rate (HR) stroke volume (SV) and arterio-venous oxygen difference (AVD) in the supine and sitting positions. Five patients with pulmonary stenosis studied at rest (open circle) and during exercise (filled circle)

The oxygen capacity of the blood averaged at supine rest for the three women 185 ml/l (range 177—197) and was 232 ml/l for the two men. It increased during exercise in both body positions and was as an average 8 % higher during sitting exercise than at supine rest.

A terio-venous oxygen difference (AVD) at rest, in the supine position, averaged 54.6 ml/l blood. One patient (no. 3) had a normokinetic circulation with an AVD of 33 ml/l This patient was considered to have a rather mild pulmonary stenosis with a systolic pressure in the right ventricle at rest of 72 mm Hg and a peak systolic pressure difference over the pulmonary valve of 42 mm Hg. The other four patients with tight stenosis had an increased AVD at rest compared to normals and thus displayed a hypokinetic circulation (range 48—84) (Fig. 13) The three patients studied at rest sitting all had a greater AVD compared to that when supine. The AVD when sitting averaged 82.4 ml/l (range 65—108) During exercise in the supine position the AVD averaged 132.9 ml/l (range 86—

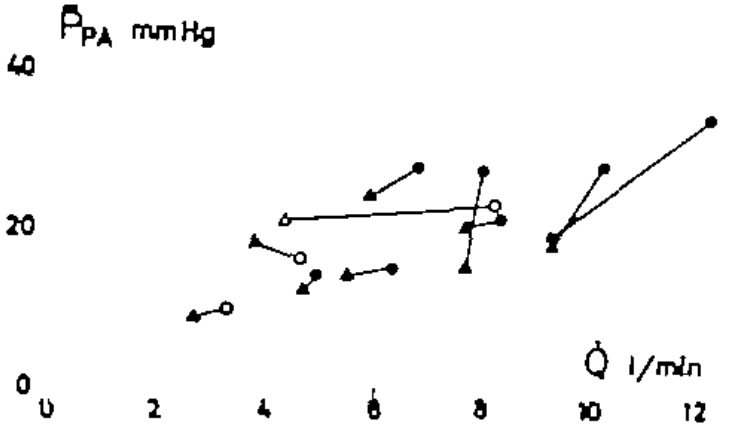


Fig. 13 Pulmonary artery mean pressure (P_{PA}) in relation to cardiac output ($\dot{Q}$) in 5 patients with pulmonary stenosis. Symbols as in fig. 2

on positional change (4—15 mm Hg) The other two patients with a lower pulmonary artery mean pressure had almost identical pressures in the sitting and supine positions (Fig. 13)

Pulmonary vascular resistance (PVR) was normal in all patients and did not change either from rest to exercise or by position. The mean value was 1.0 unit.

The end-diastolic pressure in the right ventricle averaged at rest 15 mm Hg (range 11—18) and during work 23 mm Hg (range 18—30) in the supine position. In the sitting position it could only be calculated in tree patients and averaged during work 12 mm Hg (range 7—20)

Respiratory data

The measured and calculated data are presented in Table IX as mean values for the four patients with tight pulmonary stenosis. The patient with a normokinetic circulation and moderately tight stenosis is presented separately

Total ventilation ($\dot{V}$) at rest was 0 5—3 3 l/min. higher in the sitting position than in the supine position (n=3) During work (250 kpm/min.) the total ventilation in the patients with tight stenosis increased to 28.8 l/min. in the supine and 29 6 l/min. in the sitting positions. The two patients also studied at 500 kpm/min. displayed a marked increase in ventilation and in patient no 5 it was 10 l/min. higher in the supine than in the sitting position. During exercise there was no significant difference in ventilatory equivalent between the two body positions. The total ventilation correlated linearly with oxygen uptake, but the regression line was steeper than in normal subjects (Fig. 25 in General discussion) The high $\dot{V}$ in patients with tight pulmonary stenosis was due to alveolar hyperventilation as is seen from $P_{a}CO_2$ during work. In the supine position at 250 kpm/min. $P_{a}CO_2$ was 34.5 mm Hg (range 32—37) and 34 3 mm Hg (range 32—36) in the sitting position in the patients with the tight stenosis. The patient with moderately tight stenosis had a normal $P_{a}CO_2$ and this was essentially the same on the transition from rest to work in both positions. At the highest work load the alveolar hyperventilation was further accentuated, particularly in patient no. 5 in whom $P_{a}CO_2$

TABLE IX. Mean value of some respiratory data in 4 patients with right pulmonary stenosis at rest and during work. One patient with moderately tight pulmonary stenosis (no. 5) is indicated separately. See list of symbols and abbreviations.

Position	Work load kpm/m			V_A ml/min.	V_E l/min	V_E/V_{O_2}	V_T l	Resp. rate cpm	V_D ml	V_D/V_T
	Rest	$\bar{X}$	4	260	7.9	30.0	0.46	18	130.8	0.31
		range		(218—301)	(5.9—10.1)	(24.7—34.5)	(0.37—0.68)	(14—24)	(78—275)	(0.21—0.41)
Sup.	250	$\bar{X}$	4	831	23.8	30.9	1.04	26	267.8	0.25
		range		(807—871)	(21.4—30.2)	(26.6—35.9)	(0.82—1.49)	(20—28)	(147—403)	(0.18—0.32)
	500	$\bar{X}$	2	1338	56.1	41.8	1.96	28	427.5	0.22
		range		(1323—1353)	(39.0—73.1)	(29.5—54.0)	(1.62—2.29)	(24—32)	(323—532)	(0.20—0.23)
	Rest	$\bar{X}$	2	239	9.6	35.9	0.57	17	240.0	0.43
		range		(191—286)	(9.2—10.0)	(33.0—34.8)	(0.51—0.62)	(16—18)	(213—265)	(0.42—0.43)
Sitt	250	$\bar{X}$	4	903	29.6	32.7	1.29	23	372.5	0.29
		range		(761—1023)	(26.0—34.4)	(25.9—40.9)	(1.23—1.49)	(20—28)	(183—528)	(0.15—0.40)
	500	$\bar{X}$	2	1285	47.5	37.0	1.95	24	494.5	0.26
		range		(1188—1383)	(41.4—53.1)	(30.0—43.9)	(1.88—2.01)	(22—26)	(375—614)	(0.20—0.31)
Case no. 3										
Sup.	Rest			267	7.3	27.3	0.41	18	136	0.33
	300			1045	28.0	26.8	1.17	24	319	0.27
Sitt	Rest			318	9.6	30.2	0.44	22	154	0.35
	300			1014	27.4	27.0	1.05	26	270	0.26

Position	Work load kpm/min			V_A l/min	$P_{A_{O_2}}$ mm Hg	$P_{a_{O_2}}$ mm Hg	$P_{(A-a)_{O_2}}$ mm Hg	$P_{a_{CO_2}}$ mm Hg	R	pH units	St.b., mEq/l
	Rest	$\bar{X}$	4	4.7	101.5	87.5	14.0	36.0	0.75	7.43	23.0
		range		(3.5—5.9)	(96—107)	(74—95)	(1—33)	(31—41)	(0.69—0.77)	(7.41—7.44)	(22.0—25.0)
Sup.	250	$\bar{X}$	4	17.9	108.5	97.8	10.8	34.3	0.86	7.39	20.0
		range		(15.4—20.7)	(105—113)	(91—104)	(8—14)	(32—37)	(0.72—0.94)	(7.38—7.40)	(19.0—20.0)
	500	$\bar{X}$	2	42.6	118.5	104.5	14.0	27.5	0.97	7.36	18.0
		range		(30.6—54.5)	(112—125)	(100—109)	(12—16)	(25—32)	(0.85—1.08)	(7.35—7.38)	(16.0—20.0)
	Rest	$\bar{X}$	2	4.7	98.5	95.0	3.5	36.0	0.70	7.45	23.0
		range		(4.4—4.9)	(97—100)	(93—97)	(0—7)	(35—37)	(0.69—0.71)	(7.43—7.47)	(22.0—23.0)
Slit.	250	$\bar{X}$	4	19.8	108.8	99.0	9.8	34.3	0.86	7.39	20.0
		range		(18.4—20.9)	(107—111)	(95—104)	(7—13)	(32—36)	(0.75—0.91)	(7.37—7.41)	(19.0—21.0)
	500	$\bar{X}$	2	33.5	115.0	102.5	12.5	30.5	0.94	7.37	18.0
		range		(32.1—34.9)	(112—118)	(98—107)	(11—14)	(29—32)	(0.87—1.01)	(7.35—7.38)	(17.0—19.0)
Case no. 5											
Sup.	Rest			4.0	93	69	26	40	0.68	7.41	23.0
	500			19.1	103	66	37	39	0.83	7.38	22.0
Slit.	Rest			5.1	99	80	19	36	0.67	7.38	22.0
	500			19.1	104	80	24	39	0.84	7.37	21.0

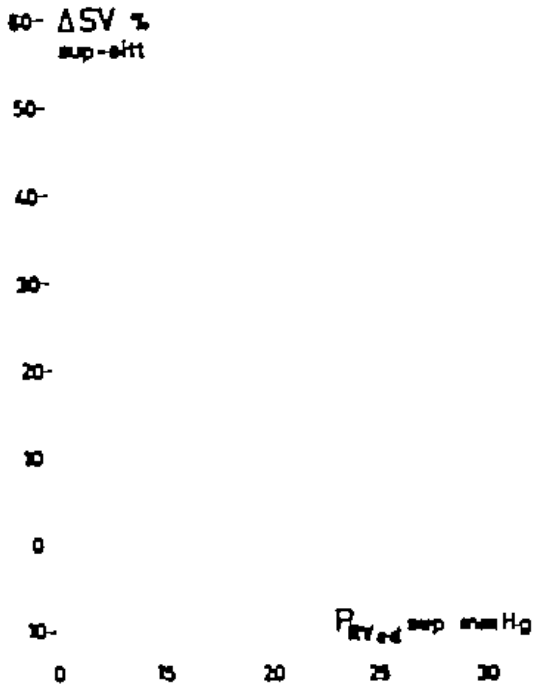


Fig. 16. Relative postural difference in stroke volume (ΔSV) in relation to the supine end-diastolic pressure of the right ventricle ($P_{RV_{ed}}$) ($r = -0.78$ *) Symbols as in fig. 1.

venous pressure in patients with heart failure. During mild exercise their venomotor tone is further augmented (Wood 1965) Although the patients with tight pulmonary stenosis were not in *failure*, the *filling* pressure for the *right* ventricle during supine exercise was within the same range as that found in patients with heart failure. This could to some extent explain the inverse correlation between right ventricular diastolic pressure and the orthostatic change in the stroke volume, as a decrease in the distensibility of the capacitance vessels would result in less blood volume displacement.

Hyperventilation during exercise in patients with pulmonary stenosis has been reported by others (Lewis et al. 1964, Gazetopoulos et al. 1966) Gazetopoulos et al. was unable to show any relationship between cardiac output and hyperventilation during supine exercise but suggested that mechanisms connected with tissue hypoxia contributed to the hyperventilation. In this study hyperventilation during exercise was also found in the patients with tight pulmonary stenosis, with no marked influence of body position. The patient (no 5) with the most hypokinetic circulation, independent of position, displayed a more pronounced hyperventilation in the supine than in the sitting position. When the maximum oxygen transport capacity is reached (mixed venous saturation in both body positions was around 12 %) the lower perfusion pressure in the exercising muscle in the supine position could be critical and support the idea of the mechanism for inducing hyperventilation to be connected with the increased anaerobic metabolism observed (St.b $= 16.0$ mEq/l)

In the four patients with a hypokinetic circulation the $P_{(A-a)O_2}$ was normal or small during exercise in both body positions, indicating small alterations in ventilation—perfusion ratios with changes in posture. Pulmonary diffusing capacity

measured during exercise, was normal and no significant difference was found after changing body position.

Hemoglobin concentration increases on changing from the supine to the upright position both at rest and during work (Beregård 1962, Strandell 1964, Ekelund 1967 Smeenk 1969) and this will increase the oxygen transport capacity per litre cardiac output. In two patients (no 4 and 5) the oxygen capacity of the blood was higher than in normal subjects (232 ml/l) at supine rest. This could be interpreted as an adaptation to increase oxygen transport capacity in a patient with a long-standing hypokinetic circulation.

IX

Postural effects on the central circulation in patients with ATRIAL SEPTAL DEFECT

Observations were made on seven patients, all of whom had an ASD of secundum type (1 6 7 18, 22, 23 24) These patients, belonging to the larger group described in Chapter V were studied separately with additional measurements of respiratory function There were 4 women and 3 men with an average age of 35 years (range 16—57) All patients were studied at rest in the supine position and then during exercise in the supine and sitting position.

RESULTS

Hemodynamic data

Mean values during exercise are given in Table X.

Heart rate (HR) at rest in the supine position was 74 beats/min. (range 66—82) It increased during exercise to 124 beats/min. (range 113—152) in the supine position and was significantly higher in the sitting position, 137 beats/min. (range 110—178) ($p<0.01$)

Oxygen uptake (V_{O_2}) at supine rest averaged 245 ml/min. and increased during work in the same position to 1105 ml/min (range 653—1600) It increased to 1219 ml (range 839—1940) at the same load in the sitting position. The difference in oxygen uptake was significant ($p<0.01$) This could partly explain the higher heart rate during work in the sitting position.

Arterial oxygen saturation ($S_{a_{O_2}}$) averaged 98.8 % (range 96 7—100) at rest and 97.8 % (range 96 0—100) during exercise in the supine position. During exercise in the sitting position the saturation decreased to an average of 96 6 % (range 94 4—97 9) This difference of 1 2 % was almost significant ($p<0.05$) The arterial oxygen tension, measured at the same time, was similar however in the two body positions. It was 89 mm Hg (range 79—97) in the supine and 86 mm Hg (range 74—97) in the sitting position

Oxygen saturation of pulmonary artery blood was on average 90.0 % (range 85 0—92 5) at rest and decreased during work in all patients to an average 74.6 % (range 61 5—82 8) During exercise in the sitting position it was significantly lower 69 2 (range 60 5—82 1) ($p<0.01$) Three patients (nos. 1 7 and 18) however had almost the same or higher saturation when sitting.

Arterio-venous oxygen difference (AVD_P) for the pulmonary circulation was 16 9 ml/l at rest and increased to 46 1 ml/l during exercise in the supine and to 55 9 ml/l in the sitting position Small variations in the determination of the high pulmonary artery oxygen saturation at rest will largely affect the accuracy of the

TABLE X. Hemodynamic data during exercise in the supine and sitting position 7 patients with atrial septal defect
See list of symbols and abbreviations

	$\bar{X}_{sup}$ (range)	$\bar{X}_{sit}$ (range)	$\bar{d}$	n	C	p
HR, cpm	144 (113—152)	137 (110—178)	−13	11	7.0	
V_{O_2} ml/min.	1105 (653—1600)	1219 (839—1940)	−114	11	6.6	
Q_P, l/min.	24.4 (16.3—33.0)	22.6 (16.8—35.8)	1.8	11	8.0	—
AVD_P, ml/l	46.1 (29.9—67.4)	55.9 (26.3—72.7)	−9.8	11	11.3	
SV_{RV} ml	198 (137—292)	169 (113—319)	29	11	13.7	
S_{aO_2}, %	97.8 (96.0—100)	96.6 (94.4—97.9)	1.2	11	1.1	
P_{RA}, mm Hg	9 (4—19)	1 (—5—5)	8	7	44.2	
$\bar{P}_{PA}$, mm Hg	34 (25—59)	27 (20—41)	7	10	18.3	
P_{PA_D} mm Hg	20 (15—27)	18 (14—27)	2	10	14.8	—
$\bar{P}_{PCW}$ mm Hg	17 (11—24)	—	—	10	—	
$\bar{P}_{Ao}$ mm Hg	110 (91—125)	103 (72—1 2)	7	9	8.6	—
PVR, units	0.7 (0.3— 2.1)	—	—	9	—	

determination of the pulmonary blood flow. No conclusion will therefore be drawn from postural differences at rest.

Pulmonary flow ($\dot{Q}_P$) at rest in the supine position was 15.6 l/min. (range 8.3—24.6). This was on average 3.2 times the aortic flow (range 1.7—5.0). During exercise in the supine position the pulmonary flow increased to 24.4 l/min. (range 16.3—33.0) and in the sitting position to 22.6 l/min. (range 16.8—35.8). This difference between values in the two body positions was not significant. The individual variation was, however, large. In three patients (nos. 1, 7 and 18) the pulmonary blood flow was higher in the sitting position (Fig. 17 a, bottom) and in the others it was lower in the sitting than in the supine position (Fig. 17 a, top).

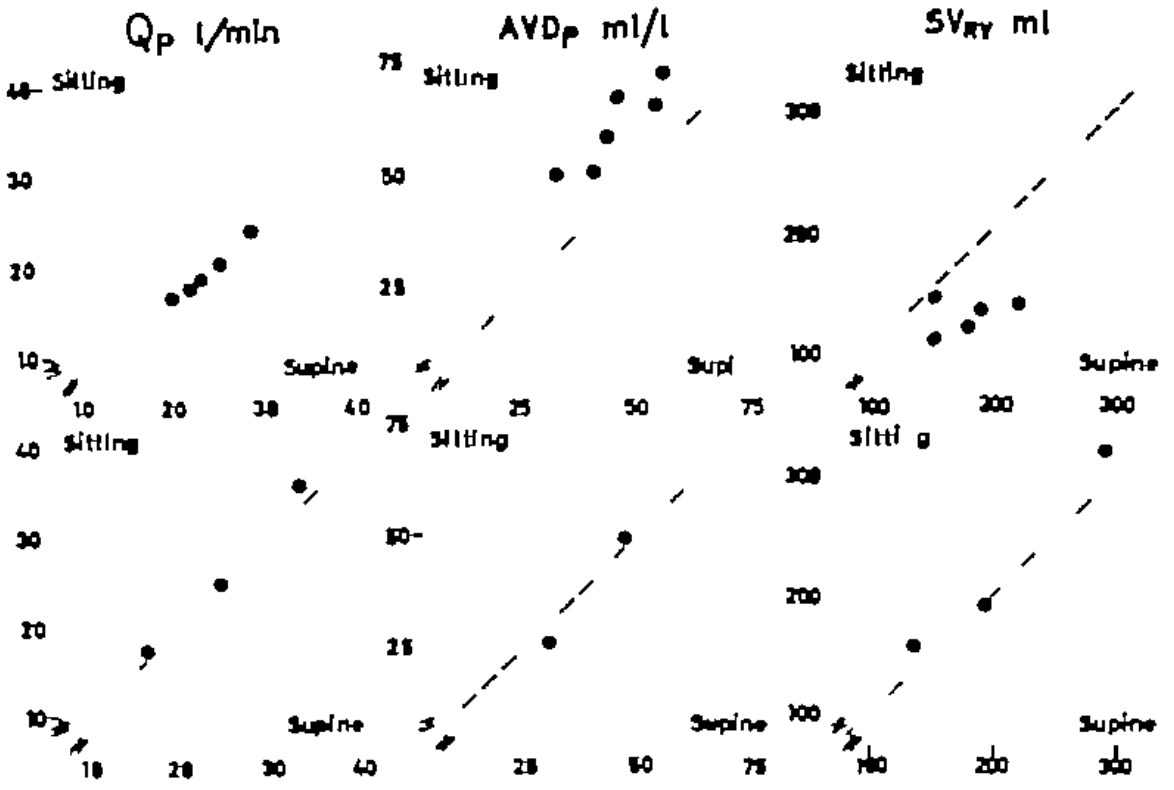


Fig. a.

Fig. 17 a and b. Pulmonary blood flow (Q_P) arterio-venous oxygen difference for the pulmonary circulation (AVD_P) stroke volume of the right ventricle (SV_{RV}) heart rate (HR) and aortic mean pressure (P_{Ao}) in the supine and sitting position Six patients with ASD studied during exercise For further explanation see text

Left ventricular stroke volume at rest in the supine position was 67 ml (range 53—91) *The right ventricular* stroke volume averaged 210 ml (range 123—312) at rest and 198 ml (range 137—292) during exercise in the supine position. This difference was not significant. In the sitting position during work it averaged 169 ml (range 113—319) The positional difference (range 4—81 ml) during exercise was probably significant ($p<0.05$) (Fig. 17 a)

Aortic pressure ($\bar{P}_{Ao}$) was within the normal range both at rest and during exercise in the supine position. The mean pressure averaged 93 mm Hg (range 90—108) at rest and increased to 110 mm Hg during exercise in the supine position In the two patients studied at rest in the sitting position the aortic mean pressure was almost unchanged. During work in the sitting position the mean pressure increased to a slightly lower level than in the supine, although the positional difference (7 mm Hg) was not significant. The individual variation was, however, large The patients could be separated into two groups according to the positional response. In three patients (nos 1 7 and 18) the aortic mean pressure during exercise was 17 to 32 mm Hg lower in the sitting than in the supine position. This was mainly due to a fall of 3—33 mm Hg in the diastolic pressure, the pulse pressure being almost unchanged. These patients all had a larger pul

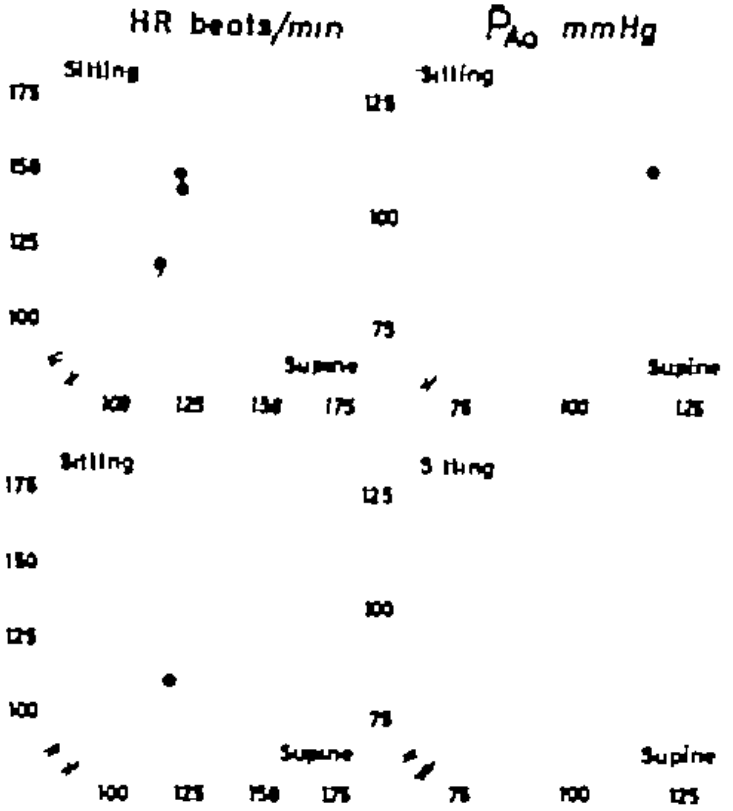


Fig. b.

monary blood flow in the sitting compared with the supine position. The heart rate was almost the same in the two body positions in this group (Fig. 17 b, lower part) In the other group (nos. 22, 23, 24) the aortic mean pressure was mainly unchanged by position during work, with small differences in diastolic and pulse pressures. The *pulmonary blood flow in all these patients was lower and the heart* rate higher (mean 16 %) in the sitting than in the supine position (Fig. 17 a, b, upper part) (No. 6 is excluded in this comparison, as the aortic pressure was not measured in the sitting position)

Pulmonary capillary venous pressure ($\bar{P}_{PCV}$) was normal at rest in the supine position. It increased normally during work in all except in two patients (nos. 1 and 7) who had slightly elevated pressures (21 and 24 mm Hg) In the sitting position $\bar{P}_{PCV}$ was not measured in all patients and so no conclusions could be drawn.

Pulmonary artery mean pressure ($\bar{P}_{A}$) increased from 21 mm Hg (range 17—31) at rest to 34 mm Hg (range 23—59) during supine exercise. In the sitting exercise the pressure was 7 mm lower ($p<0.05$) (Fig. 18)

Right ventricular end-diastolic pressure and *right atrial pressure* were elevated in the two patients (nos. 1 and 7) who also had increased values for $\bar{P}_{PCV}$ The other patients had values which were within the normal range in the supine position both at rest and during exercise. The atrial pressure was lower in the sitting position in all patients. The postural difference during exercise was 8 mm Hg

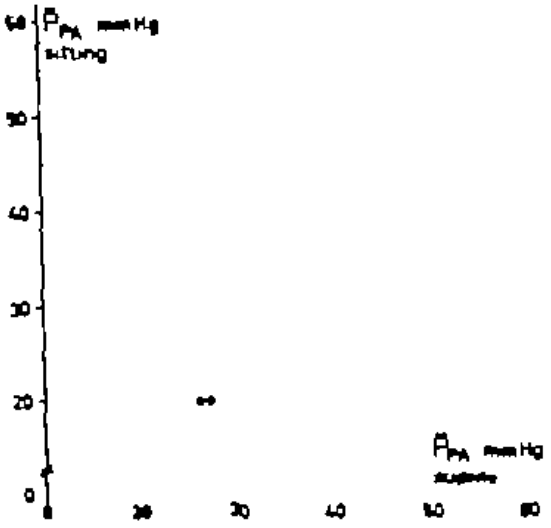


Fig. 18. Pulmonary artery mean pressure ($\bar{P}_{PA}$) in the supine and sitting positions in patients with ASD. Figures refer to case no. 1 and 7. Symbols as in fig. 1.

Pulmonary vascular resistance (PVR) was less than 1 unit in all except in one patient (no. 7) in whom it was 2.3 units. This patient had the smallest left-to-right shunt (pulmonary systemic flow ratio = 1.7) and the most elevated $\bar{P}_{Pcv}$ and $\bar{P}_{PA}$. These pressures increased much less during work in the sitting than in the supine position. Her pulmonary artery mean pressure was 59 mm Hg and 34 mm Hg respectively in the two positions (Fig. 18). The PCV pressure dropped from 24 mm Hg to 11 mm Hg. PVR was unaltered by change in body position.

COMMENTS

In patients with ASD the size of the defect is less important in determining the magnitude of the left-to-right shunt than the difference between wall compliances of the stiffer left ventricle and the more compliant right ventricle when subjected to equal filling pressures (Rowe et al. 1961). The systemic blood flow and left ventricular stroke volume have been found to be normal or slightly low in ASD at rest, a result supported by the common finding of a narrow aortic root. During exercise the systemic blood flow increases normally so that patients with low resting values have correspondingly low values on exercise (Davies and Gazetopoulos 1966). The explanation for a low working capacity in a patient with left-to-right shunt is probably a low left ventricular output, there being no correlation between left to-right shunt and physical working capacity (Jonsson et al. 1957). Physical working capacity is of the same order in the supine and sitting positions (Jonsson et al. 1957) but with a great variation in the working capacity and a marked discrepancy between this and the degree of disability described by the patient at the work test. This fits well with the findings in the 24 patients studied here in whom there was no significant change in working capacity on altering position.

Most studies concerning the hemodynamics in ASD during exercise have been done in the supine position, when the left-to-right shunt either remains unchanged or decreases (Jonsson et al. 1957, Swan et al. 1958, Davies and Gazetopoulos 1966,

Peterssen 1967 Siltanen 1968) Bruce and John (1957) described in 4 patients with ASD an increase of 21 % in pulmonary blood flow at rest on changing from the supine to the upright position This increase was proportional to the extent the oxygen consumption was greater in the upright position No comparison was made during exercise in the two body positions.

No patient in the present group had signs of right-to-left shunt in either the supine or sitting position. Davies and Gazetopoulos (1966) described, in patients with ASD with low pulmonary vascular resistance, a parallel increase in systemic and pulmonary flow during supine exercise, with little change in the magnitude of the left-to-right shunt

In patients with a large atrial septal defect, the atria can be looked upon as one chamber with a common filling potential for both ventricles, and stroke volume for each is determined mostly by the difference in diastolic filling resistance. On changing body position to the sitting position there is some change in intrathoracic pressure and a hydrostatic shift of blood, but this would act equally on both ventricles. If the systemic and pulmonary pressures are not markedly changed the outputs of both ventricles would not be affected by altering body position.

There were inter individual variations in the two types of response. Three patients had lower systemic pressures during exercise in the sitting position. Probably they all had lower peripheral resistances in this position, revealed by a dominant fall in diastolic pressure. Contrary to the normal response the heart rate was not increased. These patients all maintained the same stroke volume and cardiac output for the right ventricle during exercise in both positions. Two of these patients

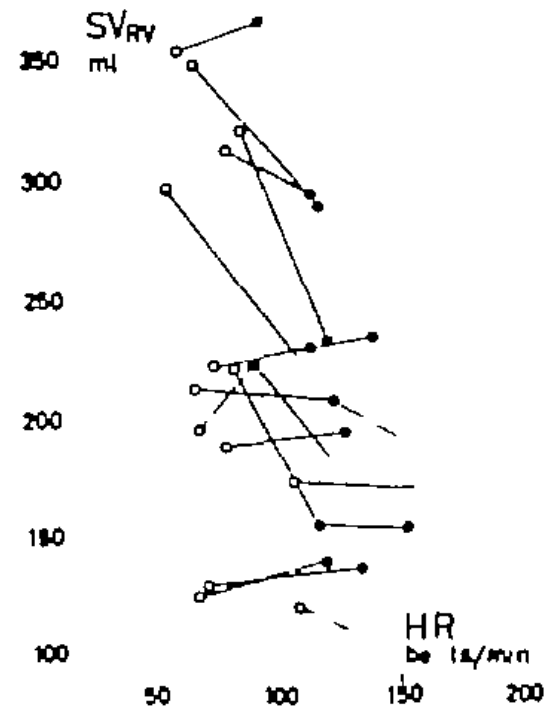


Fig 19 Stroke volume of the right ventricle (SV_{RV}) in relation to heart rate (HR) at rest (open circle) and during exercise (filled circle) in the supine position, in 14 patients with ASD

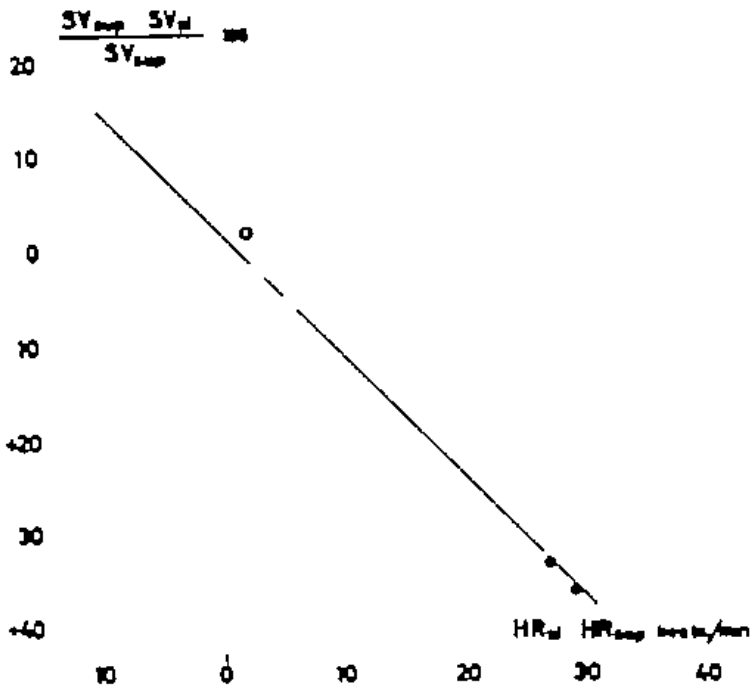


Fig. 20. Relative postural difference in stroke volume of the right ventricle (ΔSV) related to the difference in heart rate between the sitting and supine positions (r= —0 95)

(1 and 7) had in supine abnormally increased atrial and pulmonary artery pressures that were lower in the sitting position (Fig. 18) The other three patients had mainly unchanged aortic pressures on changing position and heart rate was higher in the sitting position. All in this group displayed a lower pulmonary blood flow and even lower right ventricular stroke volume when sitting The response of the latter group was, concerning the regulation of the systemic circulation, more normal.

Jonsson et al. (1957) pointed out that in ASD with a large diastolic flow through the tricuspid valve a significant filling of the right ventricle also takes place during the last part of diastole, contrary to the situation in normals in whom most filling probably takes place in the early part of diastole. The lower right ventricular stroke volume during supine exercise, seen particularly in those with a large stroke volume at rest, could be explained by the shortening of diastole at high rates This would decrease the filling of the right ventricle while filling of the left ventricle could be unchanged (Fig. 19) The difference in heart rate between the sitting and supine positions was correlated with the relative change in stroke volume of the right ventricle (Fig. 20) which may indicate that the difference in pulmonary blood flow on changing position could be secondary to the different reactions in heart rate Why some patients showed a decrease in systemic pressure without a rise in heart rate (in this response being similar to patients with postural hypotension) could not be explained. The lower aortic pressure during sitting exercise did not cause any symptoms. A sustained vaso-vagal reaction which was not counteracted by exercise cannot be excluded as one possible explanation of the absence of increasing heart rate on changing posture.

X

Ventilation alveolar gas exchange and D_{LCO} during exercise in patients with ATRIAL SEPTAL DEFECT with special reference to body posture

All patients described earlier were studied during exercise in the supine position. In three patients (nos. 22, 23 24) the D_{LCO} was not measured. In sixteen patients observations were also made during sitting exercise at the same load as that studied in the supine position. At rest the patients were only investigated in the supine position. Seven patients had been diagnosed preoperatively by heart catheterization in other hospitals.

RESULTS

Hemodynamic data

The pulmonary blood flow ($\dot{Q}_P$) at rest varied between 8 3 and 46.5 l/mm. and this was 1 7—9 3 times the systemic flow In most patients the pulmonary blood flow increased during exercise (Fig 21)

The pulmonary vascular resistance (PVR) was within the normal range in most patients and in only 2 patients was it above 2 units

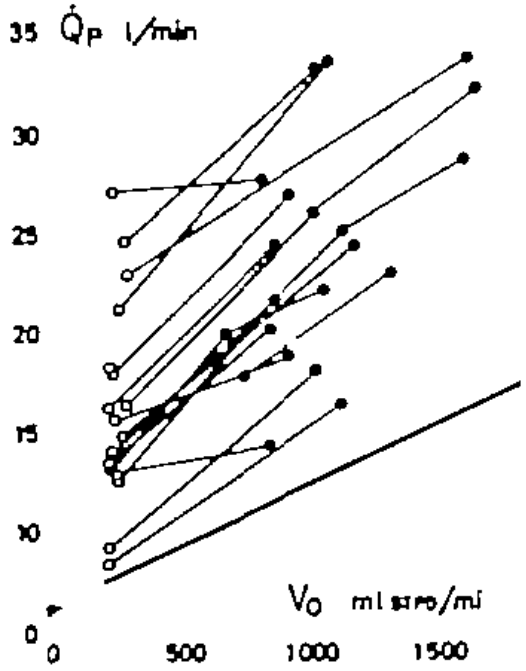


Fig. 21 Pulmonary blood flow (Q_P) in relation to oxygen uptake (V_{O_2}) in the supine position. Thick line represents the normal regression ($Q = 0\,0063\ V_{O_2} + 6.17$)

TABLE XI Effect of body position on respiratory data during exercise in 16 patients with atrial septal defect analysed as differences between data (sitting minus supine) See list of symbol and abbreviations

	$\bar{X}_{sup}$	$\bar{d}$	SD_d	C	n	p
HR, cpm	127	9	11	6 3	[illegible]	
Resp rate, cpm	30	−3	5	12.5	22	*
V_D, l/min.	31 2	2 6	3 5	7 1		
V_T l	1 08	0.22	0 19	11 5	[illegible]2	
V_{o_2} ml/min.	960	55	69	4.9	22	
V_E/V_{o_2}	3 7	−0 1	[illegible].8	6.0	2[illegible]	—
R	0 96	0 02	0 08	6.0	2	—
V_D, ml	225	59	57	15.9	22	
V_D/V_T	0 21	0 01	0 03	11 0	22	—
P_{aCO_2} mm Hg	55 7	0 2	2 1	4.2	1	—
P_{aO_2} mm Hg	88 2	2.5	7 9	6 2	22	—
P_{AO_2} mm Hg	112.2	−0 1	2.5	1 6	2[illegible]	—
$P_{(A-a)O_2}$ mm Hg	24.0	−2 6	7.8	24.3	22	—
pH, units	7 40	−0 02	0.02	0 19	22	
St.b mEq/l	21.2	−0.4	0.9	3 1	21	
D_{LCO} ml min^{-1} mm Hg^{-1}	34 9	1 9	4 1	8 1	15	—

The pulmonary artery mean pressure ($\bar{P}_A$) varied between 13 and 33 mm Hg at rest and 18 and 64 mm Hg during exercise. In eleven patients the pressure was above 30 mm Hg during exercise

Comments All patients had increased pulmonary blood flow without marked vessel changes in the lungs The hyperkinetic blood flow was accompained by a slight pulmonary hypertension in about half the patients.

Respiratory data

Mean values during exercise are given in Table XI

Oxygen uptake (V_{O_2}) averaged 243 ml/min at rest in the supine position. During exercise in the same position the average increase was about 3 times the resting value to 960 ml/min. In the sitting position it was still higher than in the supine position and the difference of 55 ml/min. was significant ($p<0.01$)

Minute ventilation (V_E) in the supine position increased from 7 8 l/min at rest to 31 l/min (range 16—40) during work. In the sitting position the ventilation was 2 6 l/min higher and this was probably due to the increased oxygen uptake in the sitting position, the ventilatory equivalent being the same in the two positions There was a linear increase of minute ventilation in relation to oxygen uptake $V_E = 0.032\ V_{O_2} + 0\ 62$ ($r = 0\ 92^{***}$) (see Fig. 25 in General discussion)

The ventilatory equivalent during exercise was 5 0 units higher in this group with ASD than in normal subjects. The increase in ventilation during work was achieved by both an increase in respiratory rate and tidal volume but there was a significant difference between the findings in the two body positions. During work in the supine the respiratory rate was higher and the tidal volume smaller than in the sitting position

Physiological dead space (V_D) was higher in the sitting than in the supine position but when related to the tidal volume (V_D/V_T) there was no positional difference during work (0.21 in the supine and 0 22 in the sitting positions respectively)

Arterial carbon dioxide tension ($P_{a_{CO_2}}$) in the supine position averaged 32.2 mm Hg (range 25—42) at rest and 35 7 (range 28—42) during exercise and was unchanged by body position.

Arterial oxygen tension ($P_{a_{O_2}}$) averaged 85.0 mm Hg (range 72—100) at rest. In six patients it was below 80 mm Hg at rest but in five of these it increased during work to over 80 mm Hg. These patients were considered not to have any right-to-left shunt through the atrial septal defect. One patient (no. 11) having the largest left-to-right shunt, with a pulmonary blood flow over 9 times the systemic flow had a $P_{a_{O_2}}$ of 75 mm Hg at rest and it decreased to 61 mm Hg and 59 mm Hg during work in the supine and sitting positions respectively In this patient it seemed probable that some desaturated blood from the right atrium could have been mixed with blood leaving the pulmonary veins. (At operation a large central secundum defect 3×4 cm was found) During exercise $P_{a_{O_2}}$ averaged 88.2 mm Hg (range 80—102) In the supine position and it was unchanged during exercise in the sitting position.

Alveolar oxygen tension ($P_{A_{O_2}}$) increased from 105 0 mm Hg at rest to 112.2 mm Hg (range 103—120) during exercise and was unaffected by body position.

Alveolar-arterial oxygen tension difference ($P_{(A-a)_{O_2}}$) averaged 20 4 mm Hg at rest (range 4—32) and 24.0 mm Hg during exercise (range 10—35) There was no significant positional difference. *pH* was on a erage 7 44 (range 7 40—7 51) at rest and 7 40 (range 7 32—7 45) during exercise in the supine position. In the sitting position it decreased to 7 38 (range 7 30—7 45) This difference of 0.02 was highly significant ($p<0.001$) The *standard bicarbonate* was slightly lower (0 4 mEq/l) in the sitting than in the supine position during exercise ($p<0.05$)

Respiratory exchange ratio (R) averaged 0 78 at rest and increased to 0 96 during exercise in both positions.

Pulmonary diffusing capacity (D_{LCO}) was measured at rest in 5 patients and averaged 28.2 ml min.$^{-1}$ mm Hg^{-1} The variation was large. In 3 patients in whom the resting value was measured both before and after exercise the difference ranged between 4.8 and 25 1 ml min^{-1} mm Hg^{-1} During supine exercise in

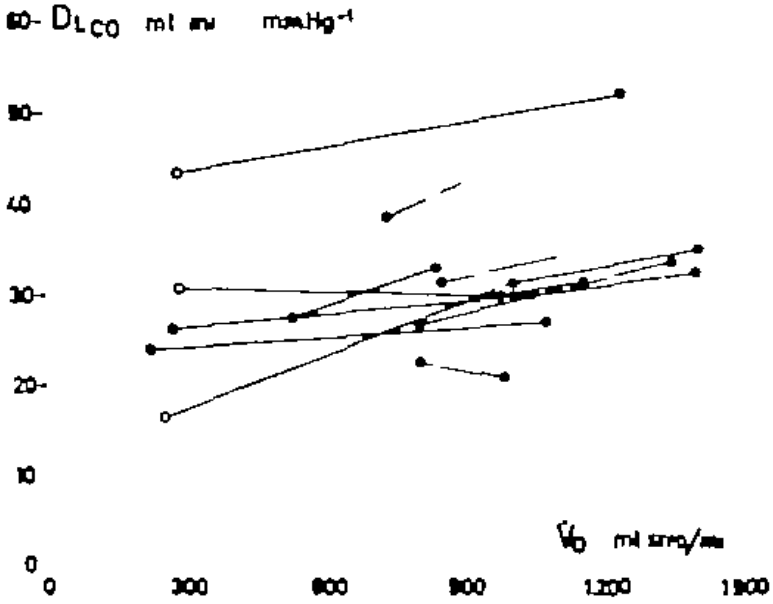


Fig. 22. Pulmonary diffusing capacity (D_{LCO}) in relation to oxygen uptake ($V_{O_2}^{l}$) at rest (open circle) and during exercise (filled circle) in 10 patients with ASD

this group D_{LCO} increased to an average of 34.2 ml min.$^{-1}$ mm Hg^{-1} This increase from the resting value was however not significant. For the whole group there was no significant positional difference during exercise. Thus D_{LCO} averaged 34 9 ml min.$^{-1}$ mm Hg^{-1} (range 26—52) in the supine and 36.8 ml min.$^{-1}$ mm Hg^{-1} (range 24—58) in the sitting position. In about half the group of patients D_{LCO} was measured at two work loads and there was a small increase ($p<0.05$) from 33 2 ml min.$^{-1}$ mm Hg^{-1} on the first load to 36.9 ml min.$^{-1}$ mm Hg^{-1} on the second load (Fig. 22) Maximal measured values for D_{LCO} were compared with those in normal subjects studied by Holmgren (1965) All cases were within the normal range, except two cases who were above (Fig. 23) For the whole group there was a significantly elevated D_{LCO} of 11 % from the predicted normal mean value.

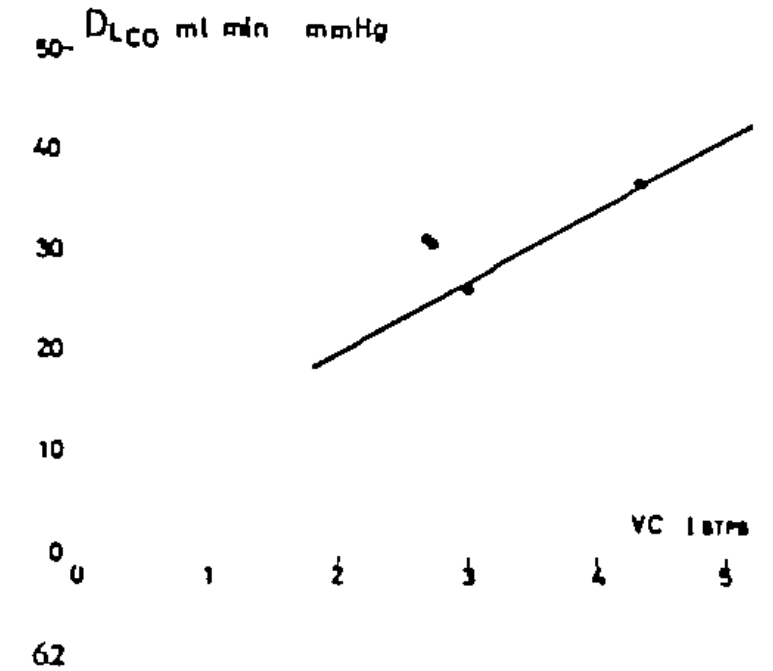


Fig. 23 Pulmonary diffusing capacity (D_{LCO}) in relation to vital capacity (VC) The maximal individual value during exercise is plotted. Lines represent the normal regression line ±2 SD. D_{LCO} =7 13 VC+5 4, SD ±4.6 (Holmgren 1965)

Effect of age on some respiratory data

The data were divided in two groups according to the age of the patient at the time for the investigation. Eleven patients belonged to the group younger than 40 years of age with a mean age of 30 5 years (range 16—39) and thirteen patients were older than 40 years with a mean age of 48 9 years (range 41—57) There was no significant difference between the groups concerning the oxygen uptake, ventilatory equivalent, V_D/V_T alveolar oxygen tension, carbon dioxide tension, pH or diffusing capacity in either position during work.

In the supine position arterial oxygen tension was lower however both at rest and during work in the older patients. The average oxygen tension in the older group was 81 mm Hg at rest and 83 mm Hg during supine exercise and this averaged 9 mm Hg and 8 mm Hg lower respectively ($p<0.01$) than in the younger group During sitting exercise there was no significant difference between the values in the two groups.

COMMENTS

All patients had a pure left-to-right shunt, except one, in whom there was suspicion of a slight right-to-left shunt due to a large secundum defect. The pulmonary blood flow was higher than normal in all patients but there was a large variation in the absolute values both at rest and during exercise. The pulmonary artery mean pressure was normal or slightly elevated due to the hyperkinetic lung circulation.

Total ventilation was found to be increased in relation to oxygen uptake due to alveolar hyperventilation (Fig. 25) This was not affected by body position although the ventilation in the supine position was achieved with a higher respiratory rate than that in the sitting position. The arterial gas tensions were mainly changed as a consequence of hyperventilation.

The arterial oxygen tension was normal in most cases during work in both body positions, suggesting that no right to-left shunt developed during exercise as a consequence of the change from the supine to the sitting position. In one patient $P_{a_{O_2}}$ decreased to the same extent during exercise in both positions. The relationship between the different ventricular wall compliances that mainly governs the amount and direction of the shunt does not seem to be markedly altered by body position.

Hyperventilation in patients with increased pulmonary blood flow has been reported earlier (Shephard 1955) Ressl et al. (1969) described in adults with atrial septal defect a correlation of resting hyperventilation to pulmonary hypertension but not to the left-to-right shunt. Gazetopoulos and Davies (1966) also demonstrated an excessive ventilatory response to exercise, but this was apparently not related directly to the level of he pulmonary arterial pressure or the magnitude of the pulmonary flow Such a relationship could not be demonstrated in this study either

The slightly lower pH, StB and base excess in the sitting position could be explained by increased anaerobic metabolism in this position.

Pulmonary diffusing capacity (D_{LCO}) measured with a steady state technique has been found to vary hyperbolically with oxygen uptake in normal subjects (Freyschuss and Holmgren 1965) The increase in D_{LCO} above an oxygen uptake of 40 % of the maximum uptake or a heart rate during exercise above 120 beats/min. was found to be small.

D_{LCO} at rest in patients with increased pulmonary flow has been found to be elevated, due to an increase in the pulmonary capillary blood volume (McCredie et al 1964) and the increase during work is less than normal (MacNamara et al 1960) or normal (Rotman and Wolf 1963)

The reproducibility of D_{LCO} at rest with the steady state technique is poor in normal subjects as has been pointed out (Bjure 1965 Holmgren 1965) and this is also verified in the patients studied During work it increases and is estimated to be 7.2 % The coefficients of variation of the D_{LCO} differences during exercise between the supine and sitting positions and between two work loads in the supine position in this material were 8.1 % and 6.2 % respectively Smaller changes than about 10 % would thus not be significant with this technique. Although there was a significant increase in D_{LCO} with increasing oxygen uptake during exercise it is of a small amount and including the resting values it seems to be an approximately unchanged D_{LCO} from rest to exercise (Fig. 22) The explanation of this may be that the pulmonary capillary blood volume is large at rest and a further increase is small The absolute value of D_{LCO} was not directly correlated with the amount of pulmonary blood flow

In patients with ASD all pulmonary vessels are probably perfused at rest with no new recruitment during work and this is not influenced by body position as was also found by Giammona and Daly (1968)

Alveolar gas change and pulmonary diffusing capacity have not been found to be restricted in patients with ASD and these cannot account for the hyperventilation usually seen. It might be due to altered lung mechanics.

XI

Ventilation alveolar gas exchange and D_{CO} in patients after corrective surgery of ATRIAL SEPTAL DEFECT

Dyspnea is the most common symptom restricting physical activity in patients with atrial septal defect The relief of this symptom postoperatively is also a most striking clinical finding Most studies of the pulmonary function in this type of patients have been under resting conditions, when hyperventilation has been found, both in children (Gamalero 1957) and adults (Ressl et al. 1969) with left-to-right shunts.

In the previous Chapter it was shown that high total ventilation due to alveolar hyperventilation is common during exercise in these patients and no differences were found in the ventilatory equivalent with change in body position. The arterial blood gases changed mainly as consequence of hyperventilation.

This chapter deals mainly with the postoperative measurements of lung ventilation, alveolar gas exchange and diffusing capacity of the lungs in 11 patients, belonging to the larger group of ASD studied earlier preoperatively

PATIENTS AND PROCEDURES

Eleven patients with atrial septal defect, operated on at the Thoracic Clinics, were selected for the postoperative study There were 6 females and 5 men with a mean age of 45 years (range 21—57) One patient (no 3) had a defect of primum type and all the others had secundum defects. Six patients were operated on with the use of hypothermia and five with an extracorporeal circulation. Two patients had atrial fibrillation both at the pre and postoperative study and one had a first degree AV-block preoperatively and atrial flutter at the postoperative investigation. All the others were in sinus rhythm at both investigations.

The heart catheterizations and all methods used were as described earlier The circulatory and ventilatory investigations were done at the same time and the same work loads were chosen for the pre and postoperative studies The patients were investigated 7 to 20 months (mean 13 months) after operation.

The catheterization studies were performed only in the supine position. In five patients who were also studied in the sitting position preoperatively the double-lumen venous catheter was withdrawn and alveolar gas exchange and diffusing capacity were studied also during exercise in the sitting position. One patient (no.

8) was not subject to heart catheterization but only examined with determination of the diffusing capacity She had radiological and clinical evidence of a closed defect postoperatively

RESULTS

Mean values for some anthropometric data are given in Table XII

Heart volume was on average 15 % smaller postoperatively ($p<0.05$) In two patients the heart volume was almost unchanged, one of these was the patient with atrial fibrillation.

Total hemoglobin and *blood volume* were 10 % and 9 % respectively smaller after operation ($p<0.01$ and $p<0.05$)

Maximal work intensity in the two body positions was on average not significantly changed after operation.

Spirometry There was no significant difference pre and postoperatively in the total lung capacity and its subdivisions The dynamic spirometry displayed an increase of 12 l/min. in the MVV_{12s} from the preoperative 103 4 l/min. ($p<0.05$) the other variables being unchanged

Hemodynamic data

Mean values for 10 patients studied with heart catheterization pre and postoperatively are given in Table XIII.

Heart rate (HR) and *oxygen uptake* (V_{O_2}) were the same before and after operation both at rest and during work. None of the patients had any residual left to-right shunt postoperatively The difference between the oxygen saturation in superior vena cava (S_{svc}) and that in pulmonary artery (S^-) ranged between —5.8 and 4.1 %. There was a significantly higher S_{svc} postoperatively compared to that preoperatively

TABLE XII Mean values for anthropometric and spirometric variables in 11 patients with atrial septal defect investigated before (pre) and after (post) operation
See list of symbols and abbreviations

	THb g	BV l	HV ml	W_{max} stat kpm/min.	W_{max} sup kpm/min	VC l	FRC l	TLC l	FEV_1 %	MVV_{12} l/min.	$MVV_{12\,ex}$ l/min.
Pre	703	5 6	1089	537	433	5 80	2.59	5 35	79.2	88.8	103 4
Post	631	5 1	924	670	523	5 78	2.59	5 27	79 0	95.2	115 4
d	72	0 5	165	−133	−91	0.02	0	0.08	0 2	−6.4	−11 9
C	5.8	6.5	12 8	23 7	28.2	5 7	5 9	4.5	5 4	9.2	9.2
n	8	8	11	10	7	9	9	9	9	9	9
p				—	—	—	—	—	—	—	

TABLE XIII Mean values for hemodynamic data at rest and during exercise in the supine position in 10 patients with atrial septal defect investigated before (pre) and after (post) operation
See list of symbols and abbreviations

	Q_T l/min.	Q_S l/min.	S_{SCV} %	SV ml	$\bar{P}_{RV_{ed}}$ mm Hg	$\bar{P}_{PA}$ mm Hg	P_{PCV} mm Hg	$\bar{P}_{Ao}$ mm Hg	PVR units
Rest									
Pre	16.8	4 6	69 1	65	6.6	20.1	9.9	98.0	0.7
Post	5 7	5 7	74.6	85	5.0	16.6	9 7	95.3	1 3
d	11.1	−1 1	−5 5	−20	1.6	3 5	0.2	2.7	−0.6
C	27 7	1_3	4.5	17 4	33.7	28.9	24.2	7.2	52.9
	10	10	8	10	8	10	10	7	10
p					—	—	—	—	
Exercise									
Pre	23.9	—	—	—	6.7	36.3	16.2	114.1	0.9
Post	9.8	9.8	—	93	6.0	28.5	17.0	112.0	1.5
d	14.1				0.7	8.0	−0 8	2.1	−0.4
C	29 4				41.0	27 3	34.6	7 7	34.6
	9				6	8	9	9	8
p					—	—	—	—	—

Pulmonary blood flow ($\dot{Q}_T$) decreased from 16.8 l/min. to 5 7 l/min. after operation at rest and from 23 9 l/min. to 9.8 l/min. during exercise. After operation there was a significantly higher systemic blood flow at rest, corresponding to an average stroke volume of 65 ml preoperatively and 85 ml postoperatively The stroke volume did not increase significantly from rest to exercise after operation.

Intracardiac and intravascular pressures averaged about the same level pre- and postoperatively The *right ventricular end-diastolic pressure* decreased in 2 patients, increased in one and was unchanged in the rest. The *pulmonary artery mean pressure* ($\bar{P}$) at rest and during work was slightly above normal in one patient preoperatively (no 3 33 and 56 mm Hg) and postoperatively all were within the normal range. The *PCV pressure* ranged from 5—19 mm Hg at rest preoperatively and from 6—17 mm Hg postoperatively and were in most patients unchanged. During exercise the findings were similar although the pressure levels were higher The range before operation was 4—28 mm Hg and after 11—29 mm Hg. The *aortic mean pressure* ($\bar{P}_{Ao}$) at rest and during exercise was unchanged by operation both at rest and during work. The *pulmonary vascular resistance* (PVR) was normal both before and after operation in all patients

Respiratory data

Mean values of ventilation, alveolar gas exchange, blood gases and diffusing capacity during exercise are given in Table XIV

TABLE XII Mean values of pulmonary data during upright exercise in 11 patients with proximal septal defect investigated before (pre) and after (post) operation

	$\bar{X}_{pre}$	$\bar{X}_{post}$	$\bar{d}$	n	C	
HR, cpm	121	117	4	14	8.9	—
Resp rate, cpm	26.9	23.5	3.4	14	9.6	
Hb g/100 ml	13.8	14.2	−0.4	14	3.8	—
$\dot{V}_{O_2}$ ml/min	982	1002	−20	14	7.4	—
$\dot{V}_E$ l/min	30.3	27.3	3.0	14	8.1	
V_T ml	1250	1 00	50	14	11.8	—
$\dot{V}_E/\dot{V}_{O_2}$	33.0	27.3	5.7	14	11.4	
R	0.98	0.91	0.07	14	9.2	—
V_D ml	255	234	21	14	20.3	—
V_D/V_T	0.21	0.20	0.01	14	17.0	—
$\dot{V}_A$ l/min.	24.4	20.8	3.6	14	9.9	
$P_{\bar{v}O_2}$ mm Hg	114.0	108.2	5.8	14	3.5	
P_{aO_2} mm Hg	87.0	90.6	−3.6	14	5.3	—
$P_{(A-a)O_2}$ mm Hg	27.0	17.6	9.4	14	22.9	
P_{aCO_2} mm Hg	34.8	38.7	−3.9	14	5.1	
pH units	7.41	7.38	0.03	14	0.37	
St.b., mEq/l	21.5	22.1	−0.6	14	6.7	—
D_{LCO} ml min^{-1} mm Hg^{-1}	32.2	24.7	7.5	14	18.7	

At rest there was no significant difference between pre- and postoperative values of total ventilation, tidal volume, alveolar or dead space ventilation. The same was found for arterial oxygen and carbon dioxide tensions and pH. During *exercise* with an average oxygen uptake of 1.0 l/min. there was a highly significant difference in *total ventilation* which was on an average 3 l/min lower after operation for the same oxygen uptake (Fig. 24 a). This was achieved with a lower *respiratory rate*, *tidal volume* being unchanged. The *dead space ventilation* was unchanged and therefore the lower total ventilation after operation was due to a significantly smaller alveolar ventilation. P_{aCO_2} increased from an average of 34.8 mm Hg preoperatively to 38.6 mm Hg postoperatively (Fig. 24 b). The *pH* was lower postoperatively although the standard bicarbonate and base excess were unchanged.

Arterial oxygen tension (P_{aO_2}) was not changed by operation (Fig. 24 c). There was a significant decrease of the $P_{(A-a)O_2}$ postoperatively and this was due to a lower $P_{\bar{v}O_2}$.

Diffusing capacity for the lungs (D_{LCO}) during supine work, decreased from 32.2 to 24.7 ml min^{-1} mm Hg^{-1} ($p<0.01$) (Fig. 24 d). This value after

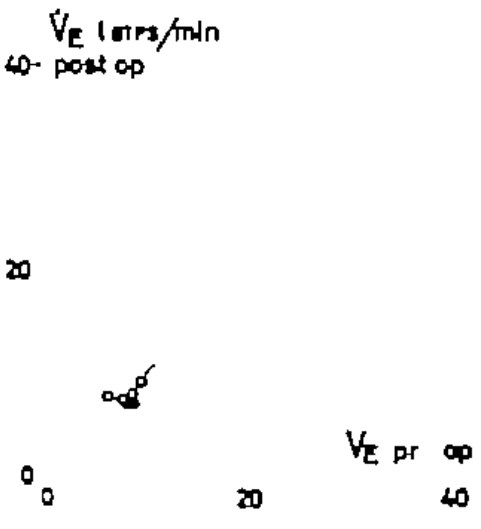


Fig. a

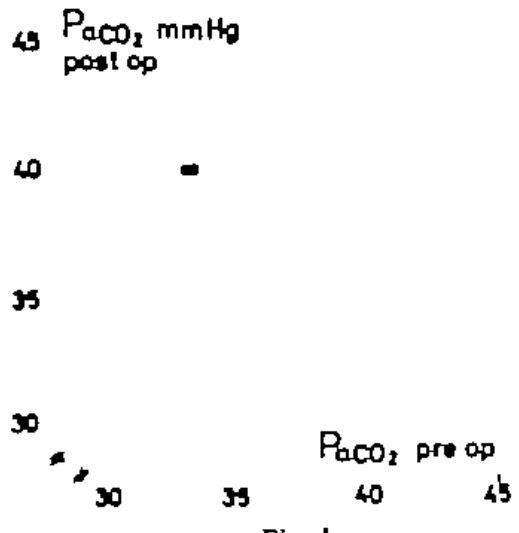


Fig. b.

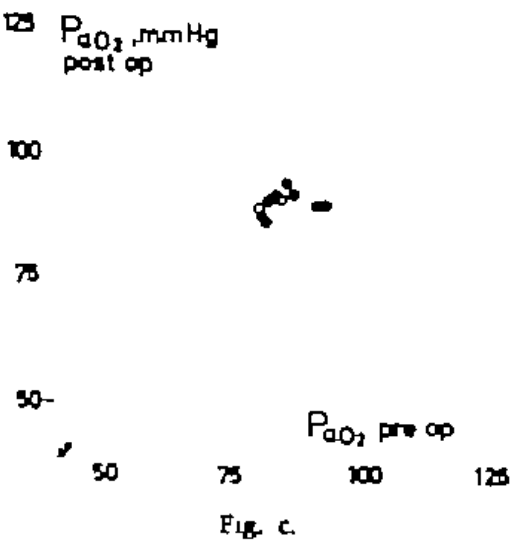


Fig. c.

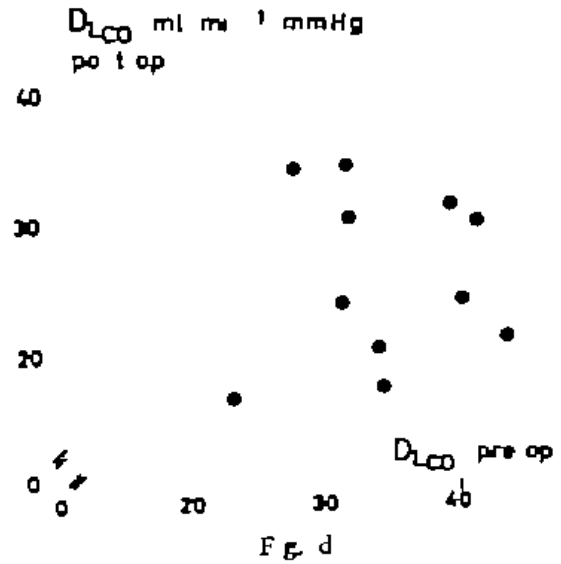


Fig. d

Fig. 24 a, b, and d Lung ventilation ($\dot{V}$) arterial carbon dioxid tension (P_{aCO_2}) arterial oxygen tension ($P_{a_{O2}}$) and pulmonary diffusing capacity (D_{LCO}) obtained at rest and during exercise at the same load pre and postoperatively Symbols as in fig 1

operation did not significantly deviate from the predicted normal mean value. In five patients studied during work in both body positions pre and postoperatively the fall in D_{LCO} after operation was even more marked in the sitting position, although the d fference between the two positions was not significant either before or after operation.

COMMENTS

The natural history for patients with ASD is usually uncomplicated, a normal physical act ty being maintained into middle age, when they often develop dyspoea on effort. The patients studied, except for one, were over 35 yea

of age. All patients had a pure left to-right shunt at atrial level preoperatively with a hyperkinetic pulmonary circulation without increased pulmonary vascular resistance. The hemodynamic disturbance is usually corrected by the operation even in older patients (Peterson 1967 Siltanen 1968) This also fits well with the findings in the present study where no residual left-to-right shunt was found postoperatively The pressures in the pulmonary circulation were in most cases within the normal range preoperatively and remained unchanged or were lower after operation. The PCV pressure during exercise increased postoperatively in some patients and this has been found earlier among older patients (Siltanen 1968 Jonsson 1969)

The systemic cardiac output at rest was greater after operation due to a significantly larger left ventricular stroke volume as was also found by Peterson (1967) Probably this is acting also during work, although it cannot be proved.

Increased ventilatory equivalent at rest and during work in patients with heart disease has been reported by many authors (Campbell et al. 1923 Gazetopoulos et al. 1966) Shephard (1955) found resting hyperventilation in patients with increased pulmonary blood flow and it was mainly due to high respiratory rate. Krantwald et al. (1962) found in patients with left-to-right shunt a good correlation between the resting hyperventilation and pulmonary artery pressure but no correlation with the magnitude of the shunt. Resal et al. (1969) reported the same and found no correlation with pulmonary blood flow or femoral venous oxygen saturation.

Increased pulmonary blood flow and pulmonary hypertension in atrial septal defect may alter the lung mechanics Davies and Gazetopoulos (1967) found a significant inverse relation between the product of pulmonary pressure and flow and the compliance of the lungs They also observed an increase in compliance after closure of the atrial septal defect. Woolf (1963) found no relation between total ventilation and compliance during exercise. However he pointed out that in patients with moderate or severe dyspnea, the abnormally high ventilation during mild exertion was associated with a high work of breathing and this dual abnormality distinguished them from patients with only slight dyspnea on exertion. Hyperkinetic pulmonary hypertension could thus be of importance for the hyperventilation in these patients A significant correlation was also found in the present study between the decrease in ventilation and the change in the product of pulmonary flow and pressure pre- and postoperatively ($r=0.60$ $p<0.05$)

In the present study no changes were found in ventilation or alveolar gas exchange at rest after the defect had been closed. During exercise, however there was a significantly lower total ventilation with a lower respiratory rate postoperatively

As the $P_{a_{O_2}}$ was unchanged $P_{a_{CO_2}}$ increased, and the pH decreased after operation, these variables do not seem to be important determinants for the hyperventilation in these patients. Impairment of systemic output has been proposed as a stimulus to ventilation (McIlroy 1959) but was not proved by others (Sloman and Gandevia 1964 Gazetopoulos et al 1966) Although the systemic output was

higher at rest after operation in this group of patients, there was no difference in standard bicarbonate during exercise, indicating any marked changes in the aneorobic metabolism pre and postoperatively

Diffusing capacity of the lungs in patients with ASD has been reported to be increased at rest (Rankin and Callies 1958) and this is mainly due to an increase in the pulmonary capillary blood volume (McCredie et al. 1964) The increase of D_{LCO} during exercise in patients with ASD has been reported to be both lower (MacNamara et al. 1960) and the same as in normal subjects (Rotman and Woolf 1963) Reversibility of the elevated D_{LCO} and pulmonary capillary blood volume after surgical repair has been observed at rest (Murao et al 1963) Tala et al (1968) reported a postoperative decrease of 25 % in D_{LCO} at rest 9—16 weeks after surgery

The present study demonstrates a postoperative decrease in D_{LCO} also during exercise on an average of 23 % This probably suggests that the pulmonary capillary bed during exercise is also reduced after surgical correction In two patients, however there was a small increase in D_{LCO} after operation, although the pulmonary flow in these patients decreased by 23.1 l/min. and 8.7 l/min. respectively

stroke volume at rest in the supine position. Shortening of diastole with increasing heart rate would interfere with the filling of the right ventricle because of its large stroke volume, while the filling time for the left ventricle would not be critical. In the present study most patients with a stroke volume larger than 200 ml at rest displayed a decrease during supine exercise, while in the others it was essentially unchanged (Fig. 19). If the relationship between the left and right ventricular filling resistances, which is the main determinant of the size of the shunt, remains essentially unchanged by exercise and posture, the stroke volume of the right ventricle and probably also the shunt would decrease at high heart rates. A highly significant negative correlation was found between the relative change in stroke volume of the right ventricle and the change in heart rate when body position was altered (Fig. 20). When the heart rate during exercise was approximately the same when supine and sitting, pulmonary blood flow was also equal in the two positions. Changes in right ventricular stroke volume in ASD after shifting body position is likely to be secondary to changes in heart rate, which are mainly controlled by the systemic circulation.

Vital capacity and total lung capacity were lower in patients with MS and ASD than in normal subjects. Both groups of patients had a significant decrease in vital capacity of 15 % from the predicted normal mean values and contrary to the findings of Siltanen (1968) the vital capacity in ASD was not changed after operation. In patients with pulmonary stenosis the lung volumes were within the normal range. Lung volumes are thus affected by increased pressure levels and/or an increased pulmonary blood flow. This is usually accompanied by a low lung compliance (McIlroy and Apthorp 1958, White et al. 1958, Woolf 1963).

Total ventilation in normal subjects is linearly correlated with oxygen uptake during submaximal work loads. At higher work loads, when the lactate concentration starts to increase rapidly, the ventilation increases more steeply with increasing oxygen uptake (Asmussen 1965). In this study the oxygen uptake was increased up to about 1.5 l/min, and within this range the ventilation in normal subjects is linearly related with oxygen uptake. This was also found in the present three patients groups (Fig. 25). The ventilation was, however, larger for any given oxygen uptake during exercise compared with that in normal subjects, particularly in patients with PS and ASD. After surgical correction of the ASD the ventilation during exercise was normal and the ventilatory equivalent was significantly decreased from 33.0 to 27.3. This decrease in ventilation for the same oxygen uptake in combination with an increase in compliance after operation, reported by Davies and Gazetopoulos (1967), correlates with the relief of exercise dyspnea, which is the principal improvement in symptoms after surgery.

The mechanism of the increased ventilation cannot be fully explained and it is probably multifactorial and not necessarily the same in the different patient groups. As the pulmonary artery pressure and flow were so different in the three groups of patients and as ventilation did not alter with change in posture, the vascular

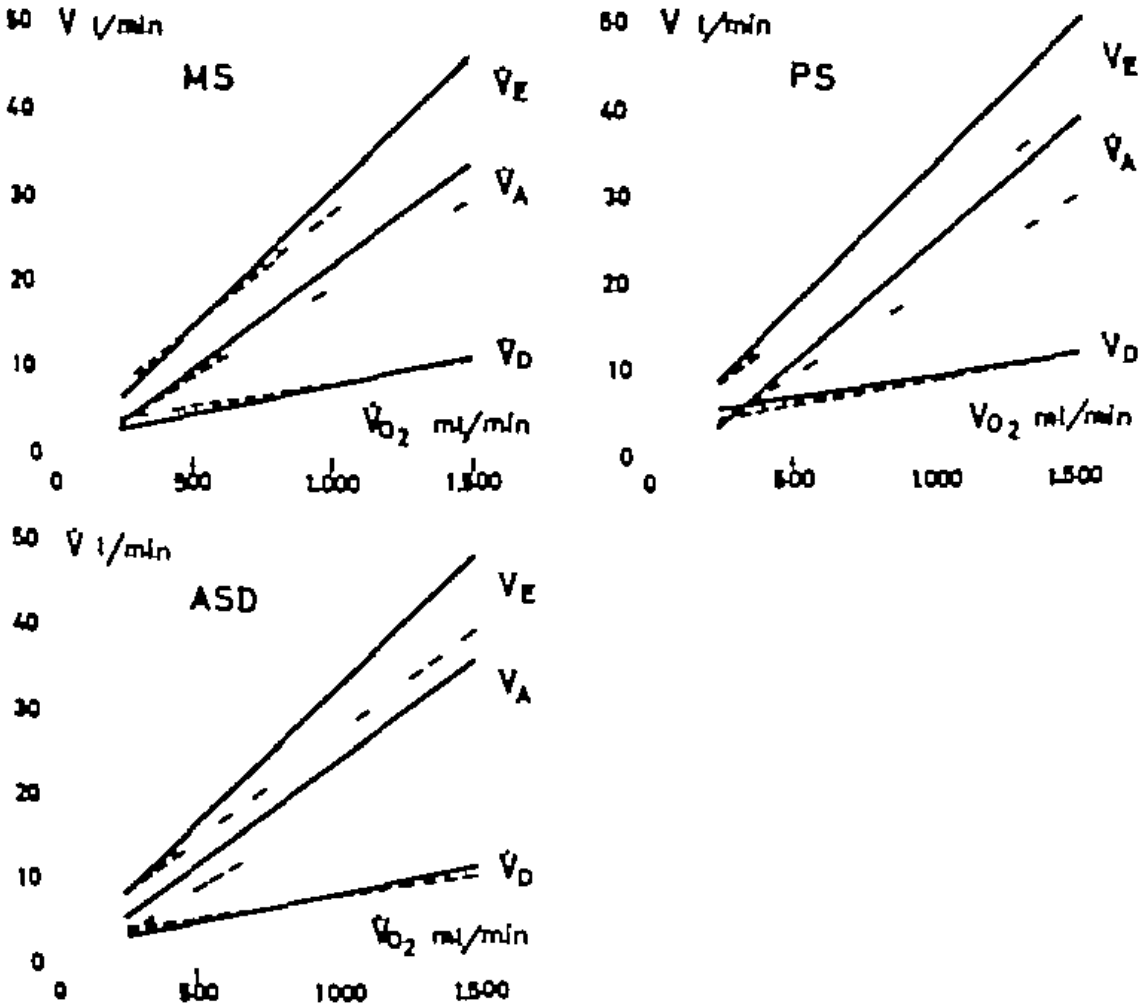


Fig. 25 Lung ventilation (V_E) alveolar ventilation (V_A) and dead space ventilation (V_D) in relation to oxygen uptake (V_{O_2}) in patients with mitral stenosis (MS) pulmonary stenosis (PS) and atrial septal defect (ASD). Broken lines represent the normal regression $V_E = 0.026\ V_{O_2} + 1.53$ (Holmgren 1967). Regression equation in MS $V_E = 0.037\ \dot{V}_{O_2} - 0.87$ ($r = 0.95$) in PS $\dot{V}_E = 0.033\ V_{O_2} + 0.19$ ($r = 0.88^{**}$) and in ASD $V_E = 0.032\ V_{O_2} + 0.62$ ($r = 0.92^{*}$)

pressures and flow in the lung do not appear to be important causes of hyperventilation. Gazetopoulos and Davies (1966) suggested a mechanism connected with tissue hypoxia. In one patient with the tightest pulmonary stenosis and the most markedly hypokinetic circulation the ventilatory equivalent was high but did not change from rest to the first load ($S_{vo_2} = 26\ \%$) but then increased markedly on the second load in both body positions ($S_{vo_2} = 12\ \%$) which may support such a mechanism. Holmgren and Strömberg (1959) found higher blood lactate concentrations in relation to absolute work load in patients with MS and ASD than in normal untrained individuals. In the patients with ASD after corrective operation the pH was lower and the standard bicarbonate unchanged at a given work l

The decrease in ventilation therefore could not be fully explained by any marked difference in anaerobic metabolism.

Variation in the alveolar arterial oxygen tension difference ($P_{(A-a)O_2}$) is dependent on three factors the diffusing properties of the lung, the distribution of alveolar ventilation perfusion ratios in the lung and the right to-left shunt. The D_{L-CO} was not markedly changed in the three groups of patients and would therefore not affect the $P_{(A-a)O_2}$ to any important extent. The other two components could not be separated in this study but constitute about half each of the gradient in normals at rest (Mellemgaard 1966) In this study the $P_{(A-a)O_2}$ was unchanged by body position in patients with PS and ASD during exercise, although at a slightly higher level in the latter group In patients with MS the $P_{(A-a)O_2}$ was lower during sitting than supine exercise. This suggests changes in the ventilation perfusion ratios in the lungs related to high capillary venous pressure (Blount et al. 1955) $P_{(A-a)O_2}$ was related to PCV pressure in the present study (Fig. 11) After closure of the ASD the $P_{(A-a)O_2}$decreased due to a lower alveolar oxygen tension as the arterial oxygen tension was unchanged and the decrease in $P_{(A-a)O_2}$ thus depends on the absence of alveolar hyperventilation.

The $P_{a_{O_2}}$ at rest decreases with increasing age (Raine and Bishop 1963) The only respiratory variable in the group with ASD that differed between patients above and below the age of 40 was the arterial oxygen tension in the supine position. This was lower in the older group, both at rest and during exercise. It decreased, however within the normal range quoted by Raine and Bishop (1963) when age was taken in account. This probably indicates that long standing high pulmonary blood flow in patients with ASD does not accelerate any structural lung changes

Pulmonary diffusing capacity during exercise was mainly within the normal range in all three patient groups, although the mean values for MS were lower and for ASD higher than the predicted normal values. This is suggested to indicate differences in the pulmonary capillary blood volume (Bates et al. 1960 Yu 1969) Bjure (1965) found a correlation between stroke volume and D_{L-CO} in normal subjects and suggested that the stroke volume approximates the volume of the functioning pulmonary capillary bed. The D_{L-CO} during exercise was, however not affected by change in body position in any of the three groups of patients studied During exercise D_{L-CO} is thus little affected by postural changes in pulmonary blood flow and pulmonary vascular pressures. In the patients with ASD there was a small difference in D_{L-CO} from rest to exercise with increasing work loads. The exercise D_{L-CO} decreased markedly after surgical closure of the defect, suggesting a smaller pulmonary capillary blood volume.

Summary

1 The present investigation was undertaken to study how changes in body posture affect central hemodynamics, lung ventilation, alveolar gas exchange and pulmonary diffusing capacity in patients with heart disease. The patients were selected to have a large range of pressures and flows in the pulmonary circulation with an almost normal vascular resistance to flow None was in heart failure.

2. High pressures and low blood flow in the pulmonary circulation were studied in 9 patients with mitral stenosis Low pressures and low blood flow were studied in 5 patients with pulmonary valve stenosis. High pulmonary blood flow was studied in 24 patients with atrial septal defect (ASD) and 11 of these were also studied after successful surgical correction.

3 To study the influence of body posture, patients were studied in the supine and sitting positions at rest and during exercise on a bicycle ergometer

4. The central hemodynamics were studied by catheterization of the pulmonary artery and the aorta, and blood flow was measured using the direct Fick technique. The pulmonary diffusing capacity (D_{L-CO}) was measured during exercise as the diffusing capacity for carbon monoxide, using the steady state technique of Filley

5 *High pressure—low blood flow (Mitral stenosis)* The 9 patients with mitral stenosis (valve area 1.2—2.3 cm^2) and a mean pulmonary vascular resistance of 1.8 units at rest had a low cardiac output in relation to oxygen uptake both at rest and during exercise in the *supine position* (i.e. hypokinetic circulation) compared to normal subjects. The mean pulmonary capillary venous pressure (PCV) averaged 20 mm Hg and the pulmonary artery mean pressure 29 mm Hg at rest in the supine position and increased during exercise to 40 mm Hg and 57 mm Hg, respectively In the *sitting position* the circulation became even more hypokinetic. The postural difference in cardiac output was 1 2 l/min. at rest and 0.8 l/min. during exercise at a mean oxygen uptake of 0 90 l/min. The pressure level in the lung vessels decreased markedly at rest and increased less during exercise in the sitting position. The pulmonary vascular resistance was unchanged by body position. The low cardiac output in the sitting position was accompanied by a reduction in stroke volume. The relative decrease in stroke volume, after changing position, was inversely related to the right atrial and PCV pressures High pressure levels in the pulmonary circulation were accompanied by large postural pressure changes and small variations in blood flow

Total lung ventilation as well as its components *alveolar* and *dead space ventilation* increased linearly with oxygen uptake during exercise in both body positions, with no postural difference. $\dot{V}/\dot{V}$ remained unchanged after shifting position *Arterial oxygen tension* was mainly within the normal range and did not

change from rest to exercise in the supine position, but was significantly higher during exercise in the sitting compared to the supine position. The *alveolar-arterial oxygen tension difference* was positively correlated with the PCV pressure and was highest during exercise in the supine position (26 mm Hg) D_{L-CO} during exercise was mainly within the normal range, although on average significantly lower than the predicted normal value. No postural difference was found.

6. *Low pressure—low blood flow (Pulmonary stenosis)* 4 patients with tight pulmonary stenosis (valve area 0.20—0 50 cm^2) had a low cardiac output in relation to oxygen uptake at rest and during exercise in the *supine position* One patient with a moderately tight pulmonary stenosis (valve area 1.20 cm^2) and a normokinetic circulation is discussed separately The mean pressure in the pulmonary artery was 15 mm Hg at rest and 23 mm Hg during exercise (the peak systolic pressure difference, at rest, between the right ventricle and the pulmonary artery averaged 126 mm Hg) In the *sitting position* the circulation became even more hypokinetic. The postural difference in cardiac output during exercise was 0 6 l/min. at a mean oxygen uptake of 0 95 l/min. At rest the difference was 0 7 l/min (2 patients) The *pulmonary artery mean pressure* was only slightly affected by changes in position. The patient with a normokinetic circulation had a decrease in cardiac output of a similar magnitude to that found in normal subjects. The pulmonary artery mean pressure also dropped more in this patient than in the others. The relative change in stroke volume after changing body position was negatively correlated with the end-diastolic pressure of the right ventricle in the supine position.

Total lung ventilation was high in both body positions in relation to oxygen uptake due to *alveolar hyperventilation* The *dead space ventilation* was normal. The *alveolar-arterial oxygen tension difference* was normal or small and was not influenced by changes in body position. The D_{L-CO} was normal and was not affected by changes in position.

7 *High blood flow (Atrial septal defect)* The twenty four patients with great pulmonary blood flow at rest, the pulmonary blood flow increased further during exercise in the supine position. In 7/24 patients the central hemodynamic was studied in both body positions during exercise. The pulmonary blood flow in the *supine position* averaged 15 6 l/min. at rest and 26.4 l/min. during exercise at an average oxygen uptake of 1 11 l/min. and the pulmonary artery mean pressure increased from 21 mm Hg to 34 mm Hg. Pulmonary blood flow during exercise was not significantly altered by changes in position, but the interindividual variation was large Changes in the right ventricular stroke volume from rest in the supine position to exercise in both patients was considered to be mainly secondary to changes in heart rate. Pulmonary artery mean pressure during exercise averaged 7 mm Hg lower in the sitting than in the supine position. In 11/24 patients, studied 13 months after surgical closure of the ASD the left ventricular output at rest was increased in relation to oxygen uptake compared with preoperative

Total lung ventilation rose linearly with increasing oxygen uptake (up to 1.5 l/min.) at a higher level than in normal subjects, due to *alveolar hyperventilation* and thus was unaffected by changes in position. *Dead space ventilation* was normal. The ventilation during exercise was lower after surgery and alveolar ventilation became normal. *Arterial oxygen tension* was normal, except in one patient, and was not influenced by body position during exercise, either before or after closure of the ASD *Alveolar-arterial oxygen tension difference* during exercise was unchanged by posture but decreased after operation due to a decrease in the alveolar oxygen tension. The D_{LCO} was high preoperatively at rest and increased by a small amount during exercise, irrespective of body position Exercise D_{LCO} decreased markedly after operation in both positions. This probably indicates that pulmonary capillary blood volume during exercise is increased in patients with ASD but is reduced after surgery

8. The investigation has shown that in patients with low blood flow and high or low pressure levels in all lung vessels, the hemodynamics are characterized by a smaller cardiac output in the sitting both at rest and during exercise than in the supine position. The postural difference, however being smaller than in normal young and old subjects. The postural difference in pulmonary artery mean pressure increases with increasing pressure level in the supine position, particularly when pulmonary artery mean pressure exceeds 25 mm Hg. The relative change in stroke volume after shifting from the supine to sitting position varies inversely with the filling pressure of the right ventricle. This is interpreted as indicating a stabilization of the capacitance vessels. In patients with high pulmonary blood flow (ASD) there is no postural change in pulmonary blood flow and only small variations in the pulmonary pressures.

Total ventilation, alveolar gas exchange, alveolar-arterial oxygen tension difference and pulmonary diffusing capacity are little influenced by changes in body position. Thus variations in pulmonary blood flow and pressure levels influence these variables only slightly The pulmonary diffusing capacity during exercise is affected by large changes in pulmonary blood flow e.g. after surgical correction of ASD

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Supplementum 348

From the Department of Histology
Karolinska Institutet, 104 01 Stockholm, Sweden

GROWTH CHARACTERISTICS OF ADRENERGIC NERVES IN THE ADULT RAT

Fluorescence histochemical and ^3H noradrenaline uptake studies using tissue transplantations to the anterior chamber of the eye.

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To the memory of
Nils-Åke Hillarp

CONTENTS

Abbreviations 7

I INTRODUCTION 9

1. Preface 9
2. Founders of the present concepts of neural ontogeny and growth 11
3 Early studies on regeneration of sympathetic postganglionic neurons 12
4 Transplantation technique 13
5 Aim of the present study 14

II MATERIAL AND METHODS 16

1 Animals 16
2. Transplantation techniques 16
3 Postoperative in vivo observations 18
4 Dissection procedures 20
5 In vivo treatments with drugs 20
6. In vitro incubations 21
7 Histochemistry 21
8. Isotope measurements 22
9 Evaluation of fluorescence microscopical judgements 22
10. Functional experiments 24

III REINNERVATION OF IRIDES TRANSPLANTED TO THE ANTERIOR CHAMBER OF THE EYE 25

1 Time course of reinnervation of iris transplants and the effects on the host irides 25
2. Control experiments 31
3 Discussion 32

IV REINNERVATION OF IRIDES FROM SYMPATHETIC GANGLIA TRANSPLANTED TO THE ANTERIOR CHAMBER 36
1 Time course of reinnervation of the iris and appearence of the ipsilaterally transplanted superior cervical ganglion 36
2. Transplantation of other sympathetic ganglia 40
3 Discussion 42

V TRANSMITTER MECHANISMS OF THE NEWLY FORMED NERVES 48
1 Iris transplants and their host irides 48
2. Ganglion transplants and irides reinnervated from them 52
3 Discussion 56

VI ATTEMPTS TO INFLUENCE THE GROWTH RATE OF THE ADRENERGIC NERVES 61
1 Outgrowth of adrenergic nerves to non-denervated irides from transplanted ganglia 61
2. Chronic treatment with some drugs affecting the adrenergic transmission 62
3 Chronic treatment with cortisone or testosterone 66
4 Effects of certain decentralization and denervation experiments 67
5 Discussion 69

VII RESTITUTION OF ADRENERGIC NEUROTRANSMISSION IN THE ANTERIOR CHAMBER TRANSPLANTS 72
1 In vivo experiments on intestinal smooth muscle transplants 72
2. In vitro experiments of portal vein and vas deferens muscle strip transplants 74
3 Discussion 77

VIII COMPARATIVE TRANSPLANTATION EXPERIMENTS USING DIFFERENT TISSUES, LOCATIONS AND SPECIES 79
1 Transplantation of other organs to the anterior chamber 79
2. Confirmatory experiments on the growth of adrenergic nerves in hamster and mouse 81

3 Heterologous transplantations 81
4 Transplantation of the anterior half of the eye to different locations 82
5 Special transplantations of the superior cervical ganglion e.g. to different locations 8
6. Transplantation of adrenal medulla to the anterior chamber 84
7 Discussion 85

IX GENERAL DISCUSSION 88
1 Methodological considerations 88
2. Normal innervation 90
3 Growth mechanisms of the adrenergic nerves 91

X SUMMARY 98
Acknowledgements 100
References 101
Microphotographs 112

Abbreviations

CA catecholamine (s)
NA noradrenaline
MAO monoamine oxidase
DMI desipramine hydrochloride
cpm counts per minute

I INTRODUCTION

1 Preface

The growth characteristics of the sympathetic nervous system are much less well-known than the growth of *e g* sensory and skeleto-motor nerves. Yet, growth of the postganglionic sympathetic adrenergic nerves can be assumed to be an important process also in the adult individual. Regeneration of adrenergic nerves after lesions of the axons as well as ingrowth of new adrenergic nerves into regenerating and transplanted tissues and organs constitutes one of the necessities for reestablishment of proper function in denervated tissues.

The relatively limited knowledge concerning growing adrenergic nerves in the organism has been due to the lack of specific histochemical methods. Earlier investigators have evaded this problem *e g* by isolating the sympathetic ganglia in tissue cultures or by using more indirect evidence for growth and regeneration such as the reappearance of the transmitter or other compounds of the axons in different organs as determined by biochemical methods. Also, non-specific nerve staining techniques have been used although it has sometimes been difficult to interprete results from such investigations.

The introduction in 1962 of the Falck Hillarp fluorescence technique for histochemical visualization of the transmitter distribution in the adrenergic nerves opened new possibilities for specific studies of these nerves in the organism. The normal extension and appearance of the adrenergic innervation apparatus could be determined with high precision at the light microscopical level. This knowledge constitutes the necessary background when growth of the adrenergic nerves is to be studied.

The present investigation is based upon transplantation of tissues to the anterior chamber of the eye in the rat (Textfig 1). When organs or tissues are transplanted, new adrenergic nerves will grow over from the host iris to the transplant. When sympathetic ganglion cell bodies are transplanted, regenerating axons will instead grow out into the iris of the host eye. This model system is investigated with the histochemical fluorescence techni-

que in combination with isotope uptake measurements and pharmacological and physiological methods.

The morphology pharmacology and function of the newly formed nerv es is described. Qualitative and quantitative information concerning rege- neration and reinnervation by adrenergic nerves in the adult animal is obtained. The specificity of the growing nerves vis-a-vi the effector organs as well as problems concerning the regulation of growth and factors that influence growth will be elucidated.

2. Founders of the present concepts of neuronal ontogeny and growth

In 1839 Schwann put forward a theory of nerve fibre formation, the s.c. "cell-chain hypothesis" stating that one nerve fibre originated by fusion of several aligned cells. This idea survived for several decades but was successively replaced by the "outgrowth theory according to which all peripheral nerves originate from the central nervous system from which the fibres (not cells) grow out into the periphery Bidder and Kupffer (1857) realized that the fibres of the white matter of the spinal cord derived from the cells of the grey matter Kölliker (1879) concluded that Schwann cells and nerve fibres were of different origin and His (1887) postulated that each nerve fibre derives from a single cell, the neuroblast. Later on Cajal definitely settled the question by giving strong evidence to the now generally accepted "neuron doctrine" formulated by Waldeyer (1891) Cajal also introduced the concept of "neurotropism — Chiefly through the monumental work of Cajal (1928) using the Golgi (1873) tech nique, the classical histological features of nerve degeneration and regene ration were established. Cajal was able to demonstrate not only axons but also dendrites and the principle organization of these elements in relation to the cell bodies in his embryological studies on the chick embryo spinal cord

generates. New nerves grow over from the host iris to the transplant.

c. Standard ganglion transplantation. The superior cervical ganglion is extirpated and transplanted to the anterior chamber of the ipsilateral eye. The original plexus of adrenergic nerves in the host iris degenerates. New nerves grow out from the transplanted ganglion and reinnerv te the host iris.

d. Ganglion transplantation to an intact eye. The superior cervical ganglion is extirpated and transplanted to the anterior chamber of the contralateral eye. The normal plexus of adrenergic nerves in the host iris is thus left intact. New nerves will grow out from the transplanted ganglion but to a much smaller extent than in model 3

(1890). Experimental neuroembryology was further developed by Harrison using amphibians and transplantation techniques. Harrison also developed the technique of tissue culture and was able to show that nerve fibres can grow out without Schwann cells or any other cellular support (Harrison 1910). Spiedel (1932, 1933) in his "studies on living nerves" fully confirmed the findings of Harrison and vividly described the ameboid movements of the advancing axon tips. Weiss introduced the concept of "contact guidance" (1941) as a complement to the theory of "neurotropism" put forward by Cajal. Weiss and Hiscoe (1948) convincingly demonstrated the axonal flow which was shown to occur continuously also in mature nerve fibres.

3 Early studies on regeneration of sympathetic postganglionic neurons

As late as 1960 Hillarp, in his comprehensive review on peripheral autonomic mechanisms, had to state: "The data hitherto reported concerning the various aspects of regeneration in the autonomic nervous system are incomplete, unsystematic and often highly contradictory". This is especially true of postganglionic sympathetic nerves because most earlier studies have dealt with regeneration of preganglionic fibres and the reestablishment of ganglionic transmission (Langley 1895, see also *e.g.* Lee 1930, Brücke 1931).

Regeneration of the postganglionic sympathetic nerves after axotomy above the superior cervical ganglion has been studied functionally by following the disappearance of Horner's syndrome (Horner 1869), the temperature changes of the ear and the response to stimulation of the preganglionic trunk in cats and rabbits (Tucket 1895—96, Langley 1897, Mashida 1929, Simeone 1938, see also review by Guth 1956). It can be concluded from these investigations that although a number of neurons succumb to the close axotomy, regeneration does occur. It takes, however, much longer time than preganglionic regeneration and is often incomplete. Histological examinations have been few. Mashida (1929) described regenerating fibres at the site of lesion above the superior cervical ganglion at the 14th day postoperatively. Ingrowth of regenerating sympathetic fibres into scar tissue was likewise observed by de Castro (1931).

Observations of growing sympathetic nerves in the living state were described by Matsumoto (1920) using vitally stained tissue cultures. Remarkably this investigator observed movements of mitochondria and vitally stained granules within the nerve fibres and stated that the granules

"exhibited considerable movement, especially in a longitudinal direction along the fibre and at the periphery" — perhaps an early description of axonal flow in growing sympathetic neurons.

Marinesco and Minea (1907) and Marinesco (1907) have described homologous transplantations of sympathetic ganglia subcutaneously or into the liver. The ganglion cells were reported to survive for a few days after which neurofibrillar degeneration began. Newly formed sprouts were not described. Similar experiments were subsequently performed by Cajal and Cardenal (Cajal 1928). To quote Cajal: "Since the experiments of Marinesco, Cardenal and ourselves have made some attempts at grafting sympathetic ganglia with not very brilliant results. In general if the ganglion comes from a young animal, the cells retain their condition well until eight or ten days after the operation. As in the spinal ganglia, the central neurons become necrobiosed; on the other hand, two or three rows of the marginal cells maintain their normal stainability and structure, and fine and pale neurofibrils are visible. The method of Nissl shows in the protoplasm delicate chromatin granules. It is difficult to establish whether among the expansions seen some are newly formed. Cajal, however concludes: "the sympathetic ganglia survive grafting, like the sensory cells of spinal ganglia. Like them also they are capable of producing, though less abundantly, sprouts, balls, and other structures and phenomena that reveal an attempt on the part of the neuronal protoplasm to accomodate itself to the abnormal environment and to turn it to the best account. Perhaps it is the satellite cells that take the initiative in this compensatory reaction; their number is much increased in the cells in which supcapsular expansions are formed."

4 Transplantation technique

The technique of tissue transplantation suggests itself as a tool for experimental *in vivo* studies when investigating the growth characteristics of adrenergic nerves.

Various transplantation techniques were tried on rats in an explorative survey of different possibilities. The purpose was to find simple, reproducible models where results could be quantitated in terms of amount of reinnervation. It should also be possible to study the influence of various experimental procedures on the nerve growth as well as the pharmacology and function of these nerves.

The anterior chamber of the eye has been widely used as a site of transplantation both in lower and higher animals. It offers a series of

advantages when compared to other areas used for transplantation such as the spleen, renal capsule, omentum rectus muscle or subcutis. The anterior chamber provides a suitable space which does not become obliterated by proliferating connective tissue. The condition of the transplants can be continuously followed in the living animal through the cornea. The aqueous humor constitutes a relatively good nutritive milieu and local injections can be performed. Furthermore, it is known since long that the eye is an immunologically privileged site (see Woodruff and Woodruff 1949 Raju and Grogan 1969 Waltman Burde and Faulkner 1969) and transplantations to the anterior chamber have therefore been used for a number of purposes such as endocrinological (e g Schochet 1920 Goodman 1934 Falck 1959) or to study heterologous tumor growth (see Krementz and Greene 1953).

It was soon found that an autologous transplantation of the left iris to the right eye in the rat performed as described in IL2 constituted a reproducible model for reinnervation studies and that the graft as well as the host iris could be easily studied with the fluorescence technique by means of stretch preparations. As described in a preliminary report (Malmfors and Olson 1967) a new adrenergic ground plexus was formed in the transplant under these circumstances. Transplantation of sympathetic ganglia were also tried initially In spite of the close axotomy the ganglion cells were found to respond with a vigourous regeneration when transplanted to the ipsilateral, and hence sympathetically denervated, iris of the host eye.

The promising results from the initial iris and ganglion transplantations prompted experiments with a large variety of other tissues (see VIII) Out of the different transplantation experiments tried it was decided to use the iris and the superior cervical ganglion transplantations to the anterior chamber for a closer investigation of some qualitative and quantitative aspects of the nerve growth.

5 Aim of the present study

Summarizing the introductory paragraphs above the following fields of interest of the present study can be listed:

1 The possibilities for growth of sympathetic adrenergic nerves into transplanted tissues or from transplanted ganglia to its local surroundings in the adult animal.

2. A qualitative morphological description of the growing adrenergic nerves reinnervating different tissues.

3 A quantitative determination of the rate of growth of the adrenergic nerves.

4 The transmitter pharmacology of the newly formed nerves with regard to the uptake, storage and release of NA.

5 The possible influence on the nervous growth rate of different experimental and pharmacological procedures.

6 The reestablishment of functional neurotransmission between the newly formed nerves and the reinnervated tissues.

II MATERIAL AND METHODS

1 Animals[1]

About 1700 adult albino rats of the Sprague-Dawley stem were used. Females weighing mostly 200 g were used except when the experiments required the use of males. In comparative experiments a few mice (NMRI) hamsters and newborn rats were used. These animals were also albinos.

2. Transplantation techniques

Autologous transplantation of the iris to the anterior chamber of the contralateral eye.

This method will be described in detail. Other methods will be described only to the extent that they differ from the following scheme. The animal was anaesthetized with ether and the vibrissae cut off. The right eye was atropinized (1 drop of atropine, 10 mg/ml, applied to the cornea). The left eye was removed and a haemostatic gelatin pad was placed in the orbit and the eyelids clipped together. The eyeball was carefully washed with cold sterile Ringer solution and the iris[2] dissected free (Malmfors 1965a) on an ice-chilled glass plate, covered with Krebs-Ringer solution.

The right eye and its surroundings were carefully washed with sterile Ringer solution. A small incision, 2—3 mm long, was made obliquely to the surface of the cornea of the right eye with a small piece of a razor blade in a peang. The incision was made slightly anterior to the corneo-scleral junction. Cutting of the iris was avoided since the atropine treatment now caused maximal dilatation of the iris.

The isolated left iris was cut radially once and carefully sucked up together with Ringer solution as a string into a thin specially grounded

1 The term *normal* will be used in the following only to define the situation in unoperated and otherwise untreated animals when compared to operated or otherwise treated while the term *control* has different meanings in different experimental set-ups.

2. In the following, "iris" refers to the iris proper and the ciliary body which were always handled as one piece.

Pasteur pipette. The oblique, blunt tip of the pipette was inserted into the corneal slit and the transplant was injected into the anterior chamber of the recipient eye by means of a rubber stall attached to the pipette. The injection of a transplant in Ringer solution approximately restored the intraocular fluid volume after the loss caused by the corneal incision. When sometimes too large a volume was injected, the excess drained away when the pipette was slowly withdrawn. As the pipette was removed, the corneal slit closed immediately and the transplant could be manipulated into the desired position along the lateral margin of the anterior chamber by application of slight pressure to the cornea.

All operative procedures were performed under dissection microscopes, using sterile instruments, preparative plates and solutions. Homologous iris transplantations (from one rat to another) were performed in a similar way

Transplantations of sympathetic ganglia to the anterior chamber

The superior cervical ganglion was transplanted either auto- or homologously and either into an intact or into a sympathetically denervated eye. The ganglion was carefully exturpated, its capsule opened and carefully peeled off and the preganglionic and the two postganglionic nerve trunks cut close to the ganglion. It was then divided longitudinally with a razor blade. The two halves were sucked up into the transplantation pipette and injected into the anterior chamber of the recipient eye as described above. They were manipulated to diametrically opposite positions over the iris and away from the corneal slit.

Other sympathetic ganglia which were transplanted in a similar way included the lumbar sympathetic chain ganglia, th coeliac ganglion and some of the small hypogastric ganglia innervating the vas deferens. Smaller ganglia were neither decapsulated nor divided before being transferred to the anterior chamber When ganglia were transplanted to intact eyes (*cf* Textfig 1) the eyes were sometimes sympathetically denervated 24—48 hrs before preparation by exturpation of the ipsilateral superior cervical ganglion. In this way only the adrenergic nerves belonging to the transplants were seen in the fluorescence microscope.

Transplantations of other tissues to the anterior chamber

Small pieces of several other tissues were prepared for transplantation and injected as described above. Adrenal medulla: After decapsulation the adrenal medulla was prepared free from the cortex. Transplants were trimmed to a size of about $^1/_1 \times {}^1/_3 \times 2$ mm. Salivary gland: Small pieces from decap-

sulated apical lobules of the submaxillary and sublingual glands were trimmed to the same size as the adrenal medulla. Smooth muscle strips: Thin strips, about 3 mm in length, were prepared from the following tissues: Small intestine: The outer longitudinal and inner circular muscle layers with the plexus of Auerbach in between was peeled off from the mucosa and used for the strips. Vas deferens: After removal of the serosa and mucosa, strips were prepared by two longitudinal radial cuts so that each strip contained a thin part of the whole width of the muscle layer. Portal vein: 5 mm longitudinal strips were taken from the vein. They included the external longitudinal muscle layer which is present in this vessel. Uterus: After removal of the endometrium, longitudinal strips were prepared from one horn of the organ.

Transplantation of the anterior half of the eye.

The eyeball was removed and divided just behind the corneo-scleral junction. The lens and lens capsule was removed from the anterior half. The remaining anterior part of the eye, including the iris, was then placed in the required position and glued to the underlying tissues by applying a small amount of plastic adhesive (isobutylcyanocrylate I. Ethicon Inc., U.S.A.) around the cut edge of the sclera, thereby connecting it to the underlying tissues. The anterior part of the eye was transplanted in this manner to 1) the decapsulated surface of the submaxillary gland, 2) the cut end of the vas deferens, and 3) the proximal stump of the cut sciatic nerve.

Transplantation of the superior cervical ganglion to other locations.

By means of the described glass pipette decapsulated ganglia were transplanted to 1) the subconjuctival tissue of the eye, 2) the sciatic nerve through a small whole in the epineurium to the inner part of the nerve.

3 Postoperative in vivo observations

By using a dissection microscope, the anterior chamber of the eye can be easily examined in the living animal keeping the animal slightly anaesthesized with ether. Thus, the condition of the aqueous humour, sclera, cornea, lens and the iris of the recipient eye as well as the transplant could be followed. The typical changes following a transplantation and the various complications which sometimes occurred were the following.

Twenty four hours after the operation most transplants had a dark blue-red colour. At the same time the plexus of blood vessels in the

host iris became more prominent while the cornea was somewhat blurred and whitish. No vascular connection between the transplant and host iris could be observed.

The pattern was similar after 48 hrs. The vascular reaction in the host iris persisted. The colour of the transplant was, however often much weaker

Four days after the transplantation, most eyes were clear and without signs of irritation. The transplants were vascularized by vessels running in thin tissue strands between the host and the transplant. The circulation of individual red blood corpuscles could be directly observed. The vascularization of the transplant became more prominent during the following days. This pattern then remained even during the longest periods studied (10 months)

Aberrations from the described scheme sometimes occurred. The complications were of varying seriousness but a few eyes (1 to 3) from each experiment (*e g* 20 eyes) were often excluded. Aberrations included

a. Faulty localization of transplant: The transplant was sometimes distorted or fastened at the pupil edge or even below the host iris, instead of being located at the ideal site above the host iris, at its peripheral border Rarely no connections at all were established with the host iris, and the transplant then became vascularized via the cornea, which in turn had become vascularized itself

b. Lens damage: This probably occurred when the edge of the razor blade injured the lens or after an infection. The lens became white and opaque and was later partially resorbed.

c. Intraocular bleeding: Bleeding was encountered if the host iris was injured with the edge of the razor blade. A small haemorrhage was soon resorbed. Heavy bleeding, apart from making the injection of the transplant difficult, caused fibrotizations and impaired nutrition of the transplant. Such eyes were always discarded.

d Infections: Sometimes the blurred cornea never cleared up after the transplantation and the content of the anterior chamber became opaque and deep red blue as a sign of an infection. The transplant remained discoloured and the cornea became vascularized. The fate of the infections varied but sometimes caused prolapses, micro-ophtalmy or macro-ophtalmy fibrous and changes of all components of the eye. Such eyes were always discarded.

4 Dissection procedures

At the time of sacrifice the eye was rapidly exturpated without compressing the eyebulb since this might reopen the corneal scar and cause the eye to collapse, especially after short post-operative periods. All dissection procedures were performed on ice-chilled plates covered with Krebs-Ringer solution. The eye was divided and the host iris and ciliary body with its attached transplant(s) freed from the anterior half of the eye in the same way as used for the routine preparations of irides (Malmfors 1965a) The host iris and the transplant were separated by tearing the two pieces apart using fine tipped watch makers tweezers. Usually this did not damage the tissues since the connective tissue bridges between the host iris and the transplant were fragile and thus broke up first. Sometimes, however a slight damage of the host iris was unvoidable especially after long postoperative intervals. When irides had been transplanted, the iris transplants had to be unfolded. This was done on a glass slide under a *high magnification stereoscopic microscope and using the fine tweezers.* By letting an identified part of the transplanted iris dry and thus fasten to the slide, the rest could usually be unfolded and stretched so that the transplant resembled a stretch prepared iris from a control animal. However a certain reduction in size of the dried and stretched specimen was always noted. If an *in vitro* incubation was to be done, the unfolding was performed after the incubation.

5 In vivo treatment with drugs

The number of animals doses and times before killing are given in the respective chapters. The following drugs were administered *in vivo* either acutely in single doses or chronically in repeated doses during several days.

(—)—NA bitartrate monohydrate (Sigma): i v

Nialamide (Niamid® Pifzer) i.p.

Reserpine (Serpasil® Ciba) i p.

H44/68 the methylester of (±)-α-methyl p-tyrosine (doc. H. Corrodi AB Hässle) i.p

H59/64 the methylester of 3,α-dimethyl tyrosine (doc. H Corrodi AB Hässle) i p.

H56/28 1-(0-allylphenoxy) 3 isopropylamino-2 propanol (doc H. Corrodi, AB Hässle) i.p

Desipramine hydrochloride (Petrofran® Geigy)

Phenoxybenzamine hydrochloride (Dibenzyline® S K F)

Phentolamin methansulfon (Regitin® Ciba)
Isoproterenol sulphate.

All drugs except nialamide and reserpine were dissolved in 0.9 per cent sodium chloride and calculated as the salts. NA was calculated as the free base.

6. In vitro incubations

In most transplantation experiments the irides of the host eyes and the transplants when these consisted of ganglia or irides and the control irides were incubated in a medium containing ^{3}H NA. The tissues were transferred to an ice-chilled medium which consisted of a Krebs-Ringer bicarbonate buffer (in g/liter: NaCl 6.92, KCl 0.35, $CaCl_2$ $6H_2O$ 0.28, KH_2PO_4 0.16, $MgSO_4$ $7H_2O$ 0.29, $NaHCO_3$ 2.10; ascorbic acid 0.2, glucose 1.8). Incubations were performed for 30 min at 37° C in a metabolic shaker using 1 ml of the medium, pH 7.4, saturated with 93,5 per cent and 6.5 per cent CO_2 and in an atmosphere of the same composition. Unlabelled medium contained 10^{-6}—10^{-7} *l* NA. Labelled medium contained 10^{-6}—10^{-7}M *d,l* NA with added *d,l* 7-^{3}H NA (New England Nuclear Corp. Boston, Mass. specific activity 1—2 and 5—10 C/mM respective). The tissues were rinsed for 10 min in isotope-free buffer. In some experiments the tissues were preincubated in 10^{-4}M DMI for 15 min before the addition of NA. For further details of the *in vitro* technique, see Hamberger 1967 and Jonsson, Hamberger, Malmfors and Sachs 1969. Following the incubation irides were prepared as whole mounts for fluorescence microscopy as described below. The ganglia incubated were directly combusted or solubilized for radioactivity determination (see below).

7. Histochemistry

The fluorescence method of Falck and Hillarp (development of the present method: Ehringer 1955; Falck, Hillarp, Thieme and Torp 1962; Falck 1962; Carlsson, Falck and Hillarp 1962; Falck and Owman 1965; Hillarp, Fuxe and Dahlström 1966; Hamberger 1967; Corrodi and Jonsson 1967) was used on two types of preparations. 1. Stretch preparations. All irides, including the transplants were treated as stretch preparations according to Malmfors (1965a). 2. Freeze-drying. All tissues except irides were freeze dried in a simple high capacity freeze-drier (Olson and Ungerstedt 1970) which gives good preservation of morphology with short drying times. Specimens were then reacted with formaldehyde of optimum humidity (Hamberger 1967) and embedded in paraffin *in vacuo* before manipulation.

of the individual tissue pieces took place The specimens were then further embedded, sectioned (8μ) and mounted for fluorescence microscopy

8 Isotope measurements

More than 95 per cent of radioactivity recovered in the rat iris after incubation in ^{3}H NA as described above is due to ^{3}H NA (Jonsson, Hamberger Malmfors and Sachs 1969) The corresponding value for ^{3}H NA incubated ganglia has been estimated to be about 80 per cent (unpublished results) The total radioactivity in the incubated specimens was therefore used as a measurement of the ^{3}H NA-content in most cases.

After fluorescence microscopy the irides were scraped off the slides with a razor blade and the radioactivity determined (Olson Hamberger Jonsson and Malmfors 1968) In most cases, specimens were combusted to water and CO_2 in an oxygen atmosphere and the radioactivity determined (Gupta 1966, Maurer 1968) in a liquid scintillation spectrometer (Packard Tri-Carb 3002) Two others means of determining the total radioactivity were also used In the early experiments, irides were homogenized in butanol/hydrochloric acid and an aliquot of the extract counted in a toluene-ethanol scintillation solution (see Jonsson, Hamberger Malmfors and Sachs 1969) This method showed the same good recovery as the combustion technique. Recently a new simple technique with high efficiency was introduced. The specimens were dissolved in 0.5 ml of a special sample solubilizer containing a strong organic base (Soluene™ 100 Packard Instr Co) directly in the counting vial. The technique was applied to the latter part of the present study It was found that dried specimens treated with paraformaldehyde gas required a longer solubilization time than fresh tissues and they were thus left over night at 50°C in the solubilizer whereafter they were usually completely dissolved. Ten ml toluene scintillation solution was then added.

9 Evaluation of fluorescence microscopical judgements

For quantitative estimations of the amount of nerves on the irides the fluorescence method was combined with ^{3}H NA incubations (see II.8 and Olson Hamberger Jonsson and Malmfors 1968) The stretch preparations were always coded often looked upon by both authors independently and always compared to irides from normal animals. The accuracy of the fluorescence histochemical estimations of amounts of nerves was tested by th following experiment

Partial denervations of irides were performed by bilateral excision of smaller or larger parts of the superior cervical ganglion in 15 rats 1 7 or 14 days before killing. The irides were incubated in ^{3}H NA and then prepared for fluorescence microscopy The amount of nerves remaining in the partially denervated irides was estimated and expressed as a percentage of the normal content (*cf* Fig 1) Finally the irides were scraped off and the radioactivity determined. Correlation coefficients were calculated for the judgements of the two authors, and for the mean percentage of nerves in each individual iris compared to the amount of radioactivity found. All these coefficients were found to be high, the latter *e g* 0.91 and the former even higher

When the three postoperative times were compared to each other the percentage estimations of remaining nerves were approximately 50 per cent after 1 day 30 per cent after 7 days and 55 per cent after 14 days. This pattern is suggestive of an initial degeneration of damaged neurons during the first week and a compensatory hypertrophy of the remaining nerves during the second week ("collateral sprouting see Murray and Thompson 1957a) Since all three groups were equally well correlated to their respective ^{3}H NA uptake values, the experiment indicates that also the growing nerves have an essentially unchanged ^{3}H NA uptake.

It thus seems that in the partially denervated irides the number of nerves can reliably be subjectively estimated by a trained observer The uptake of ^{3}H NA was also very well correlated to the estimated amount of nerves in these partially denervated irides within the whole range from 0 to 100 per cent, which further support the accuracy of the quantitative estimations of the number of fluorescent nerves. It cannot *a priori* be assumed that the uptake of NA in the growing nerves of *e g* the iris transplants and the reinnervated irides is the same as that of the fully developed nerves but the partial denervation experiments make such an assumption likely Thus, if there is a high correlation between the two parameters also in the transplantation experiments, the uptake of ^{3}H NA can be used as a measurement of the amount of nerves. The isotope-parameter is easily obtained from irides, and is the only one easily obtainable in other organs than those which can be stretched as whole mounts.

In the host irides of th iris transplants it was early observed that the plexus of fluorescent fibres was denser than in a normal iris. This hyperinnervation had to be estimated and correlated to the uptake of ^{3}H NA. These estimations were more difficult to perform than those performed after partial denervation. The hyperinnervation was, however

of the individual tissue pieces took place. The specimens were then further embedded, sectioned (8μ) and mounted for fluorescence microscopy

8 Isotope measurements

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III REINNERVATION OF IRIDES TRANSPLANTED TO THE ANTERIOR CHAMBER OF THE EYE

Initial experiments showed that an iris grafted to the anterior chamber of the contralateral eye received a new plexus of adrenergic nerves which grew out from the adrenergic nerve plexus of the host iris (Malmfors and Olson 1967) It was decided to use this experimental model in a closer investigation of the fluorescence morphology of the outgrowing adrenergic nerves and to quantitate the rate of outgrowth both with the histochemical and the biochemical techniques described. Furthermore, the observed hyperinnervation of the host irides called for a closer examination. For *in vivo* observations of the eye after transplantation refer to II.3

1 Time course of reinnervation of iris transplants and the effects on the host irides

Fluorescence microscopy (see Textfig 2)

Two days after the transplantation no clear effect on the host iris was noted. It was thus seldom possible to differentiate between host and control irides in the coded slides (Fig 2, 3) No fluorescent nerve fibres could be found in the transplanted iris at this time.

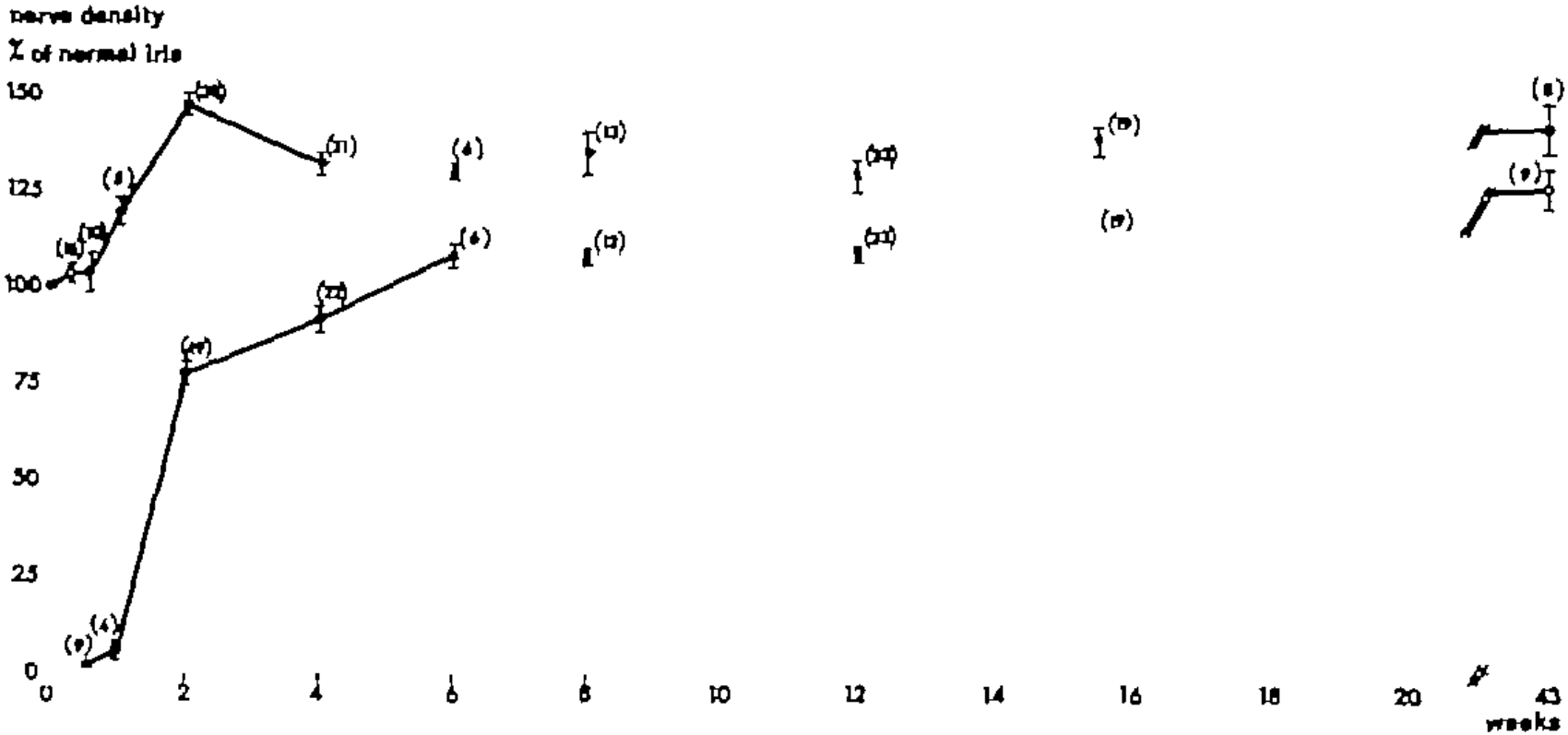
At four days, a reactive change in the host iris could be seen. While some of the host irides already showed a tendency of hyperinnervation (see II 9) *i* a somewhat denser plexus of fluorescent fib es than normally found, others had instead areas where the plexus of fluorescent nerves was less pronounced. Moreover the fluorescence intensity of the host irides was often slightly lower than normally The first few arborizing fibres were constantly found in the 4 days old transplant. At this time, a new circulation was also reestablished as described in II.3 The ingrowing fibres looked thinner and had a weaker fluorescence intensity than the adrenergic nerve terminals of the host iris. They were rather smooth with irregularly spaced small ovoid enlargements and were sometimes found to terminate with small drop-like enlargements.

One week postoperatively a slight hyperinnervation was evident in host irides. In the transplants, small areas of plexus forming nerves were found at about 2—6 different sites, and were judged as representing roughly 5—10 per cent of the normal number of nerves (Fig 5) The nerves forming a plexus now looked more like the varicose nerve terminals found in irides from normal animals.

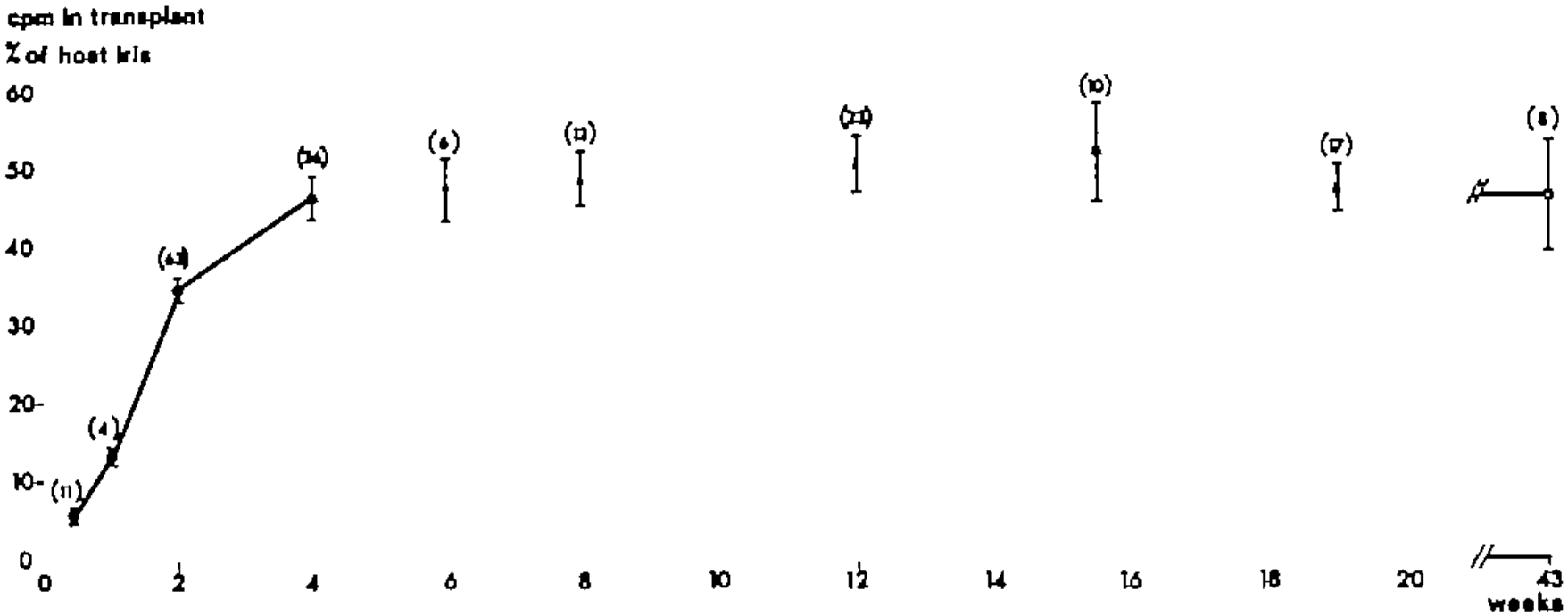
After two weeks the hyperinnervation of the host irides was judged as representing 150 per cent of the normal density (Fig 7 9) The network of nerve terminals was denser and in addition to this a second, wide-meshed plexus of fluorescent axon bundles and single axons was observed, especially towards the sphincter margin. The iris transplants had a well developed plexus of varicose adrenergic nerves. The plexus covered the whole iris in most specimens. The variation in density observed was due to the differing size of the meshes in the fibre network and to the number of fibres in each strand. The average density of the plexus was judged as about 80 per cent of the normal density with a range of 50 to 100 per cent (Fig 6 8) Axon bundles were found in the host irides. They could be followed through the connective tissue strands between the host and transplants when stretch preparations were made without separation of the two irides *cf* (Fig 4 5) These bundles were seen giving off branches over the transplant. The fluorescence morphology of the individual non terminal axons in the connections was characterized by bulgy and somewhat irregular ovoid enlargements with varying, but comparatively short, thinner parts in between The fluorescence intensity of the axons was almost as high as that found in the nerve terminals of normal irides.

At two weeks it was still relatively easy both to separate the transplant from the host iris and to unfold the transplant and make a stretch preparation. The sphincter margin the dilator area, the ciliary body and parts of the choroid were easily identifiable also in the transplant. The adrenergic innervation at the border between the ciliary body and the choroid is very specifically organized with short, radially running, almost parallel single fibres which end without any ramifications and all at the same distance from the ciliary body This pattern was exactly reestablished in the transplants (*cf* Fig 36)

In preparing and stretching the transplants it was invariably noted that the total areas of the transplants had decreased in spite of good stretching This decrease became more marked at longer times and was probably mainly due to the difficulties in unfolding and stretching older transplants. An actual loss of specific tissue mass might however also very well have occurred



2. Time course of reinnervation of irides autologously transplanted to the anterior chamber and the effect on the host irides as observed in the fluorescence microscope. The density of the nerve plexus after different times was estimated on coded slides as per cent of the density in normal irides (see II.9). At each point the mean ± s.e.m. is given. Number of observations within brackets. Upper curve: Host irides. Lower curve: Iris transplants. The first few fluorescent fibres were found in the transplants after four days (seven out of nine transplants)



4 Ratio between the radioactivity recovered in irides autologously transplanted to the anterior chamber and their respective host irides after incubation in ^{3}H NA (10^{-5}M 30 min plus 10 min rinsing). From each eye, the cpm found in the transplant is expressed as per cent of the cpm in the host iris. At each time point, the mean ± s.e.m. is given. Number of eyes within brackets. When the values are expressed in this way the small variation in the mean ^{3}H NA uptake of normal irides between different experiments does not influence the curve (*cf* Textfig 2, 3 5 and 6).

the relative growth rates of the nerves in the two irides. After a rapid increase this ratio levels off between 45 and 50 per cent from the fourth week on.

Homologous iris transplants were reinnervated in the same way as the autologous transplants described above. In a few experiments four irides were transplanted to one eye. All four transplants became reinnervated. After two weeks the total amount of nerves in the quadruple transplants clearly exceeded that of a single transplant.

2. Control experiments (see Table 1)

The hyperinnervation reaction of the host irides was first detected after iris transplantations. It was soon realized, however that the host irides reacted with a hyperinnervation following all types of transplantations, even when using non innervated tissues although these transplants remained non innervated (except for the vessel innervation). Furthermore, eyes showing signs of mild infection often revealed a more pronounced hyper innervation. However a serious and lasting infection led to a "less than normal" picture regarding all the three parameters, *i e* the number of nerves, their fluorescence intensity and the ^{3}H NA uptake.

Table 1

^{3}H NA uptake and density of the adrenergic nerve plexus in irides of control experiments.

Results are expressed as per cent of the corresponding values from irides from untreated animals in the same experiments ± s.e.m

Operation	Postoperative time (days)	number of irides	open	nerve density
Sham operations	14	8	100±14	114±8
Control side of unilateral homologous iris transplantation	28	6	104±9	100±0

It seemed feasible that at least a part of the increased number of nerves was due to the fibres growing over to the transplant. It could also be speculated that the same "factor" that caused nerves to grow into the transplant and make new terminal arborizations there also affected the nerves in the host iris. Finally as judged from the response to mild infections it seemed possible that even in the absence of any transplant the eye could react to a local irritation with an increase in the density of the adrenergic nerve plexus in the iris.

Two types of experiments were performed

1 Sham operations. These included all steps of the normal transplantation procedure except that the pipette contained only the Ringer solution when introduced into the eye.

2. Unilateral homologous iris transplantations where the irides of the unoperated eyes were compared to irides from normal animals.

The described operations did not cause any noticable mean change of the total uptake of ^{3}H NA in the irides (Table 1) A slightly increased variation between individual irides was however often encountered. This increased variation was reflected also in the fluorescence microscopical judgements. In the case of unilateral homologous iris transplantations the irides of the non-operated side were completely unaffected.

3 Discussion

The *in vivo* observations made it possible to follow the posttransplantation changes that occurred in the anterior chamber of the eye as well as in the transplant. Except for severe infections in a few cases, the tissue reactions were minor and fairly constant. During the first one to two days the transplant apparently had to rely upon nutrition from the aqueous humor while later a blood circulation was reestablished in the transplant. Any permanent changes in the transplanted tissue which might have occurred during the period of circulatory arrest was not examined in detail. It could however be expected that the thin iris would survive the transplantation well and there was no marked reduction in size of the transplant after longer times. The morphology of the transplant was always recognizable. Furthermore, also the thicker transplants used in this study clearly showed survival of e g smooth muscle cells (see VII) Not only autologous, but also homologous transplants survived well and showed no macroscopical signs of immunological responses.

The detachment of the iris to be transplanted from its normal site causes complete axotomy of all its nerves since no nerve cell bodies have been observed within the rat iris. Axotomy is known to be followed by a rapid disappearance of both adrenergic and cholinergic nerves (Malmfors and Sachs 1965a, Ehinger and Falck 1966, Roth and Richardson 1969) and probably also of other types of nerves possibly present. Consequently two days after transplantation, no fluorescent nerves could be found in the transplant.

Concomitantly to the reestablishment of circulation the first fluorescent nerves appeared in the transplant around the fourth day. These nerves could thus be safely regarded as *new* nerves. In fact, the new nerves could sometimes be followed all the way from the host iris through the connective tissue strands to the transplant.

Fluorescent growth cones (*cf* Blümcke and Niedorf 1965, Dahlström 1965, Malmfors and Olson 1967) were sometimes observed at the growing tips but the advancing fibres showed great morphological variations. It seems highly probable, that the fluorescence method reveals also the growing sympathetic nerves to their full extent (see Olson 1969).

From the attachments with the host iris the nerves grew to different parts of the transplanted iris. There seemed not to be any preferential direction or localization of the growing nerves. After the establishment of vascularization during the first week the growth rate increased to reach its highest level during the second postoperative week. After two weeks the iris transplants were well supplied with nerves and the growth slowed down as if the stimulus for growth had now decreased. It is less likely that a limited growth capacity caused the growth rate to slow down as judged from the experiment where four irides were transplanted to one eye. The total amount of nerves found in the four transplants of one eye clearly exceeded that found in the control with one transplant after the same time.

The exact origin of the new nerves could not be determined. It seems likely however that they were formed anywhere along the axon trees of the host iris, either as arborizations or as elongations of the very ends of the terminals (*cf* Fig 1).

At six weeks, the density of the nerve plexus in the transplant was similar to that of a normal iris while the uptake studies indicated only 70 per cent of the normal amount of nerves.

However the discrepancy between the fluorescence and isotope uptake parameters found can be explained if one considers th t the iris transplant had decreased somewhat in size. It therefo e contained a normal density

of fluorescent nerves but a smaller amount of fibres. After six weeks there was no further growth of the nerves. It thus seems as if steady state was reached around this time.

A conspicuous increase in the amount of nerves in the host irides was observed already in the preliminary experiments and it was therefore decided always to investigate the host irides together with the transplants in the present study. The density of the ground plexus varied from slightly less than normal to slightly more than normal during the first postoperative week. The fluorescence intensity and the NA uptake was somewhat decreased. These changes could be interpreted as reactions due to the operation which could cause disturbances in the homeostasis of the anterior chamber regardless of the transplant. After two weeks hyperinnervation (see II.9) was quite obvious, even if the exact number or amount of nerves could not be estimated. The NA uptake was also increased. The amount of nerves in the host irides seemed to reach a maximum after two weeks. The amount of nerves remained above the normal level during the whole period studied. The NA uptake was maximal after 4—8 weeks and then decreased somewhat. In both cases the level reached was about 25 per cent above that of a normal iris. — For a discussion of the correlation between the fluorescence findings and the radiometric results see General Discussion.

There are several possible explanations to the hyperinnervation. Some precautions and experiments were made in order to rule out certain of these alternatives. Normal irides were always taken from mates of the same sex, age and size as the operated animals. The increase in NA uptake which takes place during growth of the rats (unpublished results) could thus not be responsible for the difference in NA uptake between host irides and controls. Furthermore, sham operations did not reveal any hyperinnervation after two weeks which excludes the possibility of unspecific changes. The control irides from unoperated eyes of animals with unilateral homologous transplants were also essentially unchanged and it must therefore be concluded that the hyperinnervation is not somehow induced by a generally distributed humoral factor. The hyperinnervation was fairly uniformly distributed over the whole iris and it therefore seems unlikely that it is *only caused by the nerve fibres growing over to the transplant.*

The hyperinnervation of the host iris might be due either to a humoral factor reaching all the nerves in the host iris or to that the same factors that causes the nerves to grow in the transplant also stimulates the branches from the same neurons within the host iris to an overgrowth. It is known that the non terminal axons branch several times on the iris

surface and that each branch gives rise to a terminal plexus innervating a smaller relatively circumscribed area (Malmfors and Sachs 1965b) Thus several smaller areas often at considerable distances from each other are innervated by one single neuron. It does not seem unlikely then to suggest that a certain proportion of the neurons just add some axonal branches innervating the transplant and that the same neuron then probably innervates both the host iris and the transplant. The hyperinnervation coincides with the most rapid growth into the transplant which indicates that it is due to the same mechanism. While the amount of nerves reach a constant level in the transplant it has a tendency to decrease in the host iris. It may be that those nerve fibres which have grown in vain within the host irides degenerate, when the stimulus for growth from the transplant decreases. However a slight hyperinnervation of the host iris is present also 12—15 weeks after the transplantation.

The nerves in the host iris extend their field of innervation to include also the transplant. Such an enlargement of the area covered by a neuron is a well known phenomen occurring after partial denervation when the remaining intact neurons sproot into the denervated areas and it is referred to as collateral sprouting (see Edds 1953 Murray and Thompson 1956, 1957a, 1957b, *cf* also II 9) Thus, reinnervation of a transplanted effector organ may be more similar to collarteral sprouting than to regeneration after axotomy where several neurons just refill their old territories.

When an iris is transplanted to the anterior chamber of the eye it becomes rapidly reinnervated by the nerves in the host iris. The appearance, distributions and amount of the new adrenergic nerves are the same as normally At the same time there is an increase in the number of nerves in the host iris.

IV REINNERVATION OF IRIDES FROM SYMPATHETIC GANGLIA TRANSPLANTED TO THE ANTERIOR CHAMBER

Since the initial experiments had proven that sympathetic ganglia survived well in the anterior chamber and showed a vigorous axonal outgrowth onto denervated host irides, it was decided to quantitate the rate of outgrowth from the superior cervical ganglion to the iris in the same way as in the iris transplantation experiments. When the basic parameters of outgrowth had been established in the standardized preparation, experiments could be undertaken in order to influence this outgrowth

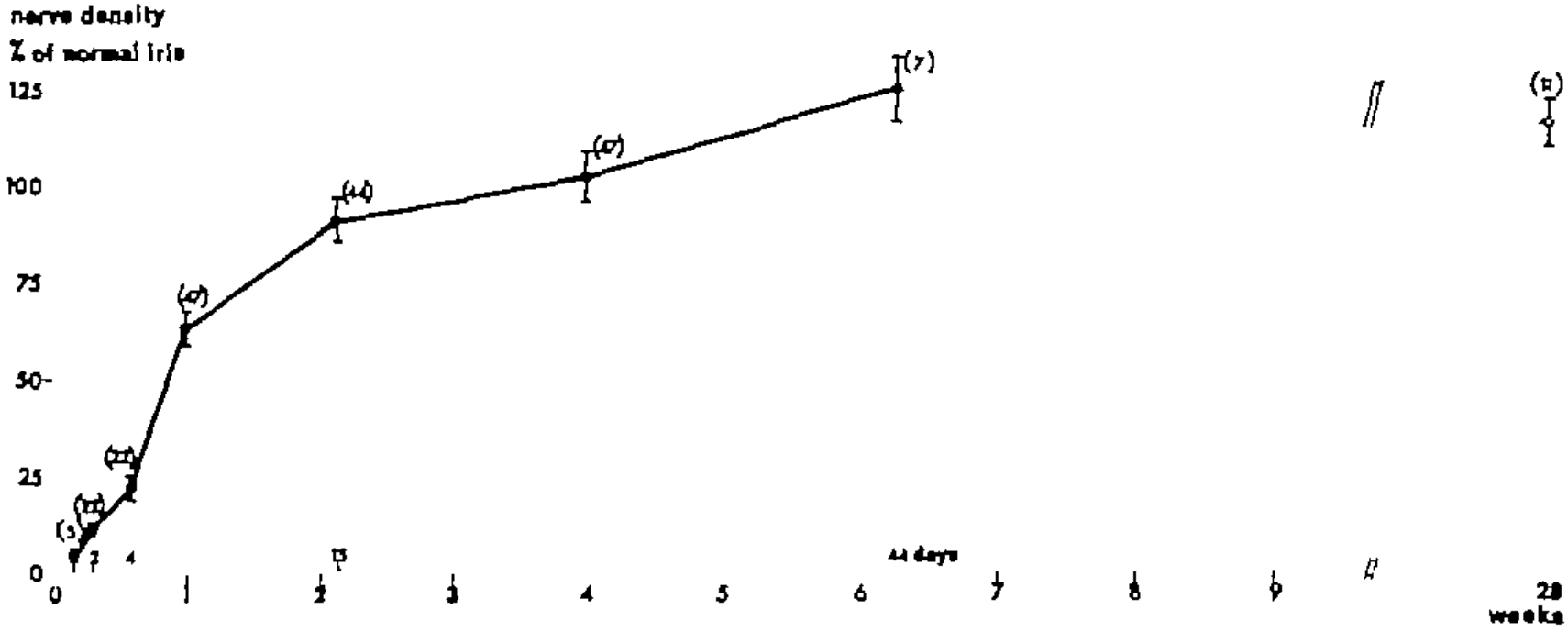
Initially several different decapsulated preparations of the superior cervical ganglion were tried including whole ganglia, cranial halves, caudal halves, and longitudinally divided specimens. Also non decapsulated ganglia were tried. Although all these preparations were found to be able to reinnervate host irides, the most consistent results were obtained by using the two decapsulated longitudinal halves of one ganglion in each eye. This preparation was therefore used in the quantitative experiments.

The condition of the transplanted ganglia followed the general scheme given in II.3 The vascularization of the two halves of the ganglion was a rapid process, evident at four days but probably present to some extent earlier

1 Time course of reinnervation of the denervated iris and appearance of the ipsilaterally transplanted superior cervical ganglion

Fluorescence microscopy (see Textfig 5).

Host irides. A new plexus of adrenergic nerves was rapidly formed on the host iris. The first few arborizing fibres were observed already at the first postoperative day (Fig 18) The new nerves were found in small circumscribed areas at the sites of attachment of the transplants. The morphology on the newly formed nerves was extremely varying. Thinner and thicker fibres, commonly with irregular enlargements and varying



5 Time course of outgrowth of adrenergic nerves in host irides after unilateral transplantation of the superior cervical ganglion to the anterior chamber of the eye as observed in the fluorescence microscope. The density of the nerve plexus was estimated on coded slides as per cent of the density in normal irides (see II 9). At each point the mean ± s.e.m. is given. Number of observations within brackets.

fluorescence intensities, were intermingled with some very thick, distorted and heavily fluorescent fibres (Fig 18) The *one day* host irides contained about 5% of the normal amount of nerves. This amount increased in a regular manner during the subsequent days (second day 12 per cent fourth day 23 per cent) The morphology of the nerve fibres was still variable on the second and fourth day but the heavy accumulations of fluorescent material had disappeared (Fig 20 21 24) At *four days* the outgrowing adrenergic nerves were centered around the sites of attachment between transplants and host irides and the innervated areas extended all from the sphincter margin to the corpus ciliare. These areas were often well radially demarcated from the neighbouring non-innervated parts and contained fluorescent plexuses of medium fluorescence intensity but often higher density than found in normal animals. Between the *first and the second week* post operatively areas with a rather regular meshwork containing more than one fluorescent fibre in each strand of the plexus were formed (Fig 25 27) Some fibres now looked similar to normal adrenergic nerve terminals, *i.e* ovoid, highly fluorescent, regularly spaced varicosities were formed (Fig 13) After two weeks the amount of nerves on the reinnervated host irides was judged as being close to the normal amount. After four weeks the varicose appearance was more pronounced than after one week (*cf* Fig 25 32)

As the amount of nerves in the host iris increased, the site of attachment of the transplants became more and more prominent. Thick, compact bundles of fluorescent axons (Fig 28) spread on the iris around the transplant in a fan shaped arrangement (Fig 35) which did not resemble any preexisting pattern of innervation. Peripheral to this central region the fluorescent axons seemed to follow the old nerve trunks (*cf* Fig 37 38)

The high initial growth rate of the outgrowing nerves, as followed by fluorescence histochemistry gradually leveled off around the 100 per cent level after one month and then remained constant for at least several months. Apart from the fan-shaped site of origin of the fluorescent axons in the newly formed ground plexus, the general structure of the ground plexus after one month was similar to the normal picture (Fig 32, 33 34) Thus, the distributions of fluorescent nerves in the sphincter margin, the dilator area, the corpus ciliare and the choroid membrane including the ciliary choroid junction (Fig 38) all resembled those of normal irides. Certain dissimilarities, however made it possible always to distinguish a host iris from a normal one also without looking at the site of attachment of the transplant. The varicosities were often less marked and of a slightly lower fluorescence intensity in the host irides, and the ground plexus was less regular

Ganglion transplants. The most conspicuous difference between transplanted and normal ganglia was the great increase in the relative amount of fluorescent nerve fibres, as compared to the amount of nerve cell bodies in the transplants (cf Fig 16, 17 with 22, 23) This was obviously due to an actual increase in the number of fibres and probably also to a decrease in the number of nerve cell bodies. The change was first observed on the second postoperative day after which it became increasingly more prominent during the first week. After that there was very little change in the relative amount of fibres in the transplants.

The first signs of differences between normal and transplanted ganglia were observed already on the first postoperative day as an increased fluorescence intensity of most nerve fibres (Fig 19) Most nerve cell bodies displayed normal fluorescence intensities at this time, but cells with an increased fluorescence intensity and cells with a very low intensity were more common in the transplants. The increased fluorescence intensity of the nerve fibres persisted, while, from the second day on, most cell bodies showed a slightly less than normal fluorescence intensity The strong fluorescence of some cell bodies thus disappeared. Small areas containing non-fluorescent cell were found. These groups of non-fluorescent cells disappeared at later stages, although single non fluorescent cells were still found. Both in some fluorescent and some non-fluorescent cells exentric nuclei were found at all times, but after about 14 days most cells had centrally located nuclei. The so called SIF-cells ("small, intensely fluorescent cells" Norberg, Ritzén and Ungerstedt 1966, Eränkö and Härkönen 1963) were found in the transplants at all times and did not differ from those found in normal ganglia as viewed in the fluorescence microscope (*cf* Fig 16,30)

On the first postoperative day both highly fluorescent, swollen fibres and very thin weakly fluorescent ones were found (Fig 19) Soon, both these structures disappeared. Later large bundles of fibres were found traversing the ganglia in all directions. The fluorescence intensity of the bundles of fibres was higher than that of the cell bodies. Fibres showing both smooth and bulgy outlines were seen. At later stages, fluorescent fibres similar to normal nerve terminals were often found to surround individual cell bodies closely (Fig 22, 23 26, 29 30, 31 45)

^{3}H NA Incubations (see Textfig 6).

Reinnervated host irides. Incubations in ^{3}H NA were performed in the same way as described for the iris transplantation experiments. The radioactivity in the host irides increased with postoperative time in the same way as the amount of fluorescent nerves. Thus a rapid initial increase during the

first week was followed by a slower increase and a steady level was reached after about one month. When compared to normal irides the radioactivity found in reinnervated host irides was found to lie at a higher level then the estimated amount of nerves.

Ganglion transplants. When incubated in a decapsulated and bisected form, the superior cervical ganglia from normal animals were found to contain less than half the amount of total radioactivity of that found in the corresponding normal irides. After 1—2 days in the anterior chamber the radioactivity recovered from the ganglia, was about four times higher than the radioactivity in decapsulated bisected control ganglia. A clearcut drop from this level occurred at the fourth day after which the radioactivity increased again to reach a maximum of about six times the original uptake of about twice as much as that found in normal irides after two weeks. The latter peak was followed by a decrease which was most marked during the third and fourth week. The original low level was not reached within the time interval studied (6 1/2 months)

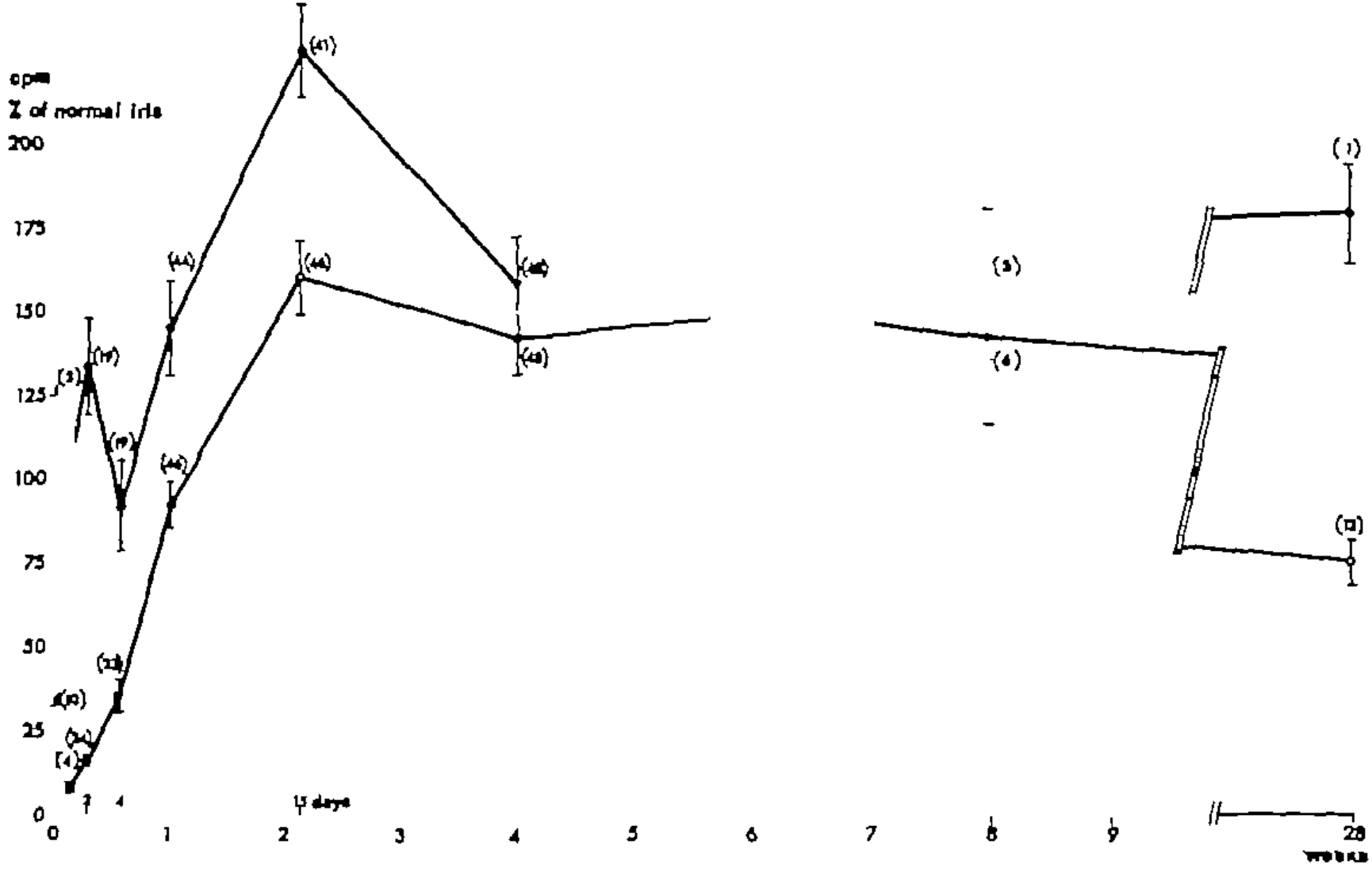
2. Transplantation of other sympathetic ganglia

The coeliac ganglion, lumbar sympathetic chain ganglia and small anterior chamber The superior cervical ganglia of these animals were extirpated in a second operation 1—2 days later The animals were sacrificed after 1,5—3 months.

A plexus of varicose adrenergic nerves, arising from the transplants, was formed on the host irides. However only the coeliac and the lumbar sympathetic chain ganglia were capable of reinnervating the whole host iris to an approximately normal amount (Fig 40) In these cases, also the blood vessels of the choroid membrane were fully reinnervated.

Typically a transplant of a small hypogastric ganglion gave rise to a small discrete area of fluorescent nerves around the site of attachment of the transplant. The rest of the iris and the choroid membrane were

6. Time course f outgrowth of adrenergic nerves in host irides after pailateral transplantation of the superior cervical ganglio as evealed by the ^{3}H NA uptake. Reinnervated host irides and ganglion transplants were incubated in H NA, 10^{-7}M, 30 min plus 10 min rinsing. Values are expressed as cpm in per cent of the cpm found in normal irides of the same batches, incubated in the same way and at the same time At each point the mean ± s.e.m. is gi en. Number of observa tions within brackets. Upper curve (left) Ganglion transpla ts. Lower curve (left) Reinnerv ted host irides.


opm
% of normal iris
200
175
150
125
100
75
50
25
0
0
1
2
3
4
5
6
7
8
9
28
weeks
15 days

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6. Time course of outgrowth of adrenergic nerves in host irides after unilateral transplantation of the superior cervical ganglion as revealed by the ^{3}H NA uptake. Reinnervated host irides and ganglion transplants were incubated in ^{3}H NA, 10^{-7}M, 30 min plus 10 min rinsing. Values are expressed as cpm in per cent of the cpm found in normal irides of the same batches, incubated in the same way and at the same time. At each point the mean ± s.e.m. is given. Number of observations within brackets. Upper curve (left) Ganglion transplants. Lower curve (left) Reinnervated host irides.

Whether the very first bundles of fluorescent fibres emerging from the transplant were accompanied by supporting Schwann cells from the transplant itself was not determined. Such an arrangement is however highly probable, since Schwann cells (forming the bands of Büngner Büngner 1891) as well as fibroblasts and possibly other cellular elements are known to proliferate vigorously after nerve injury (see Thomas 1966, Thomas and Jones 1967 Haftek and Thomas 1968)

The varicosities of the fluorescent fibres were less pronounced during the first week. This might be interpreted as immaturity since developing adrenergic nerves also lack the typical varicose appearance (Enemar Falck and Håkanson 1965 Friedman, Pool, Jacobowitz, Seagren and Braunwald 1968 Ehinger Gennser Owman, Persson and Sjöberg 1968 Schiebler and Heene 1968 Winckler 1969 Olson, de Champlain, Malmfors and Sachs 1970) The lack of typical varicosities, from which the transmitter is supposed to be released, indicates a lower NA content and perhaps also a smaller amount of amine storage granules in the outgrowing nerves. It is not known at present how the absence of distinctly fluorescent varicosities is related to the distribution of the amine storage granules. It may be that the high growth rate of the nerve fibres makes the amount of granules insufficient for a proper accumulation in the varicosities. When the growth rate slowed down after 2—4 weeks the varicosities became more distinct, which would be consistent with the above idea

From the site of attachment of the transplant, the new nerves grew in all directions and all parts of the iris became well innervated. After somewhat longer times fluorescent nerve fibres could be observed also in the anterior part of the choroid. The very typical appearance and distribution of the innervation of the border between the ciliary body and the choroid was inseparable from the normal pattern This fact strongly indicates that outgrowing adrenergic nerves grow to the same positions as the original adrenergic nerves. If this is due to guidance from the remaining Schwann cells or to specific attraction from the effector tissue or to information within the adrenergic neuron remains to be disclosed. The last alternative seems unlikely as ganglion cells not normally innervating these areas easily repeat the same reinnervation pattern.

The resemblance between th reinnervation pattern and the normal distribution especially concerning the trunks of non terminal axons in the dilator muscle makes it likely that the outgrowing nerve fibres from the transplanted ganglion reached the remaining trunks of non-terminal cholinergic axons where they became enclosed in the old Schwann cells. Adrenergic nerves can be found much proximal to the attachment of the transplant

ed ganglion indicating that the new axons can grow in a direction opposite to that of the original fibres. This has also been shown when ganglia were transplanted to the sciatic nerve (Olson 1969 see also VIII.2)

Within four weeks the density of the ground plexus reached that of the *normal iris* However the areas close to the attachment of the transplant had a much denser plexus due to the fibres radiating from the site of attachment. This indicates that the retardation of the growth is at least not solely due to a limited capacity of the Schwann cells to harbour the nerves but probably to another regulating mechanism, possibly more directly coupled to the effector tissue.

The uptake of ^{3}H NA in the host iris was as high as in a normal iris already after one week when the amount of nerves was estimated to be only about 60 per cent. This discrepancy existed also at longer times and to the same extent. The most feasable explanation might be that the extra trunks of non terminal axons emerging from the ganglion transplant caused the increased NA uptake as they were not considered when estimating the density of the terminal ground plexus.

The growth rate of the nerves coming from a transplanted ganglion was similar to that of the nerves growing into a transplanted iris. Thus the transplanted ganglia showed a vigorous ability to reinnervate the denervated host iris in spite the fact that the axons were cut close to the nerve cell bodies. From the histological examination of the transplants it is clear that the ganglia underwent marked changes after transplantation Firstly the cut axons showed accumulations of NA (Dahlström and Fuxe 1964a, Owman 1964 Härkönen 1964) due to the continuous axonal flow (Weiss and Hiscoe 1948). During the first two days these accumulations obviously extended all the way to the cell bodies (Dahlström and Fuxe 1964a, Owman 1964 Jacobowitz and Woodward 1968) Secondly there was a progressive increase in the relative number of fluorescent fibres in the ganglion, obviously due to sprouting Thirdly several cell bodies showed the well-known sign of chromatolysis (see Bodian and Mellors 1945) and excentrically located nuclei. This observation and the somewhat decreased fluorescence intensity from the second day on might have been due to the initiation of regenerative cycle (*cf* Bodian and Mellors 1945), but possibly also to impaired nutrition before restitution of blood circulation. In some cases the changes must have been irreversible as there *seemed to be a marked reduction of the number* of cell bodies after longer times.

Several authors have studied the effect of postganglionic axotomy upon the superior cervical ganglion *in situ* The initial changes thus described

were found to be quite similar to the changes observed in the present investigation after transplantation of the ganglion to the anterior chamber of the eye.

Close postganglionic axotomy of the superior cervical ganglion is known to cause a marked decrease in the density and probably also number of nerve cells in the ganglion (*e g* Hillarp 1946, Shashurina 1963 Härkönen 1964 Nagata and Tsukada 1967). The superior cervical ganglion of the rat undergoes swelling and increases both its dry and wet weights about 50 per cent one week after cutting of its major postganglionic nerve as shown by Nagata and Tsukada (1967) The latter authors also described the apparant decrease in the number of nerve cells and ascribed the swelling to a marked proliferation of Schwann cells and other fibrous elements following the axotomy This shift in the ratio of nerve cell density to density of supportive tissue and fibrous elements is consistent with the fluorescence microscopical findings of the transplanted ganglia in the present investigation. The transplanted ganglia of course underwent an almost total axotomy leaving only the possibly existing intraganglionic axons and/or collaterals (Norberg and Sjöqvist 1966, Jacobowitz and Woodward 1968) intact. Surprisingly Nagata and Tsukada found the axotomized ganglia to have the same amino acid metabolism as normal ganglia, which is characterized by a dominance of aspartic and glutamic acids. This suggests that nevertheless a considerable number of neurons indeed remained in the ganglia after axotomy

Histometrical analysis of the actual number of nerve cells were neither performed in the present investigation nor by Nagata and Tsukada but it is obvious that the swelling of the ganglia will dilute the neuronal component thus making the nerve cells appear less frequently A peripheral zone of intact nerve cells were actually described by the latter authors and the present fluorescence histochemical results clearly demonstrates the presence of a number of noradrenaline neurons sufficient fo a more than complete reinnervation of the iris. Furthermore, the large increase in the number of fluorescent axons must add to the amount of nerve cell cytoplasm present after axotomy thus partially making up for th lost neurons.

The histochemistry of the axon reaction of th superior cervical ganglion following postganglionic axotomy has been thoroughly studied by Härkönen (1964) The lowered NA fluorescence intensity of most cell bodies described by Härkönen s in accordance with the present findings. However more servere and long lasting decreases of NA as well as of MAO and cholinesterases were noted by the latter author and led him to

suggest that "when contact with the effector organ is severed, these substances loose their importance and the energy normally required for their synthesis is perhaps then used for protein synthesis in the interest of regeneration" The present experiments does not support such an hypothesis as far as NA is concerned since, in the case of transplanted ganglia, complete disappearance of the transmittor from the cell bodies was rarely observed and reappearance of normal fluorescence intensities faster Furthermore, the axons of the transplanted ganglia were strongly fluorescent at all times. It has also been shown that sympathicoblasts display a considerable fluorescence intensity very early during ontogenesis (Enemar Falck and Håkansson 1965 Olson, de Champlain, Malmfors and Sachs 1970) when these cells hardly can be supposed to need NA for neuromuscular transmission. Thus, the accumulating evidence suggests that the synthesis and storage of the transmittor is an intrinsic mechanism of the neurons basically unrelated to and independant of the growth regulating mechanisms. Further support for this concept will be presented (see VI)

Although there was a reduced number of ganglion cells surviving the transplantation those that remained were more than enough needed for reinnervation of the iris. This is of course to be expected since the iris is normally only a part of the innervation area of the superior cervical ganglion. However when very small pieces of the ganglion were transplanted the ground plexus did not become as dense and complete as when the whole ganglion was transplanted. This demonstrates that a single adrenergic neuron indeed has a limited capacity for growth.

Not only the ipsilateral superior cervical ganglion from the same animal had the ability to reinnervate the iris when transplanted into the anterior chamber of the eye. Other autologously and homologously transplanted ganglia reinnervated a denervated host irides in a similar way No quantitative difference was observed between autologously and homologously transplanted superior cervical ganglia which illustrates the good compatibility between *adrenergic neurons and effector organs from different animals* of the present rat strain. Furthermore, the similarity in outgrowth between ganglia from different locations shows that there is no topographical specificity of the adrenergic neurons. Whether neurons from different types of sympathetic ganglia (paravertebral, prevertebral or even more peripherally located) have slightly different growth properties is unclear The fact that the new nerves coming from transplants of the small hypogastric ganglia formed a normally dense plexus only in close neighbourhood of the attachment while small pieces of the superior cervicale ganglion of approximately the same size gave rise to a plexus which extend-

ed over a larger area but was less dense might indicate a difference in properties between the two different types of ganglion cells. The nerve cells of the hypogastric ganglion have been called "short neurons" (see Sjöstrand 1965).

When any sympathetic ganglion is transplanted to the anterior chamber of the eye many nerve cells survive and axon regeneration starts. If the adrenergic nerves in the host iris have degenerated the host iris is rapidly reinnervated by the transplanted ganglion. The new adrenergic nerves are quantitatively and qualitatively similar to the normal ones independantly of which ganglion that has been transplanted.

V TRANSMITTER MECHANISMS OF THE NEWLY FORMED NERVES

The adrenergic transmitter mechanisms synthesis storage, release, uptake and metabolism of NA have been throughly studied in the adult organism with biochemical and isotope methods (for references see Axelrod 1964 Carlsson 1965 Iversen 1967 Jonsson, Hamberger Malmfors and Sachs 1969 Sachs 1970) and in the adrenergic nerves with the fluorescence histochemical method (Malmfors 1965a, Norberg 1965 Hamberger 1967) Similar information has recently been obtained about growing adrenergic nerves during ontogenesis (Iversen, de Champlain Glowinsky and Axelrod 1967 Sachs, de Champlain Malmfors and Olson 1970). The purpose of the present experiments was to study the transmitter mechanisms of growing noradrenaline nerves in the adult animal. This information was needed to be able to evaluate the functional experiments (see VII) and for a proper interpretation of the results of the ^{3}H NA incubations performed.

Iris transplants together with their host irides (two weeks) and ganglion transplants together with their reinnervated host irides (one and four weeks) were examined following *in vivo* treatment with the NA synthesis inhibitor H44/68 or reserpine and/or nialamide. Most experiments were carried out using the combined *in vitro* isotope-fluorescence method (Olson, Hamberger Jonsson and Malmfors 1968)

1 Iris transplants and their host irides

Fluorescence microscopy (see Table 2)

Twenty four hours after inhibition of NA synthesis with H44/68 only a very faint fluorescence was left in the adrenergic plexuses of the two irides while decentralization of the ipsilateral superior cervical ganglion, performed at the same time as the H44/68 injection, counteracted the depletion, leaving the nerves with essentially unchanged fluorescence intensities. Electric stimulation of the preganglionic sympathetic trunk (10 impulses per sec) during 80 min beginning one hour after H44/68 pretreatment caused a marked reduction of the fluorescence intensity compared to the

unstimulated side. This reduction was prominent in the varicosities of both transplants and host irides while the smoother nonterminal axons and the somewhat more beaded axons connecting the host iris and transplant were very little affected by the stimulation (*cf* Fig 51 52)

Table 2

Schematic representation of the fluorescence intensities in the newly formed adrenergic nerves and cell bodies following different in vivo drug treatments

Two weeks old iris transplants and their host irides and four weeks old ganglion transplants and the reinnervated host irides were used. Each estimation is based on 2—10 observations.

Treatment[1])	iris transplants and host irides	cell bodies of ganglion transplants	nerve fibres in ganglion transplants and reinnervated host irides
none	+++[2])	+++	+++
H44/68 24 hrs	(+)	+	++
+ decentralization[3])	++(+)		
+ NA	+++)		
140 min	+++		
+ electrical stimulation[4])	+[5])		
reserpine	0	(+)	0
+ NA	0-(+)	+	0-(+)
+ nialamide	0	0	0
" + NA	+++[2])	+++	+++

[1]) The doses and times before killing when not indicated in the table were *H44/68* 500 mg/kg i.p.; NA; 0.2-0.5 mg/kg, 15 min reserpine 10 mg/kg, 4 hrs, i.p.; nialamide 100 mg/kg, 2 hrs, p.

[2]) Symbols for the average fluorescence intensities +++ strong, ++ moderate; + weak; 0 none. The number of plus signs are not quantitatively related, but are comparable to controls in the same experiment. +++ corresponds roughly to the fluorescence intensity of an iris from an untreated rat. When the fluorescence intensity of an individual iris preparation could not be definitely determined as belonging to one intensity grade or to the next, this was indicated by bracketing the last plus sign of the higher grade. See also Olson, Hamberger Jonsson and Malmfors 1968.

[3]) for detailed description see text.

[4]) varicose appearance of fluorescent nerve terminals.

[5]) varicosities seemed preferentially depleted.

[6]) smooth appearance of fluorescent nerve terminals.

When an H44/68 treated rat was given NA (0.2—0.5 mg/kg i.v. 15 min before death) or the transplant and the host iris if such an animal were incubated in NA (10^{-7} — 10^{-6}M) the nerves regained a fluorescence intensity and appearance comparable to that of a control iris treated in the same way (Fig 41 42)

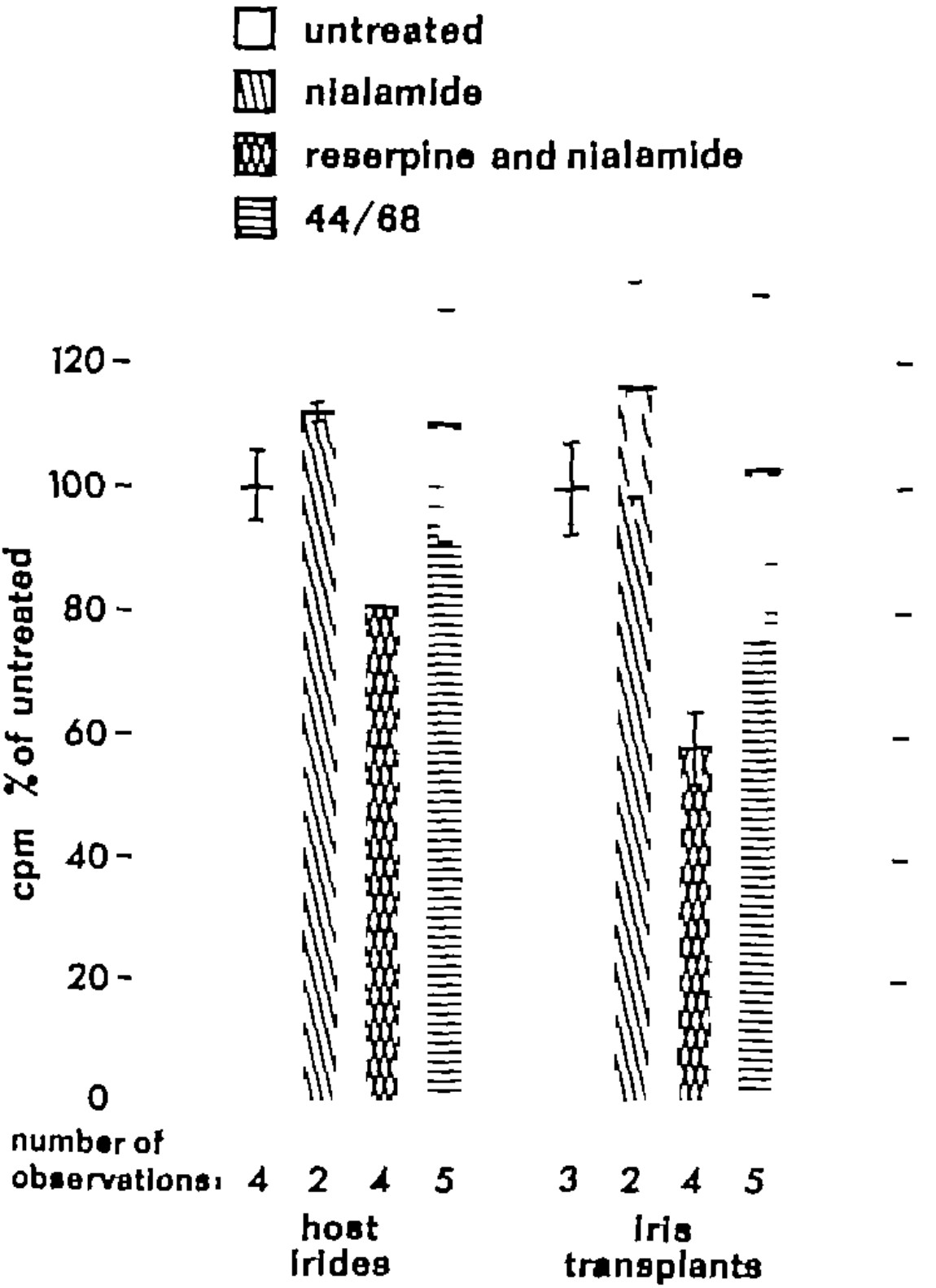
Reserpine (4—24 hrs) either used alone or in combination with nialamide (1—2 hrs) caused a complete disappearance of the neuronal fluorescence of both transplants and host irides. In tissues from animals treated with only reserpine no or almost no fluorescence restitution could be seen after *in vivo* or *in vitro* administration of NA. The addition of nialamide (1—2 hrs before the NA) caused a marked restitution of the fluorescence (Fig 43 44). However the appearance of the nerves after the reserpine-nialamide-NA treatment was different from normal. After the *in vivo* administration of NA, the nerve terminals showed the same smooth appearance and the non-terminal axons the same strong fluorescence in the host iris as earlier described for non-operated eyes (Malmfors 1965a). In the transplant the adrenergic nerve terminals were smoother than in the untreated but not as smooth as in the host iris. Furthermore, the non terminal axons, especially those in the connections between the host iris and iris transplant, had a somewhat beaded fluorescence, which was comparable in form to that of normal iris but of a stronger fluorescence intensity. When NA was administered *in vitro* the appearance was principally the same but the varicosities were somewhat more pronounced than after the *in vivo* treatment in the host irides, the transplants and the controls (*cf* Hamberger and Malmfors 1967).

The amount of nerves regaining fluorescence after reserpine and nialamide or H44/68 was about the same as in transplants and host irides from untreated rats. After preincubation in DMI (10^{-6}M) no restituted fluorescence could be observed following incubation.

^{3}H NA Incubations

Textfig 7 shows the radioactivity found, after ^{3}H NA incubation in the host irides and the transplanted irides after certain drug treatments. Nialamide or

7 Radioactivity recovered in iris transplants and host irides incubated for 30 min in ^{3}H NA (10^{-6} M) and rinsed for 10 min. Animals were treated or pretreated with nialamide 100 mg/kg 1—2 hrs, reserpine 10 mg/kg 4 hrs and nialamide 100 mg/kg 1—2 hrs, or H44/68 500 mg/kg 24 hrs. Values are expressed as cpm in per cent of cpm found in treated iris transplants and host irides respectively. The means ± *s.e.m.* are given.


untreated
nialamide
reserpine and nialamide
44/68
cpm % of untreated
120
100
80
60
40
20
0
number of observations:
4 2 4 5
3 2 4 5
host Irides
Iris transplants

H44/68 slightly increased the radioactivity while reserpine and nialamide slightly decreased it as compared to the untreated control. This was especially true for the transplants.

When tissues from untreated animals were further rinsed for three hours in NA free buffer after the initial ^3H NA incubation there was a marked decrease in the radioactivity both in control irides and in iris transplants and host irides (Textfig 8) The wash-out rates in the host irides and transplants were similar and thus loss was somewhat greater than in the control irides. Separation of the transplant from the host iris before the three hours wash did not seem to influence the disappearance from the host irides but the amount of radioactivity found in the transplants was reduced after detachment from the host irides. The reduction may be due either to lack of transport of radioactivity from the host iris to the transplant or to a leakage from the cut nerves of the detached transplant. — If the control iris was divided into four pieces the loss of radioactivity was greater than in the intact iris.

2. Ganglion transplants and irides reinnervated from them

Fluorescence microscopy (see Table 2, 3)

After H44/68 (24 hrs) the fluorescence was weak in the cell bodies of the intact and transplanted ganglia and in the terminals of the intact iris. How

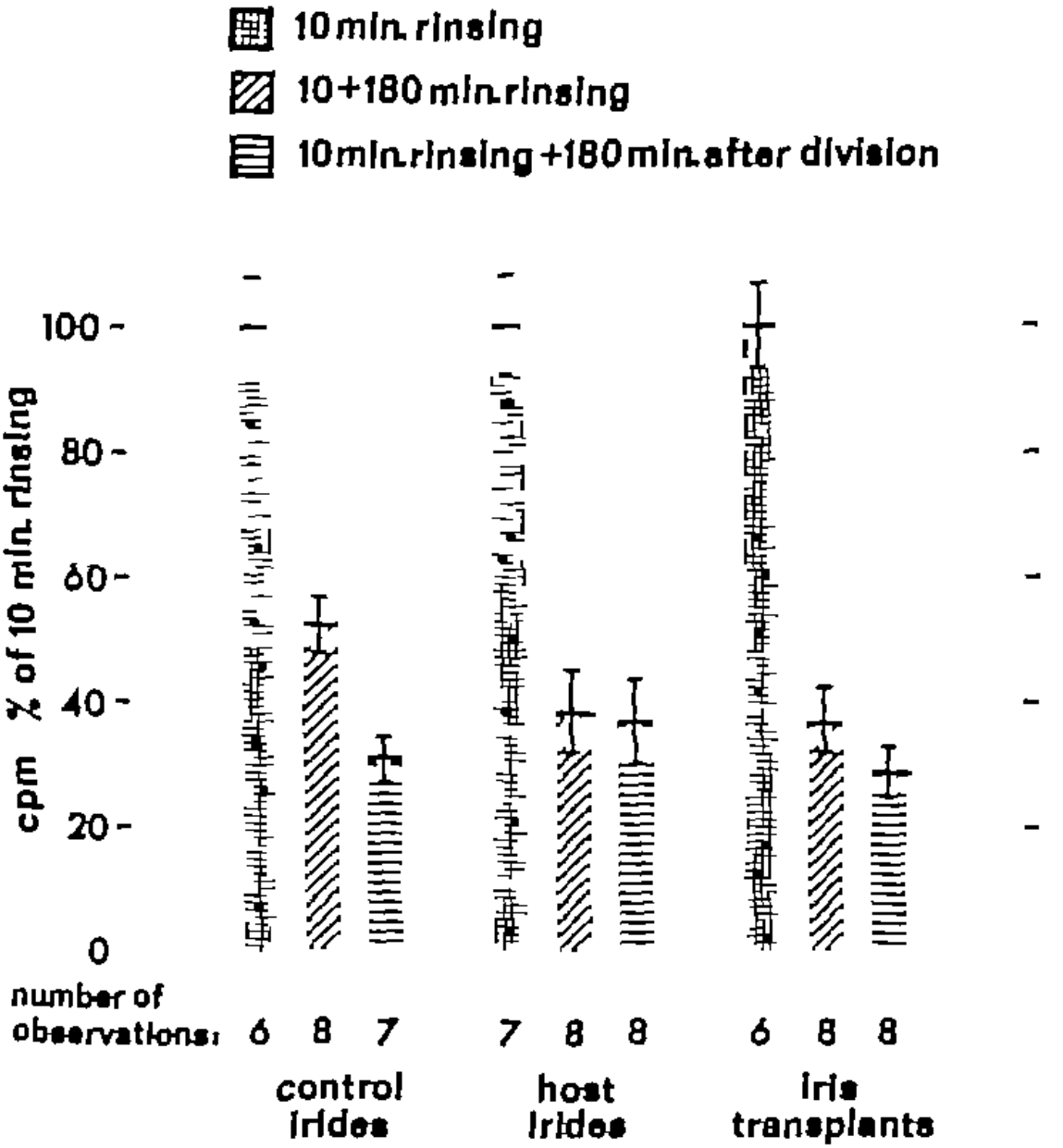
Table 3

Schematic representation of the fluorescence intensities in the nerve cell bodies of ganglion transplants and nerve terminals of the reinnervated host iris at different times after reserpine

Reserpine was given in a dose of 10 mg/kg to animals with one or four weeks old transplants. The results from the two postoperative times were similar and are described together Each estimation is based on 5—10 observations.

time after reserpine injection	*Cell bodies*	Terminals
4	(+)[1])	0
16—24	+	0
48	+++	+
96	+++	++(+)

[1]) for symbol see table 2.



8. Radioactivity recovered in control irides, host irides and iris transplants after incubation for 30 min in ^{3}H NA (10^{-7}M) and rinsed for 10 min. One group of control irides were further rinsed for 180 min undivided while in another group the irides were divided into four pieces and then rinsed for 180 min. Host irides and iris transplants were incubated in ^{3}H NA attached to each other. After the first 10 min rinsing one group of preparations were left intact during further rinsing for 180 min while in another group the transplants were separated from the irides before the 180 min rinsing. Values are expressed as the cpm in per cent of the cpm found after only 10 min rinsing in the three different groups.

ever the axons in the transplanted ganglia (Fig 47) and the nerve terminals in the reinnervated host irides had about the same fluorescence intensity as untreated controls. The difference between the nerves in the intact iris and those in the reinnervated one was probably due to the lack of nerve activity in the latter (*Corrodi and Malmfors* 1966)

Reserpine (10mg/kg) was given at different times (4—96 hrs) before death to animals killed one or four weeks after unilateral transplantation of the ipsilateral superior cervical ganglion. In the terminals and axons of the iris and the cell bodies both in the transplanted ganglia and the intact ganglia the fluorescence was very weak or absent four hours after the reserpine administration. After 16 and 24 hrs there was an increase in the fluorescence of the cell bodies especially around the nucleus (*cf* Dahlström 1967) while no fluorescence could be detected in the axons of the ganglia (Fig. 48) or in the nerve terminals of the irides. After 48 hrs the cell bodies had regained a fluorescence intensity similar to that of the untreated controls while the axons and the nerve terminals in the reinnervated irides showed a weak but distinct fluorescence. Thus the intensity of the cell bodies was higher than that of the axons during the recovery 16 to 48 hrs after reserpine which clearly illustrates that the NA fluorescence recovers faster in the cell bodies than in the axons. The fluorescence in the cell bodies of the transplanted ganglia 48 hrs after reserpine was sometimes even stronger than in the untreated controls, especially four weeks after transplantation. Apart from this there were no obvious differences between one and four weeks old transplants and reinnervated irides. After 96 hrs the fluorescence of the terminals in the reinnervated iris was moderate to nearly as strong as in untreated controls. Axons in transplanted ganglia showed a stronger fluorescence than the cell bodies as was the case also in control transplants.

A high dose of NA (0.5 mg/kg i.v 15 min) caused a general increase in the specific fluorescence, both in intact and transplanted neurons but most marked in the non terminal axons and the cell bodies. If NA was given to animals pretreated with reserpine (four hours) there was no or almost no restitution of the fluorescence in the nerve terminals, while there was a slight restitution of the fluorescence of the non-terminal axons and the cell bodies. In reserpine-nialamide pretreated animals NA caused a marked restitution of the fluorescence The nerve terminals in the intact and reinnervated irides had about the same fluorescence intensity as in untreated animals but they were smoother (*cf* Malmfors 1965a) The non-terminal axons were more intensely fluorescent than those in untreated animals. In the transplanted and intact ganglia the cell bodies had about the same or a somewhat higher fluorescence intensity than in untreated

controls. The axons were brighter than the cell bodies in the transplants giving a fluorescence distribution pattern similar to that of untreated controls (*cf* Fig 45 46)

In untreated animals the nerve terminals in the reinnervated host iris one week after the transplantation lacked pronounced varicosities. After four weeks the varicosities were more prominent. The same difference persisted after *in vivo* administration of NA (Fig 49 50) but if the NA was preceded by reserpine and nialamide no such difference could be observed.

The morphology of the nerves in the reinnervated host irides after *in vitro* incubation in ^{3}H NA (see below) was changed in the same way as described above for the nerves in the iris transplants. However the varicosities did not become as pronounced as in the iris transplants and the non terminal axons were never beaded.

^{3}H NA incubations (see Textfig 9)

After DMI-preincubation the amount of radioactivity found was very low Also four hours after treatment with reserpine the radioactivity found was low

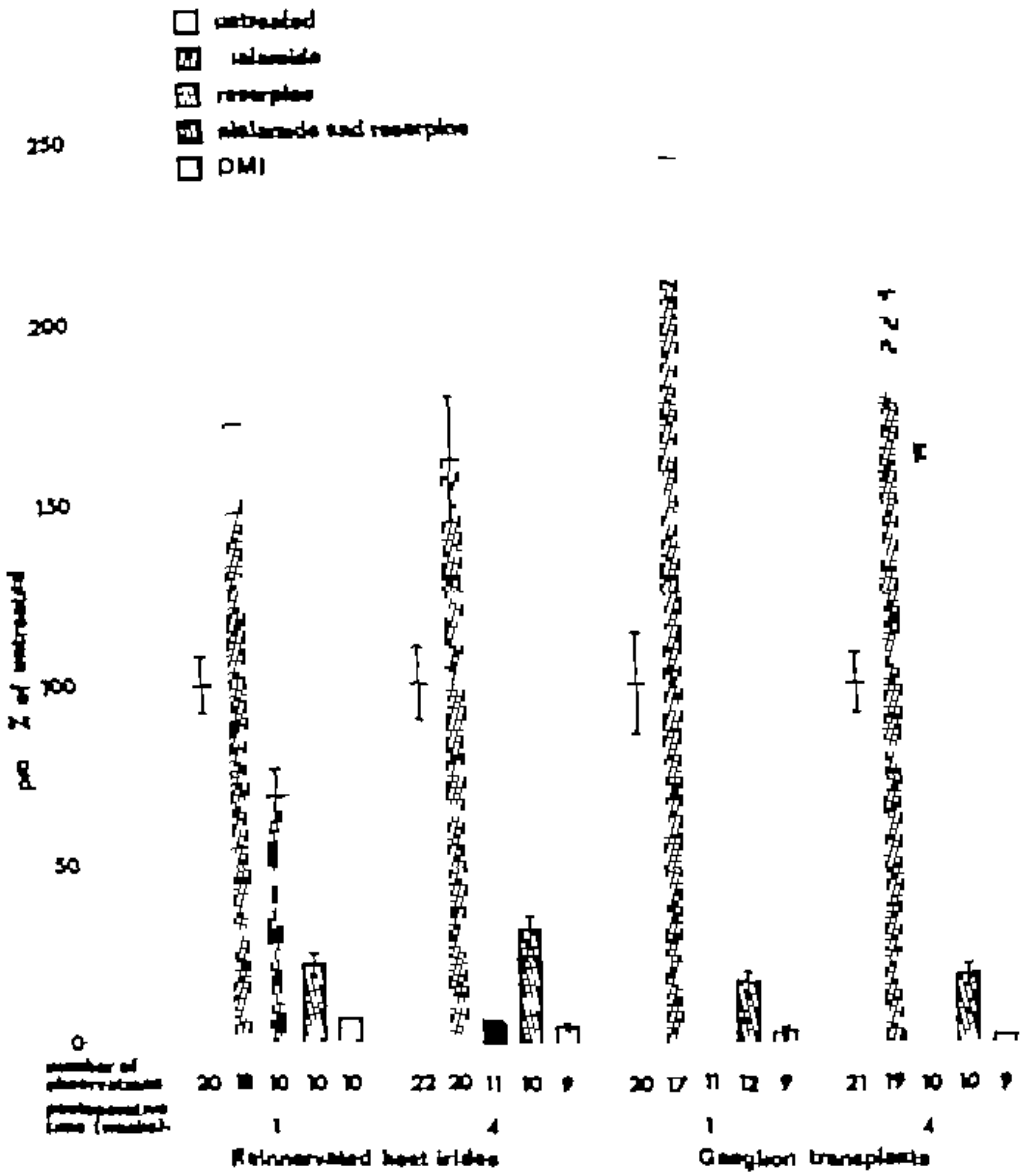
After nialamide treatment (one hour) the radioactivity found was higher than in the untreated animals, especially in the transplanted ganglia.

When the animals were given both reserpine (four hours) and nialamide (one hour) the radioactivity was higher than after treatment with reserpine alone, but less than after nialamide alone.

The recovery of the ability to take up ^{3}H NA after reserpine was followed in young and older ganglion transplants and their host irides (Textfig 10)

In the untreated animals the radioactivity was about the same in the four different tissues examined. In intact ganglia the relative amount of radioactivity was markedly higher than in the other tissues during th first 48 hrs after the reserpine treatment and reached maximum, near 100 per cent, already after 48 hrs. In the other tissues th radioactivity found up to 36 hrs after reserpine was low and there was a more rapid increase between 36 and 48 hrs than between 48 and 96 hrs.

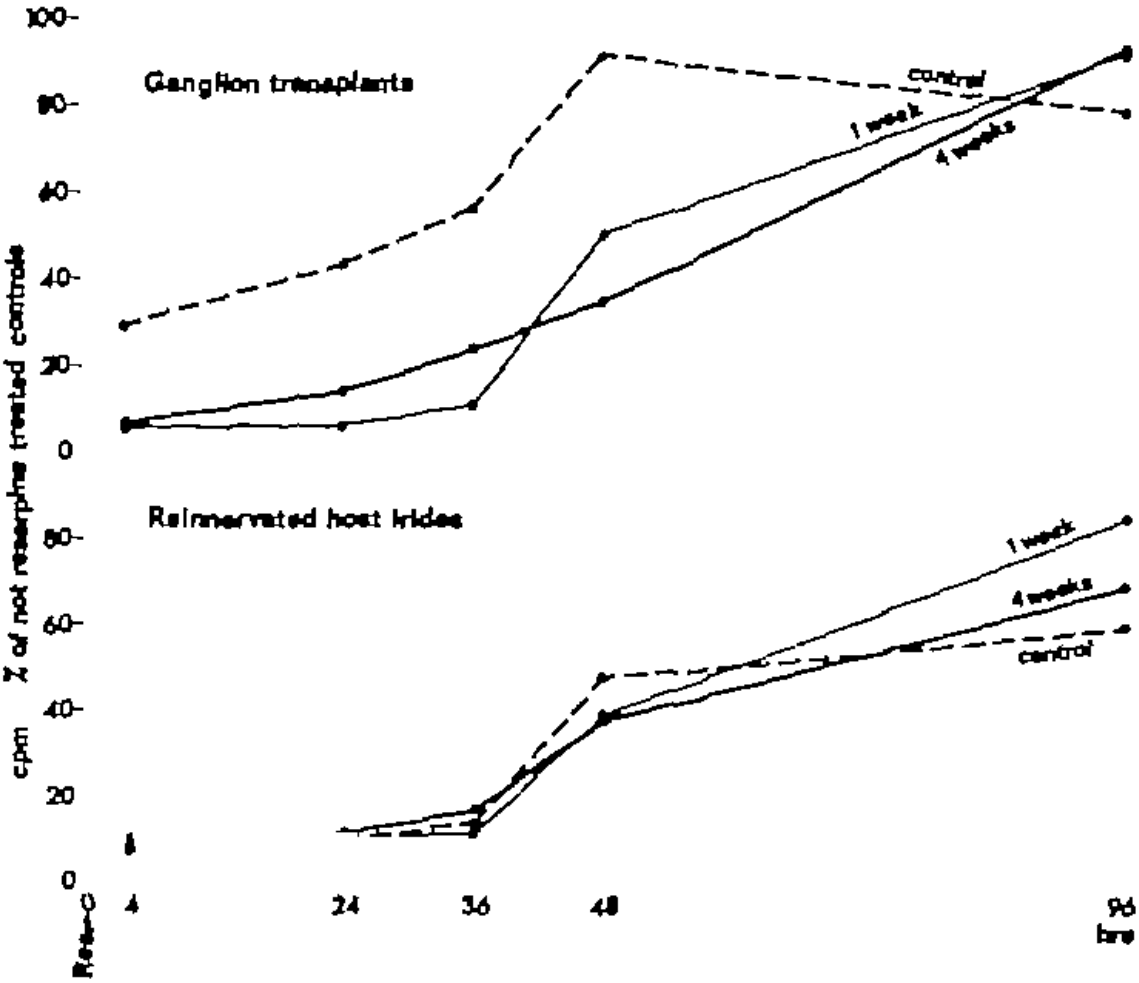
Between 36 and 48 hrs the val for the intact iris ran bout parallel to the other while between 48 and 96 hrs the increase in the intact iri was markedly slower than in the transplanted ganglia and reinnervated irides. At 96 hrs the transplanted ganglia had reached the highest level while the reinnervated irides were higher than the intact irides. The ratio between the amount of radioactivity found in the transplants and the host irides was the sam 48 and 96 hrs after eserpine as in untreated controls.



9 Radioactivity recovered in ganglion transplants and reinnervated host irides after incubation in ^{3}H-NA (10^{-7}M) for 30 min and 10 min rinsing. Animals were untreated or pretreated with nialamide 100 mg/kg 1—2 hrs and/or reserpine 10 mg/kg 4 hrs. In one group the tissues were preincubated in DMI (10^{-5}M). Values are expressed as cpm in per cent of the cpm found in untreated host irides and ganglion transplants.

3 Discussion

The material used for this part of the study was chosen in order to obtain the most favourable conditions for detection of any alternation in the transmitter mechanisms of outgrowing nerves. The iris transplants and their host irides were studied after two weeks when the number of nerves



10. Radioactivity recovered in a) reinnervated host irides and control irides and b) transplanted ganglia and control ganglia. Ganglia after incubation in ^{3}H NA (10^{-7}M) for 30 min and 10 min rinsing. The animals were given reserpine (10 mg/kg) at different times before the preparation. Values are expressed as cpm in per cent of cpm found in control irides and ganglia respectively

in the transplants was sufficiently high. The transplanted ganglia and the reinnervated irides were studied one week after the transplantation when there was a substantial amount of nerves which were fairly young and with an appearance that differed from that of normal nerves. They were also studied after four weeks when the nerves had an almost normal appearance. The preparations thus contained outgrowing adrenergic nerves from different origin, stage of growth and age.

The total radioactivity found in the ^{3}H NA incubated tissues has been shown to consist mainly of ^{3}H NA both in iris and in the upper cervical ganglion in adult animals (Jonsson Hamberger Malmfors and Sachs 1969 and unpublished observations) as well as in developing animals (Sachs,

de Champlain Malmfors and Olson 1970) Therefore, the total radioactivity recovered can be considered as a reliable measurement of the ^{3}H NA in most cases.

When the endogenous NA is greatly reduced with reserpine the uptake of exogenous NA in the adrenergic nerves can be directly observed as a restitution of their specific fluorescence provided that the MAO has been inhibited (Malmfors 1965a) No signs of NA fluorescence was detected in the nerves after preincubation in DMI, which inhibits the neuronal uptake (Hertting, Axelrod and Whitby 1961 Hamberger 1967)

It is reasonable to believe that most of the radioactivity found after preincubation with DMI represents ^{3}H NA or initiated metabolites located outside the nerves but not visible in the fluorescence microscope. Thus the difference in radioactivity between tissues not incubated in DMI and those incubated in DMI represents ^{3}H NA located in the adrenergic neurons. The extraneuronal uptake was very small compared to the neuronal uptake also in tissues containing growing nerves. These findings agree with similar studies in adult tissues where the extraneuronally located NA has been determined after sympathetic denervation (Hertting, Axelrod, Kopin and Whitby 1961 Jonsson Hamberger Malmfors, Sachs 1969 Sachs 1970) The total radioactivity in tissues not preincubated with DMI may thus be considered representative for the adrenergic nerves.

The qualitative properties of the adrenergic nerves in taking up and accumulating NA are well developed very early during the outgrowth both in nerves formed in the iris and in a transplanted ganglion after cutting its axons close to the nerve cell bodies. It has been assumed that the membrane mechanism for uptake of NA is established at the moment of formation of the membrane (Iversen, de Champlain, Glowinski and Axelrod 1967) However the quantitative development of the separate functions such as the uptake, storage and release mechanisms is difficult to estimate when all parameters may change simultaneously By selectively affecting the different mechanisms with drugs it might be possible to elucidate the relative importance of the different steps involved during the outgrowth.

NA is normally taken up accumulated by the membrane mechanism (Hamberger Malmfors, Norberg and Sachs 1964 Malmfors 1965a, Hamberger 1967 Malmfors 1967) and then incorporated and stored in the amine storage granules mainly by the ATP Mg^{++} dependent mechanism (Carlsson Hillarp and Waldeck 1963 Stjärne 1964 Carlsson 1966) The storage capacity of the amine granules is of minor importance for the initial uptake of NA but very important for the total storage capacity by protecting the NA from being deaminated by MAO (Jonsson, Hamberger Malmfors and Sachs

1969). The ATP Mg^{++} dependent uptake and storage of the amine storage granules can be effectively blocked by reserpine (see Carlsson 1966) which does not affect the membrane mechanism (Malmfors 1965a, Hamberger 1967) To demonstrate the NA uptake after reserpine a MAO-inhibitor must be given in order to protect NA from being deaminated intraneuronally The relative importance of the membrane mechanism and of the granular uptake in the out growing nerves was found to be similar to that of mature nerves. The same has been found also for nerves during ontogenesis (Sachs, de Champlain, Malmfors and Olson 1969) The quantitative relations between the membrane mechanism, the amine storage granules and the MAO is thus established very early This is further illustrated by the fact that no certain differences can be found when comparing the reinnervated irides one and four weeks after the ganglion transplantations. The ability of the newly formed nerves to retain NA was about the same as shown by rinsing the irides in an amine free solution for three hours.

In intact and transplanted ganglia, the fluorescence from endogenous NA reappeared in the cell bodies already 24 hrs after the administration of reserpine, markedly earlier than in the non terminal axons and adrenergic terminals (*cf* Norberg 1965). At the same time intact ganglia were able to accumulate NA but not the transplanted ganglia. In the transplanted, the host, and the reinnervated host irides the fluorescence of the nerves reappeared 48 hrs after the administration of reserpine. Simultaneously there was marked increase in NA uptake in the irides compared to the corresponding value 24 hrs after reserpine, when no fluorescence could be observed. Thus the ability to accumulate NA was well correlated with the reappearance of fluorescence.

The recovery of the storage function after reserpine might be achieved either by newly formed amine storage granules or recovery of the old ones. This problem has been very much discussed in combination with the axonal transport of new-formed granules from the cell body (see Dahlström and Häggendal 1969). The amine storage granules are formed in the nerve cell body and transported down the axon at a rate of 5—10 mm/hr in the rat (Dahlström and Häggendal 1966). Axonal flow is illustrated also in the present paper as the fluorescence recovered first in the cell bodies and later in the non terminal axons and terminals. Also the initial accumulations in the severed axons is explained to the axonal flow The rapid recovery of the granular storage function in the intact ganglion seems to be linear between 4 and 48 hrs. The corresponding recovery of the storage capacity in the intact iris is very low up to 36 hrs. The recovery of the NA uptake in the transplanted ganglia was slower than in the intact

ganglia. The transplanted ganglia were instead more similar to the irides during the first 4—36 hrs. These observations are explained by the fact that the transplanted ganglia contain fewer nerve cell bodies and more nerve fibres than the intact ganglia. In the reinnervated iris the recovery of the storage capacity was about the same between 36 and 48 hrs as in the intact iris but then it was faster and reached a greater part of the normal value after 96 hrs than the intact iris. The one week old reinnervated iris reached a higher level than the four week old one. The reasons might be several for these observations. There might be

a more rapid formation and transport of newly formed granules in growing nerves than in mature nerves or

a greater recovery of young granules from reserpine block or

a proportionally higher uptake in new formed granules in growing nerves after reserpine.

The adrenergic transmitter in the newly formed nerve terminals is mainly released from the varicosities as earlier observed in adult terminals (Malmfors 1965a)

The fluorescence of the enlargements of the non terminal axons in the connections between the host iris and the transplant were not affected by the stimulation. Thus because they are not sites of transmitter release they do not seem to be true varicosities.

The transmitter mechanisms of newly formed adrenergic nerves in adult rats are similar to those in normal adult ones and to those in developing and growing animals.

VI ATTEMPTS TO INFLUENCE THE GROWTH RATE OF THE ADRENERGIC NERVES

1 Outgrowth of adrenergic nerves to non-denervated irides from transplanted ganglia

It was shown in the previous chapters that, under certain experimental conditions, the rat iris could easily harbour a larger amount of adrenergic nerves than it normally does. It would then seem of interest to investigate the outgrowth of new nerves from ganglia transplanted to host irides with an intact adrenergic innervation as compared to the outgrowth from ganglia transplanted to denervated irides (*cf* IV) The former situation can be obtained either by autologous transplantations of one ganglion to the contralateral eye or by homologous transplantations. Since no indications of any differences between auto- and homologous transplantations have been found in the present experiments these two types were used simultaneously according to the various experimental needs.

Ganglia transplanted to sympathetically innervated eyes became vascularized in the same way as when transplanted to denervated eyes. No differences could be detected between the two types of experiments when examined *in vivo*

The animals with ganglia transplanted to eyes with innervated host irides were divided into two groups. In one group the adrenergic ground plexus derived from the normally located superior cervical ganglion was left intact, while in another group this plexus was removed by exturpation of the ganglion 48 hrs before sacrifice. The latter procedure was used to detect only those fluorescent sympathetic nerves which had derived from the transplanted ganglion.

The principal finding of these experiments was that adrenergic nerves grew out to a much lesser extent into innervated irides than into denervated ones. This finding was evident using both types of experiments and could be demonstrated both by fluorescence microscopy and by isotope measurements (Fig 55, 56)

The total amount of nerves found in intact irides after eight days was only slightly larger than that found in the control group after the same

time (Textfig 11) Removal of the original ground plexus two days before sacrifice revealed that the amount of nerves coming from the transplant was only about 40 per cent of the values from the control group Almost exactly the same relations between the three experimental groups were found after 30 days. Furthermore, the isotope measurements and histochemical estimations were almost identical. Also the radioactivity found in the transplants was lower in the intact groups.

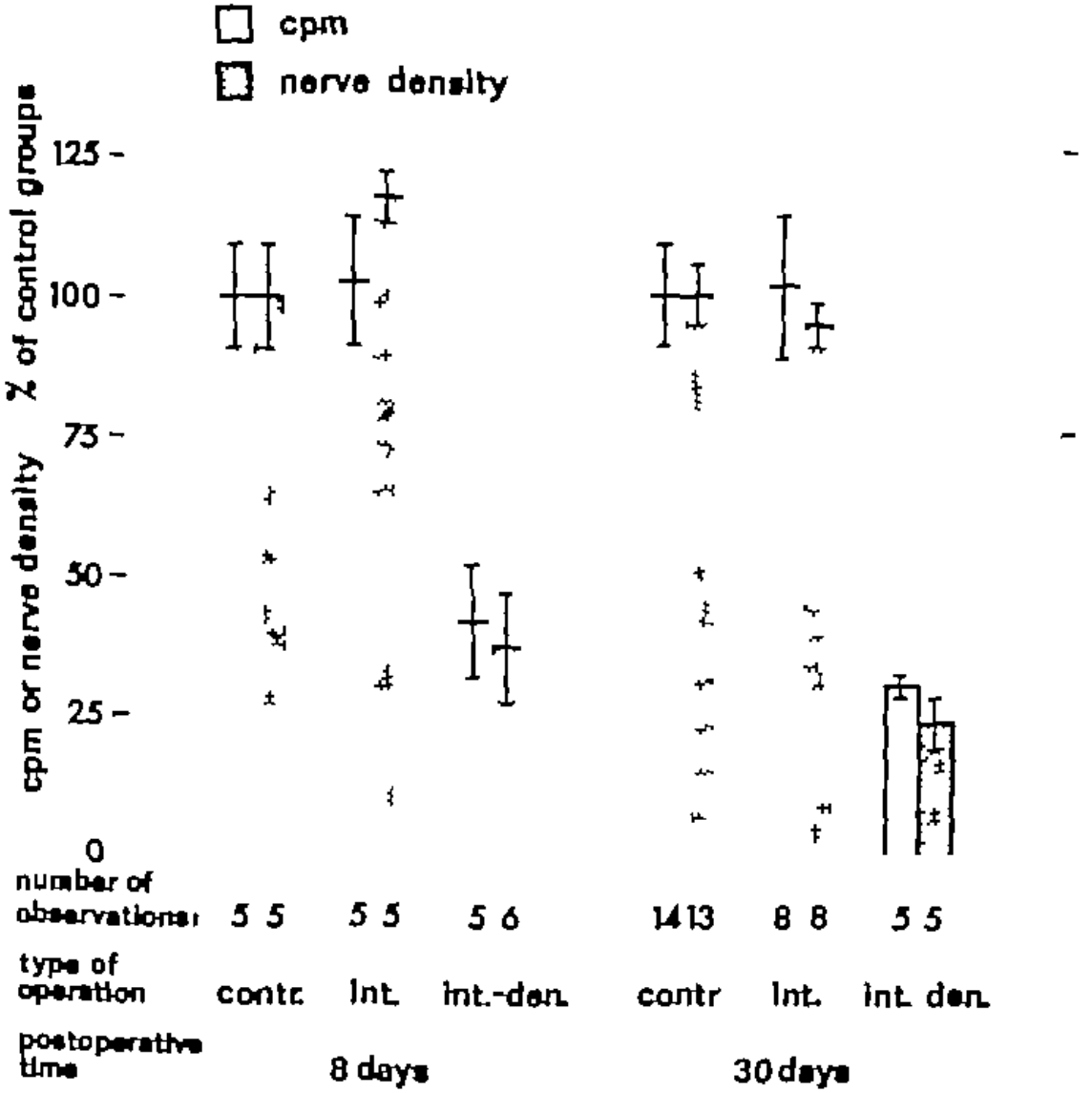
The fact that the acutely denervated groups contained more than the intact group minus 100 per cent indicated that the ganglion transplants started to grow during the last two days. To elucidate this possibility another experiment was designed where a 14 days intact group was compared to a group where the host iris was denervated after seven days and sacrifized seven days later It was clear from this experiment that such a delayed growth from the ganglion transplants as suggested in the first experiments really could be induced. Thus, the host irides which were denervated after seven days and left only with their transplants during the following seven days contained almost as large an amount of nerves as these denervated at the time of transplantation.

2. Chronic treatment with some drugs affecting the adrenergic transmission

Little is known concerning the importance of undisturbed transmitter mechanisms for the regulation of growth of the adrenergic nerves. Chronic treatment with drugs known to interfere with the synthesis, uptake, storage and metabolism of noradrenaline was therefore screened for their possible effects on outgrowth of new nerves into transplanted irides. Also the α-receptor blocking drugs phentolamine and phenoxibenzamine and the β-receptor stimulant isoproterenol were tried. The results of incubations are summarized in Textfig 12. The important conclusion from these experiments is that none of the chronic drug treatments used influenced the outgrowth of new nerves into the iris transplants to any great extent. The results of the fluorescence histochemistry parallelled the isotope uptake data.

The low uptake observed in host irides after chronic tyrosine hydroxylase inhibition was unexpected and cannot be satisfactory explained for the time being However these host irides did not differ from untreated controls when examined in the fluorescence microscope.

Almost all host irides and transplants contained somewhat less radioactivity after chronic drug treatment when compared to the chronically treated control irides than did the untreated host irides and transplants



11 Impairment of outgrowth from transplanted superior cervical ganglia when transplanted to host irides with an intact adrenergic ground plexus.

The following three groups were compared 8 or 30 days postoperatively

control: Host irides denervated at the time of transplantation. This is the type of transplantation used in chapter IV when determining the time course of the reinnervation.

Intact: The normally present adrenergic ground plexus left on the host irides until sacrifice.

Intact-denervated: The normally present adrenergic ground plexus left on host irides until 2 days before sacrifice. At this time the host irides were denervated by extirpation of the normally located superior cervical ganglion.

Each staple represents the mean ± s.e.m. from 4—14 observations expressed as per cent of the mean values from the control groups. Cpm found after ^{3}H-NA incubation and nerve density was determined in each iris.

when compared to normal irides. It should be mentioned in this connection that animals in most chronically treated groups were markedly affected by their respective treatments. Their general condition was impaired, evident as a decrease in body weight, drowsiness etc. Animals receiving α-blocking agents showed constant ptosis.
treated control irides than did the untreated host irides and transplants

A combination of α- and β-blocking agents was tried chronically on ganglion transplants. Ganglia were homologously transplanted to innervated eyes and left for one week. During the second week half of the animals received both phentolamine (50 mg/kg twice daily) and the β blocking drug H56/28 (25 mg/kg once daily) Both treated and untreated groups were further divided by adrenergic denervation of the host irides in half of the animals in each group after one week. Ptosis was observed in drug treated animals.

A small but constant decrease of ^{3}H NA uptake and amount of nerves was found in reinnervated host irides of drug treated animals (values being 10—30 per cent lower than corresponding untreated) The decrease could be observed both in irides with intact normal innervation and in those denervated after one week. No clear effect of the drug treatment was observed in unoperated animals.

3 Chronic treatment with cortisone or testosterone

Animals with autologous iris transplants were treated daily with hormones for two weeks beginning at the day of operation. They were then sacrificed and analyzed by the combined fluorescence-isotope technique

Cortisone, in a high dose (25 mg/kg) led to a body weight decrease of 10—15 per cent after 14 days in the treated animals. In the same period an increase of 10—15 per cent took place in the untreated animals. No difference in the rate of vascularization of the transplants were observed in between the cortisone treated and control animals. No effects of the cortisone treatment were observed on the NA uptake (Table four) or with the fluorescence microscope.

Testosterone was given in a dose of 5 mg/kg to male rats, castrated four days before the transplantation and the beginning of the drug treatment. The effectiveness of the different treatments were evaluated by measuring the weight changes of the seminal vesicles. Castrated groups decreased their seminal vesicle weight to 15 per cent of the untreated controls while the castrated testosterone treated groups increased the weight of the seminal vesicles 250 per cent. No effects of the testosterone treatment were obtained on the NA uptake (table 4) or in the fluorescence microscope.

Table 4

Effect of chronic treatment with cortisone or testosterone on the ^{3}H NA uptake in host irides and iris transplants

Hormones were given daily during the whole postoperative period, which was 14 days. All irides were incubated in ^{3}H NA 10^{-7}M and analyzed by the combined isotope fluorescence technique. Effectiveness of hormone treatments were monitored by following the body and seminal vesicle weights. Each value is the mean ± s.e.m. expressed as per cent of the cpm found in ^{3}H NA-incubated normal irides. Number of observations within brackets.

treatment	body weight / of start weight		weight of seminal vesicle /o of normal		cpm of normal irides		
	controls	operated	controls	operated	control irides	host irides	iris transplants
none	116	113			100±11 (6)	124±9 (4)	38±7 (4)
cortisone (25 mg/kg once daily)	89	85			100± 8 (6)	130±7 (6)	41±2 (6)
castration 4 days before transplantation			12	15	102± 7 (6)	116±8 (6)	36±3 (5)
castration + testosterone 5 mg/kg once daily)			236	259	106±11 (6)	121±7 (7)	38±4 (7)

4 Effects of certain decentralization and denervation experiments

It has been suggested that a normal impulse flow is requested for proper regeneration of certain types of nerves (Emmelin and Perec 1968 Hoffman 1952) and that the outgrowth of new nerves is stimulated by factors liberated from degenerating nerves (see Edds 1953). The following experiments were performed in order to find out f these suggestions would apply also to adrenergic nerves.

Decentralization of the superior cervical ganglion was obtained by excision of approximately f ve mm of the cervical sympathetic trunk below the ganglion. All decentralized an mals developed ptosis immediately and the

Table 5

Effects of certain decentralization and denervation experiments on the NA uptake and the amount of adrenergic nerves in two weeks old iris transplants and their host irides

Irides were incubated in ^{3}H NA and analyzed by the combined isotope-fluorescence technique. Values are expressed as per cent of the cpm and nerve density respectively found in control host irides and transplants not exposed to any additional decentralization or denervation. Each value is the mean ± s.e.m. Number of observations within brackets.

type of operation	time after transplantation weeks	cpm % of control		nerve density % of control	
		host iris	transplant	host iris	transplant
decentralization of the host iris at the time of transplantation	2	100±10 (8)	99±11 (7)	82±5 (7)	66±13 (7)
	15	134±11 (9)	137±15 (9)	90±3 (9)	100 (9)
decentralization of the iris to be transplanted 4 weeks before transplantation	2	95± 5 (6)	111± 8 (6)	100±4 (6)	99± 4 (7)
denervation of the iris to be transplanted 4 weeks before transplantation	2	85± 6 (8)	87± 9 (8)	95±3 (8)	67± 8 (8)

ptosis was present also at the longest postoperative time studied indicating that no or almost no reinnervation of the ganglion had occurred. Denervation of the eye was obtained by exturpation of the whole ganglion. Also denervated animals developed a persistant ptosis. Neither decentralization of the host iris nor chronic denervation or decentralization of the iris to be transplanted, caused any dramatic changes in the isotope or nerve density parameters (Table 5). Most values are relatively close to the corresponding controls, no change exceeding 40 per cent. It is noteworthy that decentralization of the host iris at the time of transplantation causes a discrepancy between nerve density estimations and ^{3}H NA uptake, the latter always exceeding the former relative to their control values.

However the NA uptake was found to increase 20 per cent without any observable increase of the fluorescent histochemical estimation of nerve density also in irides from animals without transplants (NA uptake in 12 normal irides 100 ± 5 0 in 12 chronically decentralized 121 ± 9 4).

The experiments indicate a somewhat delayed outgrowth of new nerves in two weeks old iris transplants following decentralization of the host iris at the time of transplantation or when transplanting a chronically denervated host iris. Ganglia were also transplanted to chronically denervated host irides (15 weeks) They were left to reinnervate the host irides during four weeks. It was found that the amount of nerves formed in the chronically denervated host irides was similar to or slightly larger than that formed in control irides after the same time. Fluorescence histochemistry and isotope measurements were similar

5 Discussion

Ganglia transplanted to eyes with an intact adrenergic innervation gave rise to a much smaller number of nerves in the host irides than when transplanted to reinnervated denervated host irides. This inhibition of the ganglionic regeneration did not seem to be merely a decreased growth rate since practically the same small amount of nerves was produced by the ganglia after 8 as after 30 days when transplanted to intact irides.

It should be mentioned that the host iris has been found always to react with a slight hyperinnervation whenever a transplant is connected to it (see VI) If this is true also for ganglion transplants the calculated amount of nerves produced by the transplant would be even smaller that is to say the growth inhibition even stronger The fact that the presence of a normal amount of nerves greatly inhibited the growth response of a balance between the outgrowth of nerves and the demands of the recip ent tissues applies also to regenerating adren rgic nerves in the adult individual. The inhibition of the ganglionic output is the more interesting when it is realized that the transplant certainly represents a large overcapacity when compared to the demands of the host iris.

The findings are in accordance with the results (IV and VII) that the ingrowth of new adrenergic n rves in an iris transplant as well as after outgrowth of nerves from a ganglionic transplant to a denervated host iris reaches a stable niveau which is close to the normal level of the receptor organ. It was also possible to induce a delayed growth response in the ganglion transplant initially inhibited by the p esence of the normal adrenergic plexus, simply by removal of this plexus.

Table 5

Effects of certain decentralization and denervation experiments on the NA uptake and the amount of adrenergic nerves in two weeks old iris transplants and their host irides

Irides were incubated in ^3H NA and analyzed by the combined isotope-fluorescence technique. Values are expressed as per cent of the cpm and nerve density respectively found in control host irides and transplants not exposed to any additional decentralization or denervation. Each value is the mean ± s.e.m. Number of observations within brackets.

type of operation	time after transplantation weeks	cpm / of control		nerve density / of control	
		host iris	transplant	host iris	transplant
decentralization of the host iris at the time of transplantation	2	100±10 (8)	99±11 (7)	82±5 (7)	66±13 (7)
	15	134±11 (9)	137±15 (9)	90±3 (9)	100 (9)
decentralization of the iris to be transplanted 4 weeks before transplantation	2	95± 5 (6)	111± 8 (6)	100±4 (6)	99± 4 (7)
denervation of the iris to be transplanted 4 weeks before transplantation	2	85± 6 (8)	87± 9 (8)	95±3 (8)	67± 8 (8)

ptosis was present also at the longest postoperative time studied indicating that no or almost no reinnervation of the ganglion had occurred. Denervation of the eye was obtained by extirpation of the whole ganglion. Also denervated animals developed a persistant ptosis. Neither decentralization of the host iris nor chronic denervation or decentralization of the iris to be transplanted, caused any dramatic changes in the isotope or nerve density parameters (Table 5) Most values are relatively close to the corresponding controls, no change exceeding 40 per cent. It is noteworthy that decentralization of the host iris at the time of transplantation causes a discrepancy between nerve density estimations and ^3H NA uptake, the latter always exceeding the former relative to their control values.

It has been suggested that an undisturbed impulse traffic is a prerequisite for collateral sprouting of parasympathetic nerves (Emmelin and Perec 1968) and that electrical stimulation accelerates collateral sprouting of motor fibres (Hoffman 1952) However decentralization of the host iris had very small effects on the outgrowth of new adrenergic nerves into an iris transplant. Similarly cultured embryonic CNS tissue will continue to grow and form complex synaptic interconnections during chronic exposure to agents which completely prevent bioelectric activity (Crain, Bornstein and Peterson 1968)

The independence of presynaptic input and, also of the presence of effector organ demonstrated by growing neurons, is in fact the necessary explanation for the successful *in vitro* culture of nervous tissues. Embryonic sympathetic ganglia readily survive in tissue cultures (chick *e g* Burdman *1968* England and Goldstein *1969* rat and mouse Sano, Odake and Yonezawa 1967 see also Levi-Montalcini and Angeletti 1968) and demonstrate efficient uptake mechanisms for NA.

The outgrowth of adrenergic nerves is inhibited by the presence of adrenergic nerves. Interference with the adrenergic transmission or transmitter mechanisms does not alter the outgrowth. Cortisone and testosterone do no affect the outgrowth of adrenergic nerves.

VII RESTITUTION OF ADRENERGIC NEUROTRANSMISSION IN THE ANTERIOR CHAMBER TRANSPLANTS

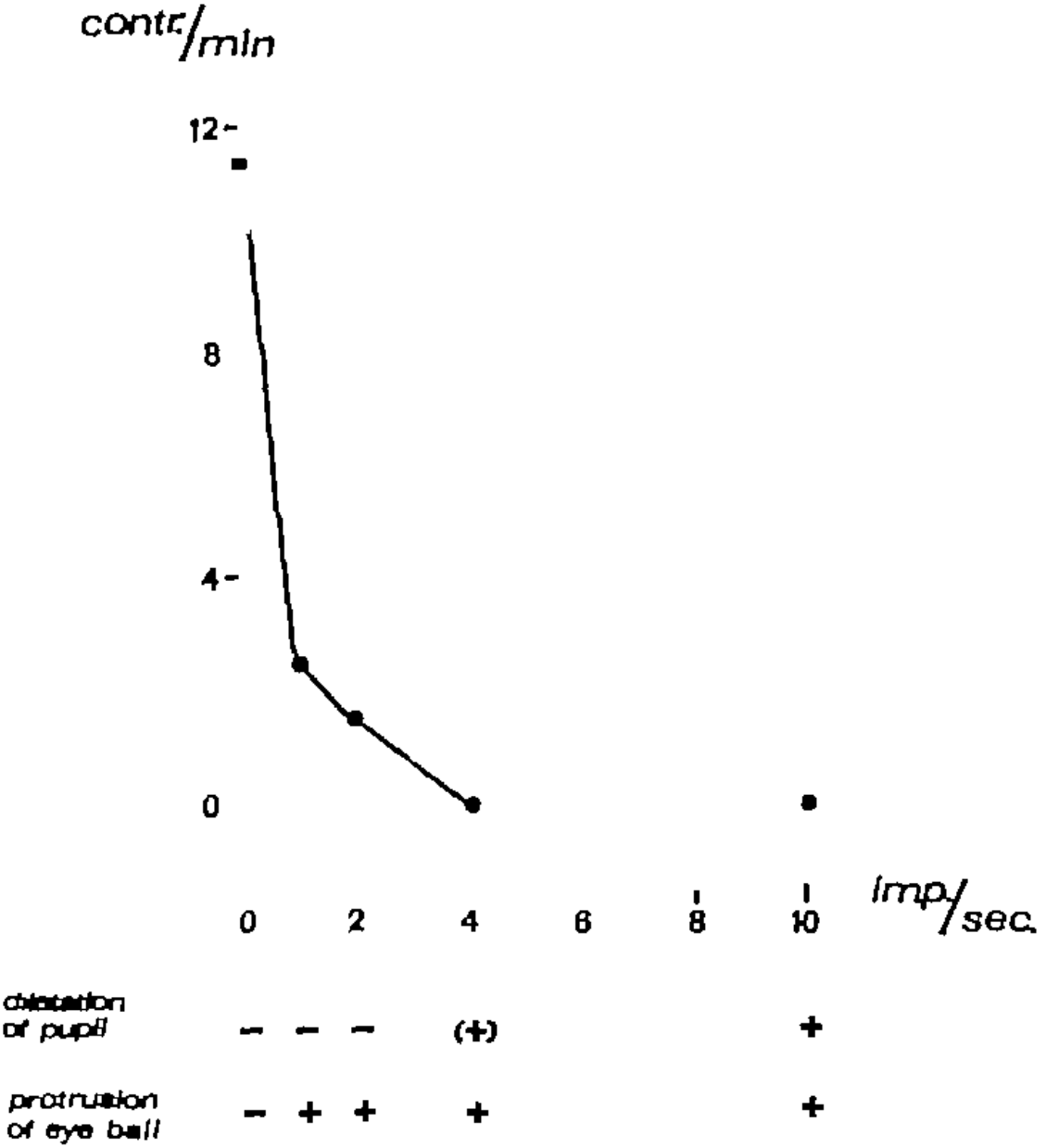
In the preceding chapters the histochemical characteristics of growing peripheral adrenergic nerves in the adult rat have been described under various experimental conditions. The reestablishment of apparently normal relationships between the nerve terminals and their effector organs suggests that return of function has also occurred. The studies of the pharmacology of the newly formed nerves demonstrated that these nerves were able to take up, accumulate and release NA. If now by electrical stimulation of the newly formed nerves, a proper response of the reinnervated tissue could be observed, the above mentioned results could be definitely linked to return of neurotransmission.

Since functional observations are technically difficult to perform in transplanted irides, three other effector organs were chosen for these studies, namely the small intestine, the portal vein and the vas deferens.

1 In vivo experiments on intestinal smooth muscle transplants

Observations of contractions, effect of electrical stimulation. Smooth muscle strips from any part of the intestine were found to survive well when homologously transplanted to the anterior chamber Duodenal strips were used for the stimulation experiments. They contained both muscle layers with the outer longitudinal layer running along the strip and the circular layer running transversally Thus, the plexus of Auerbach situated in between the muscle layers, was included.

Spontaneous contractions were observed in some transplants within one week postoperatively and in several after 14 days. Later most of the transplants developed spontaneous contractions. Those which contracted did so during the whole postoperative period (more than six months) The rate of contractions and mode of rhythmicity were determined in non stimulated animals under ether anaesthesia The range of the rate of contraction was 5—25 per min. Some transplants showed bursts of activity con



13 Effect of electrical stimulation of the cervical sympathetic trunk on spontaneous contractions of a duodenal smooth muscl strip transplanted to the anterior chamber of the eye as observed *in situ*.

sisting of 2—6 contractions at a rate of approximately one per sec with up to 30 sec intervals while others exhibited regular contractions. It was noted that handling and restraining the animals under too light ether anaesthesia sometimes caused cessation of contractions as well as dilatation of the pupil.

Electrical stimulation of the sympathetic trunk at a frequency of 4—6 per sec or more caused a prompt inhibition of the contractions of all active transplants. Textfig 13 demonstrates a typical experiment with a 15 days

old transplant. An almost total inhibition of the contractions was obtained with stimulation at 2 impulses per sec when no or almost no dilatation of the pupil was observed. Protrusion of the eye ball was evident, however at this frequency The effect could be easily repeated and was evident, at higher stimulation frequencies, even before the beginning of the dilatation.

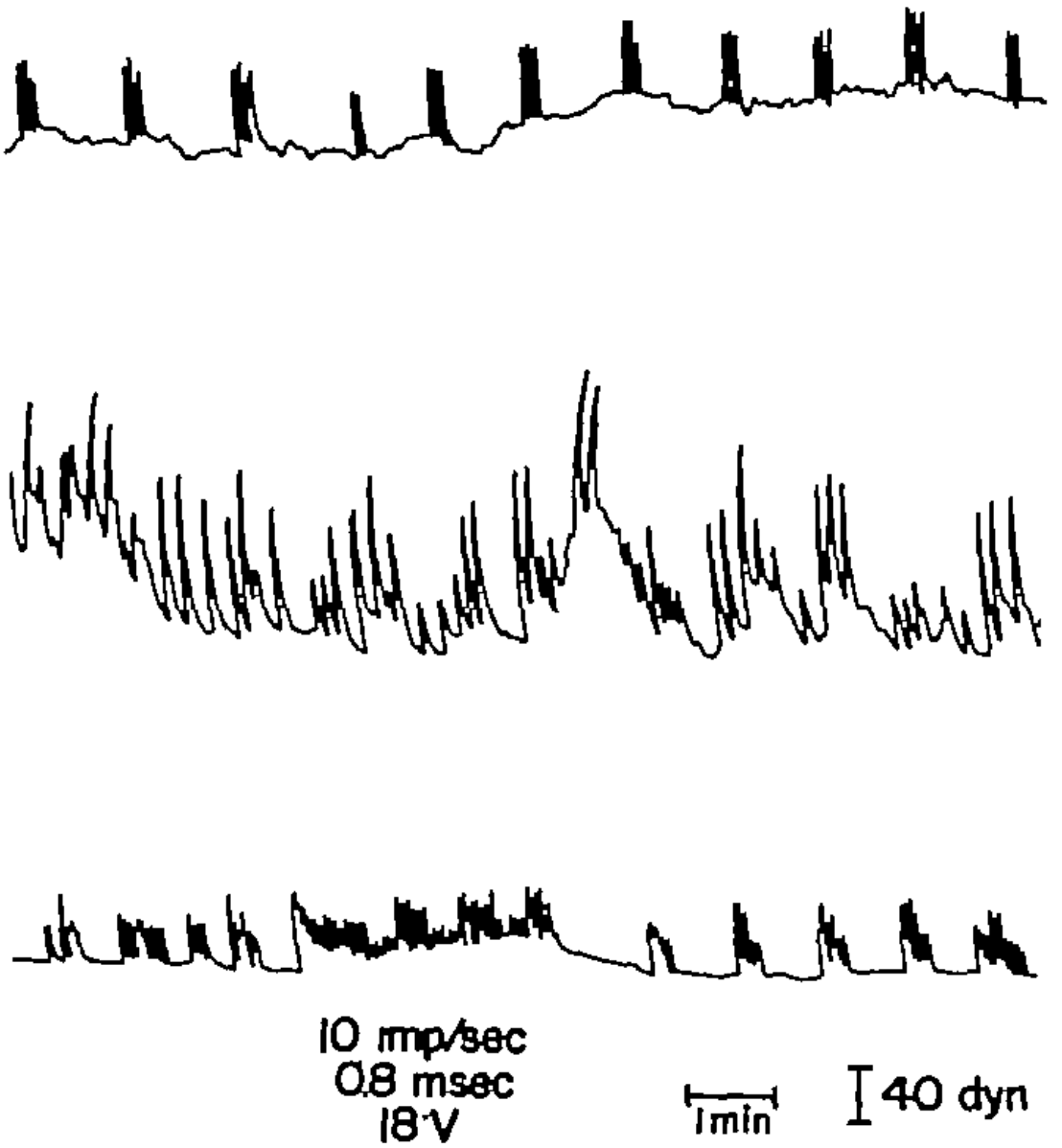
It seemed as if the muscle strips contracted at higher rates during "off minutes" following "on minutes with higher frequences than after low frequency stimulation.

Fluorescence microscopy Muscle transplants showed an essentially normal gross organization of their two muscle layers and the intervening plexus of Auerbach, as revealed in the fluorescence microscope (5μ serial sections) They were well supplied with fluorescent nerve fibres from the 14th day on (Fig 54) Fine, varicose NA nerves were always found surrounding the non fluorescent cell bodies of Auerbach s plexus, as in the intact animal (Norberg 1964) A certain amount of fluorescent fibres were also found among the smooth muscle cells without any direct relation to the nerve cell bodies. Some of the latter fibres were less varicose and exhibited a lower fluorescence intensity than the former

2. In vitro experiments of portal vein and vas deferens muscle strip transplants

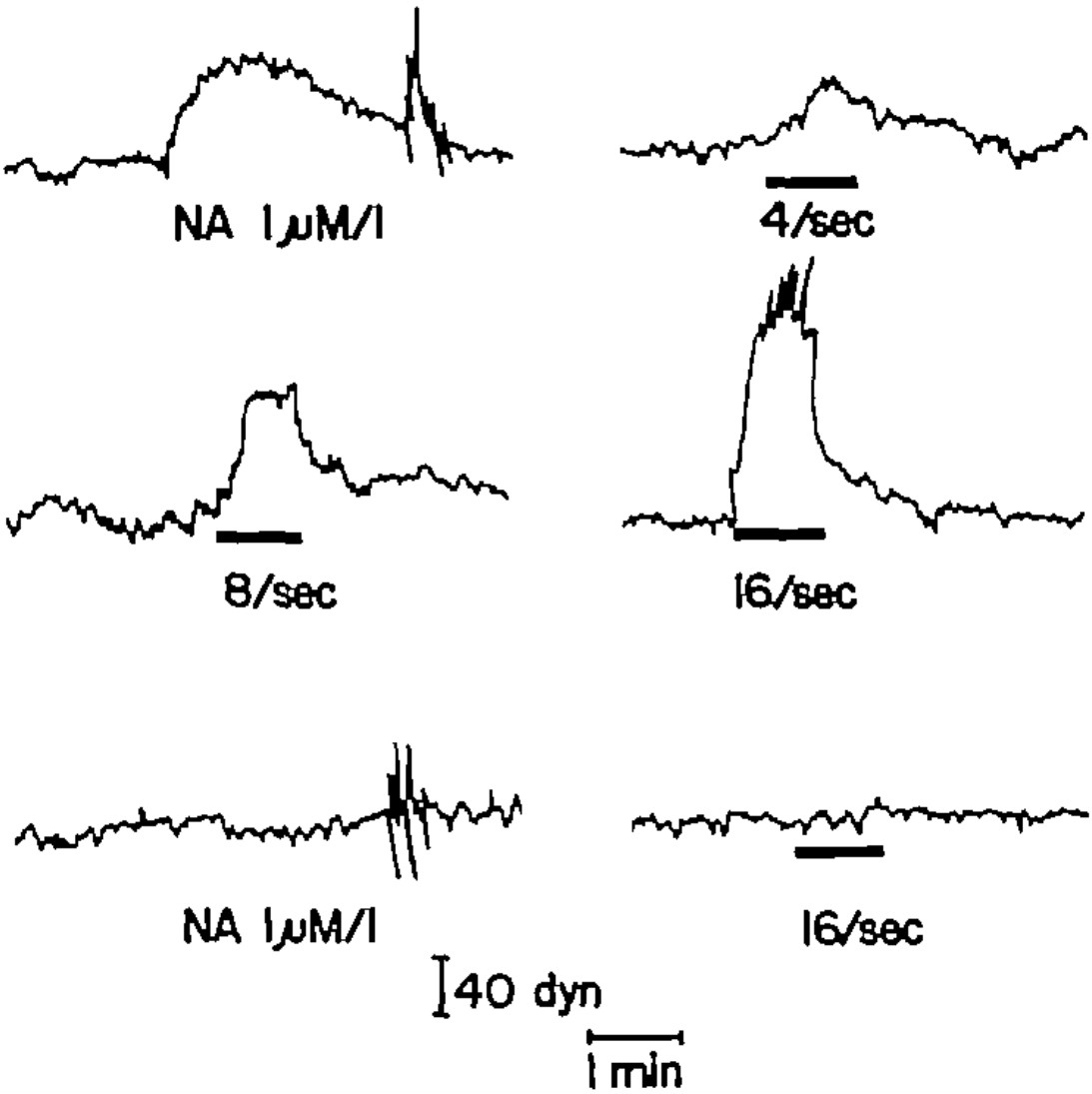
Portal vein. The longitudinal smooth muscle of the rat portal vein has a spontaneous rhythmic activity which can be well demonstrated *in vitro* (Funaki and Bohr 1964 Axelsson, Wahlström Johansson and Jonsson 1967) This activity was found to be present also in the transplants although some contracted more irregularly when examined after 2,5 months in the anterior chamber A prompt and persistant contraction could be elicited by field stimulation (Textfig 14) and this effect could be completely inhibited by adding phenoxybenzamine (10^{-6}M) to the bath Fluorescence microscopy of the transplanted vessel musculature revealed a reinnervation by adrenergic nerve fibres which resembled the normal pattern (Johansson, Ljung, Malmfors and Olson 1970)

Vas deferens. The smooth muscle of the vas deferens has a rich adrenergic innervation and is easily accessible for physiological recordings of contractions *in vitro* Longitudinal strips from the muscle wall were found to survive well in the anterior chamber and could then be tested *in vitro* with the technique similar to that used for the portal vein



14 Spontaneous contractions recorded isometrically *in vitro* from three transplants of the portal vein musculature after 2,5 months in the anterior chamber Note variation in mode of contraction, the upper and lower transplants contracting at fairly regular intervals whil the middle one contracts in more irregular manner The effect of field stimulation is demonstrated on the lower transplant. This effect wa abolished by phenoxybenzamine (10^{-6}M)

The transplants responded with prompt and sustained contractions both to added noradrenaline (10^{-6}M) and to f'eld stimulation. These effects were abolished by phenoxybenzamine (10^{-6}M) A representative experiment is shown in textfig. 15 where 13 months old transplant was used.



15 Contractions recorded isometrically *in vitro* from a vas deferens transplant after 13 months in the anterior chamber
Uppe left The effect f added noradrenaline
Upper right and middl left and right The effect f field stimulation
Lower left and right Inhibition of both the NA-effect and the ffect of electrical stimulation by α-blockade.
(Rapid pen movements following NA in the bath are running artefacts)

When examined in the fluorescence microscope, the vas deferens muscle transplants were found to contain a rich adrenergic innervation Strongly fluorescent varicose nerve terminals were distributed throughout the muscular tissue generally running parallell to the muscle fibres (Fig 53) The innervation pattern was thus similar to that normally found.

3 Discussion

The functional studies in the present work serve two different purposes. It was desired firstly to test the response of the effector cells, mainly smooth muscle cells, and secondly to study the restitution of the adrenergic neurotransmission by the newly formed nerves. The *in vivo* observations of e g transplanted intestinal smooth muscle strips, which showed spontaneous contractions, clearly indicated that the smooth muscle cells survived and retained their contraction ability after the transplantation. Furthermore, the isolated transplant of portal vein showed spontaneous activity even if more irregularly than normally and vas deferens transplants contracted when NA was added. Thus there is at least no qualitative change of the properties of the smooth muscle cells a long time after the transplantation. The vas deferens and the portal vein transplants became reinnervated to about the normal extent. The normal innervation of these organs has been described (Falck 1962, Norberg and Hamberger 1964 Ungvåry and Donáth 1969 Johansson, Ljung, Malmfors and Olson 1970)

The response in the isolated portal vein and vas deferens transplants to electric field stimulation was with all certainty due to NA released from the adrenergic nerves in th transplants as the effect was blocked by phenoxybenzamine. The experiments clearly show that these nerves are capable of restituting the adrenergic neurotransmission. Thus the portal vein and the vas deferens transplants become reinnervated both morphologically and functionally by excitatory adrenergic nerves.

An extensive network of adrenergic terminals have normally been found around the ganglion cells in the nerve plexuses of the intestinal wall. The amount of adrenergic nerve terminals directly innervating the muscle layers is generally less although great variations exists between different parts of the intestinal tract and between different species (Norberg 1964 1967; Norberg and Sjöqvist 1966 Hollands and Vanov 1965 Jacobowitz 1965 Cabella and Costa 1967 Baumgarten 1967; Baumgarten and Lange 1969 Read and Burnstock 1968 a, b, c). The plexus of Auerbach in the duodenal transplants became reinnervated by adrenergic nerves and a few nerve fibres were found also in the muscle layers.

The sympathetic nerves of the intestine are generally inhibitory in function and t has been proposed that this inhibitory effect is mediated via the adrenergic innervation of the nerve cells in the plexus of Auerbach (Norberg 1964)

The smooth muscle transplants developed spontaneous contractions in the eye. These contractions were promptly inhibited by preganglionic electrical stimulation of the superior cervical ganglion The contractions stopped nearly completely at a frequency as low as four per sec which caused only a slight bulb protrusion and pupil dilatation. At ten imp per sec there was a clear protrusion and dilatation as well as relaxation of the transplant. Although these results clearly linked the activity of the superior cervical ganglion to inhibition of contractions in the transplant, the interpretation of this finding is somewhat unclear NA, from the adrenergic nerve terminals around the ganglion cells or within the muscle layers or possibly from the adrenergic ground plexus of the host iris, can then act either via the ganglion cells or directly on the muscle tissue.

However the most prominent factor in inhibiting the mobility of the intestine has recently been claimed to be a system of neurons in the intestinal wall, separate from both the adrenergic and the cholinergic neurons. It has been called the non adrenergic inhibitory system and has been shown also in other organ systems. Activation of the neurons in this system by transmural stimulation at rather low frequencies (1—4/sec) stops the spontaneous contractions of the intestinal wall (Burnstock, Campbell, Bennet and Holman 1963 1964 Holman and Huges 1965 Bennet, Burnstock and Holman 1966; Burnstock, Campbell and Rand 1966, Furness 1969) Due to the sensitivity of the transplants to very low frequency stimulation the presence of this non adrenergic inhibitory system in the transplants cannot be excluded. The non adrenergic inhibitory component has recently been demonstrated in muscle transplants from the guinea pig taenia caeci to the anterior chamber of the eye (Burnstock Gannon Malmfors and Rogers 1970)

Thus the true nature of the relaxation induced by sympathetic stimulation in the duodenal transplants remains to be disclosed but the present model system should be useful in further experiments.

The newly formed nerves in adult rats are able to restitute the adrenergic neuromuscular transmission.

VIII COMPARATIVE TRANSPLANTATION EXPERIMENTS USING DIFFERENT TISSUES LOCATIONS AND SPECIES

The purpose of the present experiments was to perform comparisons of the growth of the adrenergic nerves after the following types of transplantations. 1 Adrenergically innervated versus non innervated organs. 2 Rats versus hamster and mouse. 3 Auto- or homologous transplantations versus heterologous. 4 The anterior chamber versus other sites of transplantation. 5 Immature versus mature adrenergic neurons.

1 Transplantation of other organs to the anterior chamber of the rat eye.

All tissues hitherto tried became vascularized and maintained in most cases structural integrity relatively well for long periods of time. No differences were observed between autologous and homologous transplants.

It seems appropriate to list here all different tissues tried, including those mentioned in earlier chapters, in a more systematic way

superior cervical ganglion, coeliac ganglion, lumbar sympathetic chain ganglia, hypogastricus ganglia

iris, choroidea

submaxillary gland sublingual gland, panchreas, kidney

portal vein, femoral artery intestinal smooth muscle layer vas deferens muscle layer uterus muscle layer auricle of heart, ventricle of heart, quadriceps muscle

adrenal medulla, ovary hypophysis, epiphysis.

The list of transplantations presented demonstrated the applicability of the method and since in fact no failures have hitherto been recorded in the taking of transplants it seems that any peripheral tissue can be used.

It is beyond the scope of the present investigation to describe in detail the ingrowth of adrenergic nerves in all the different receptor organs tried. Instead some general conclusions will be made which are valid for all transplants. A few experiments which demonstrate specific points of interest will be described in more detail.

transplants. The ganglia appeared normal in all respect
tion. They contained nerve cell bodies without specific fl
fluorescent nerve fibres were found inside the ganglia.

2. Confirmatory experiments on the growth of adrenergic nerves in hamsters and mice

Albino hamsters: Iris and ganglion transplantations were performed. The results were in all respects similar of those obtained in rats. Vascularization was rapid. Iris transplants became reinnervated in the same way as in rats. The organization of the adrenergic nerves in the hamster iris is very similar to that of the rat. This pattern was copied by the growing nerves in the hamster transplants (Fig. 59, 60). Thus, for instance the blindly ending nerves extending from the ciliary body radially for a short distance into the choroid membrane were reformed in the transplants. The hamster host irides became hyperinnervated.

Ganglion transplants rapidely innervated denervated host irides. The appearance of the new nerves formed by ganglion transplants resembled that seen in rats. They were thus somewhat smoother than the normal ground plexus after two weeks.

Albino mouse: Iris transplantations were performed. The small size of the mouse eyes was not found to cause any difficulties. Transplants became vascularized in the usual way. The organization of the adrenergic ground plexus of the mouse iris differs from that of the rat in having fewer fluorescent fibres in each strand of the plexus. No detailed analysis was performed on mouse iris transplants but it seemed as if the characteristics of the normal mouse plexus was kept in the transplant. Mast cells were frequent on the host irides.

3. Heterologous transplantations

Mouse irides and superior cervical ganglia were transplanted to the anterior chamber of the rat eye. The heterologous transplants caused more severe and long lasting reactions of the host eyes. Miscoloration was often persistant and vascularization was delayed. It seemed however as if ganglia were better tolerated than irides.

Most animals were sacrificed after 14 days. The mouse iris transplants had then become reinnervated by rat nerves (Fig. 60). The extent of innervation varied, but plexuses were often formed only on limited areas. Interestingly

the newly formed plexuses were more similar to the normal mouse plexus than to the normal rat plexus (*cf* Malmfors 1965a) The host irides were swollen and contained large amounts of cells which were interpreted as being macrophages. These cells were readily identified in the fluorescence microscope due to their heavy cytoplasmic inclusions of brownish-fluorescence granules. Some host irides were hyperinnervated while others had less nerves than normally Eventually the transplants were invaded by macrophagic cells from the host irides and had begun to desintegrate.

Superior cervical ganglia from the mouse were capable of reinnervating denervated rat irides (Fig 62) The initial outgrowth rate was similar to that found after auto- or homologous transplantations in the rat. Large areas of the rat iris could be found innervated after 14 days but the fluorescence intensity of the outgrowing nerves was low

4 Transplantation of the anterior half of the eye to other locations

The anterior half of the eye was unilaterally transferred as described (II.2) to the submaxillary gland, the vas deferens and the sciatic nerve. *In vivo* observations could be performed only before sacrifice by free-dissection of the corneal surface. In successful cases the cornea was clear the anterior chamber filled with a clear fluid and the iris visible in its original position. It was then easy to prepare the iris for fluorescence microscopy

At all three locations, a new plexus of fluorescent varicose nerves was established in the transplanted iris which was similar to the normal adrenergic plexus. A certain hyper reinnervation of the transplanted iris was often noted after transplantation to the submaxillary gland (Fig 63) or the vas deferens (Fig 64) when the transplanted irides were compared to the control eyes of the same animals.

5 Special transplantations of the superior cervical ganglion e.g. to different locations

Transplants of different parts of the superior cervical ganglion.

A comparison was made between the outgrowth from the cranial and the caudal ends of the ganglia by transplanting small pieces of about the same sizes to the anterior chamber It was found that both ends easily reinnervated the host irides as did the longitudinally cut halves which was usually used. The apical pool, however seemed to give rise to a more sparse innervation than the caudal one.

Superior cervical ganglion transplants from newborn rats: Whole ganglia from newborn rats were transplanted to the anterior chamber of adult animals. The host eyes were sympathetically denervated at the time of transplantation. The transplants survived well in the anterior chamber but no increase in size could be observed *in vivo* during the postoperative time studied. On the contrary many transplants seemed to have decreased their size during the longest intraocular period which was six months, while corresponding ganglia left *in situ* during the same time had grown to adult size.

Although no macroscopical growth was observed the transplants re innervated the denervated host irides. The early course of this growth was not closely followed but new nerves were observed *e.g.* at the ninth day. Detailed examinations after three and six months revealed a relatively well developed plexus of fluorescent nerves in the host irides (Fig 65). It covered 50—100 per cent of the iris. The density of the meshwork was sometimes less than normal but the nerve fibres were indistinguishable from fibres produced by adult ganglion transplants. Thus, both typically varicose terminals and beaded non terminal axons were found. The choroid membrane was reinner vated to a varying extent.

Small transplants were sometimes left on the host irides during stretch preparation. Single fluorescent nerve cell bodies could then occasionally be observed. The initial part of the axon could be followed. It was smooth ly outlined and had the same fluorescence intensity as the cytoplasm of the large rounded cell body. Also the small intensely fluorescent so called SIF cells typical of normal sympathetic ganglia, were found in the transplants. They were arranged in small clusters and had a few short processes.

Transplantation of the superior cervical ganglion to other locations. Ganglia were bilaterally transplanted under the bulbar conjunctiva of the ipsilateral orb t. The transplants and its local surroundings, the iris of the corresponding eye and the salivary glands were examined at postoperative times *up to six months. The ganglia survived and could be recovered from* the submucosa also after six months (Fig 66). The cell bodies generally showed a low fluorescence intensity and often excentric nuclei. The transplant was surrounded by large numbers of strongly fluorescent bulgy fibres, many of which was encircling the whole transplant. An increased number of mast cells was found around the transplant. The adjacent rudimentary nictitating muscle of the rat was sometimes reinnervated. Fluorescent nerves, which could be safely regarded as coming from the transplant, were not found in the iris or choroid membrane.

The superior cervical ganglion was also autologously transplanted to the sciatic nerve. In spread preparations of the nerve 14—28 days later a large number of axons were found emanating from the ganglion and running in both directions of the nerve (Fig 67). The fibres had the same appearance as the normally present non-terminal fluorescent axons of the nerve. The normal population of fluorescent fibres was removed by compression of the sciatic nerve cranial to the site of transplantation either at the time of transplantation or 1—2 days prior to sacrifice. It was found that the presence of the normal amount of fluorescent nerves inhibited the outgrowth from the transplant to a certain extent. This inhibition was difficult to estimate, but was by no means as great as that obtained by transplanting the ganglion to the anterior chamber of an adrenergically innervated eye.

6 Transplantation of adrenal medulla to the anterior chamber

The fluorescence technique cannot disclose any possibly existing adrenergic ganglion cells within the normal adrenal medulla, due to the extremely high fluorescence intensity of the endocrine cells, but it was hoped that if some nerve cells were present, fluorescent nerve fibres would be produced by the transplant.

As far as can be judged from the fluorescence histochemistry the endocrine adrenal medullary cells themselves gave rise to fluorescent nerve fibres reinnervating the denervated host iris (see Olson 1970).

The transplants were similar to the normal adrenal medulla, showing the characteristic strong yellowish fluorescence of the NA cells and the somewhat weaker but still quite strong yellow green fluorescence of the adrenaline cells. The transplants were often less compact than the normal medulla and many cells were elongated and showed fluorescent processes. (Fig 68).

Small groups of adrenal medullary cells were always found attached to the host irides when the main transplants had been pulled away and the irides stretch prepared (Fig 69). The cell clusters were often found at considerable distances from the sites of attachment of the transplant. In several cases, fluorescent fibres could be followed in their whole length from an origin in the soma of the medullary cells to the peripheral ramifications. These fluorescent fibres were capable of forming plexus like meshworks over large areas of the host irides and were in all respects similar to the normal iris nerves with the one exception of their peculiar cellular origin. The fluorescent cells attached to the iris surface had the

same appearance as those found in the transplants. Thus they were smaller than adrenergic ganglion cells and had a much stronger fluorescence intensity

Due to the remarkable findings described, it was not possible to definitely answer the initial question whether true adrenergic nerve cells existed in the medulla. Cells similar to the normal adrenergic nerve cells were however not found, although it can be assumed that it would have been easier to detect them among the often more loosely arranged medullary cells of the transplants or on the host irides than in the normal adrenal medulla.

7 Discussion

All peripheral organs transplanted to the anterior chamber became reinnervated to an essentially normal extent. The important conclusions from these results are twofold

1 The amount of ingrowing nerves is specifically and precisely regulated by the effector organ The transplantation of the adrenergically innervated submaxillary gland as compared to the non innervated sublingual gland (Norberg and Hamberger 1964 Norberg and Olson 1965 Fujiwara, Tanaka, Hoojo and Okegawa 1965) illustrates this fact. Although the glandular parenchyma underwent marked changes (*cf* Andén, Norberg and Olson 1966) it was clear that the submaxillary gland became well reinnervated, while the sublingual gland remained devoid of parenchymal innervation. It is interesting to note that fluorescent nerves from the host iris encircled the sublingual transplant but were unable to penetrate into it.

2 Organs normally innervated by adrenergic nerve from different parts of the sympathetic nervous system became equally well reinnervated by the iris plexus. Thus, there is no specificity between the various para and prevertebrally sympathetically innervated organs in this aspect. These findings are complementory to the results of transplanting various adrenergic ganglia to the anterior chamber (see IV) which demonstrated that any part of the sympathetic nervous system could reinnervate the iris.

The remarkable results of transplanting the adrenal medulla prompted a more detailed fluorescent histochemical study which is reported elsewhere (Olson 1970) The outgrowth of fluorescent fibres, in all respects similar to normal adrenergic nerves from the medullary cells is an indication of the ontogenetic relationship between these cells and adrenergic nerve cells. It could not be determined from the present experiments if

IX GENERAL DISCUSSION

1 Methodological considerations.

The histochemical fluorescence method The chemistry and specificity of the fluorescence histochemical method is well established and has been discussed at length in several papers (Falck 1962, Falck Hillarp Thieme and Torp 1962 Corrodi and Hillarp 1963 1964 Corrodi, Hillarp and Jonsson 1964 Corrodi and Jonsson 1965 1966, 1967 Caspersson, Hillarp, Ritzén 1966, Jonsson 1966 1967 a, b c, 1969 Ritzén 1966 1967 Norberg, 1967) The practical performance including freeze-drying, air-drying, paraformaldehyde reaction and the optical equipment needed has also been described in detail by several authors (Dahlström and Fuxe *1964b* Malmfors *1965a*, Falck and Owman 1965 Hamberger Malmfors and Sachs 1965 Hamberger 1967 Norberg 1967 Eränkö 1967 Olson and Ungerstedt 1970)

It can be concluded with certainty from the references above that the formaldehyde-induced yellow-green fluorescence derives from cellular stores of biogenic monoamines or some of their immediate precursors (see especially discussion by Hamberger 1967 and the review by Corrodi and Jonsson 1967) Thus in the case of the postganglionic sympathetic adrenergic neurons which were studied in the present investigation, the specific fluorescence observed is due to NA

It is very likely that the fluorescence technique reveals the axon ramifications of the adrenergic neurons in their full extent in the pharmacologically untreated animal also during the conditions of growth studied here. This assumption was discussed by Olson (1969) in the case of regenerating non-terminal axons in rat sciatic nerve and is supported by the following facts:

1 The adrenergic neurons contain adequate concentrations of NA for histochemical visualization very early during the ontogenesis (see de Champlain Malmfors, Olson and Sachs 1970)

2 The *number* of visible fluorescent nerves does not significantly change following pharmacological treatments which increase the fluorescence *intensity* of the already visible nerves (*cf* de Champlain Malmfors, Olson and Sachs 1970)

3 The rate of transport of NA storage granules (Dahlström and Häggendahl 1966) exceeds by far the nerve growth rates and electron microscopical studies demonstrates the presence of such amine storage granules in growth cones (Blümcke and Niedorf 1965) and axonal sprouts (Kapeller and Mayor 1966)

4 The morphology of the growing nerves studied by the fluorescence technique is similar to the classical descriptions of various regenerating nerves using classical staining methods (*e g* Cajal 1928)

Quantification of nerve growth All possible precautions were undertaken in order to obtain reliable quantitative estimations of the amount of nerves in the iris preparations. ^{3}H NA uptake measurements were always combined with fluorescence microscopy (Olson, Hamberger Jonsson and Malmfors 1968) on the same irides and the fluorescence microscopical estimations of amount of nerves were performed on coded slides, sometimes independently by the two authors. The experiments described in II 9 proved that the two estimators reached almost identical estimations of nerve amount in partially denervated irides and that these estimations were very well correlated to the radioactivity recovered in the same irides after ^{3}H NA incubations. Using the present technique of ^{3}H NA incubation the total radioactivity recovered represents almost exclusively ^{3}H NA taken up into the adrenergic nerves (Jonsson, Hamberger Malmfors and Sachs 1969) It can be concluded that the ^{3}H-activity recovered very well reflects the amount of nerves in the irides under these circumstances. Furthermore, it was shown (see V) that the transmitter mechanisms of the newly formed nerves were similar to those of normal adrenergic nerves in the adult animal. Assuming an essentially unchanged uptake and retention of ^{3}H NA, the radioactivity parameter would be a good measurement of the total amount of nerves also during growth A good correlation between the ^{3}H NA uptake and fluorescence histochemical estimations was in fact found also in the growing nerves.

While the ^{3}H NA uptake measurements possibly better reflects the total amount of nerves in certain situations such as *e g* in hyperinnervated host irides, the fluorescence histochemical estimations are needed for the exact morphological description of the nerve growth.

Quantitative aspects of the fluorescence intensity The fluorescence histochemical method can provide semiquantitative measurements of changes in CA content. Estimations of fluorescence intensities after different pharmacological treatments as compared to controls have been used *e.g.* by Malmfors (1965a) Norberg (1965) and Hamberger (1967)

A good correlation between the NA content and the fluorescence intensity of the adrenergic nerves has later been demonstrated up to a certain NA

concentration both when observing the fluorescence intensity with the eye (Olson, Hamberger Jonsson and Malmfors 1968) and when using microfluorometric techniques (Jonsson 1969) Jonsson states "if changes in fluorescence intensity as compared with the control are observed this reflects true changes in amine concentrations, but changes in amine concentration may escape detections when the concentration of amine is so high that quenching occurs"

The transplantation technique The anterior chamber of the eye has been utilized as a site of transplantation for a variety of purposes during the last 50 years. The specific advantages of this technique when studying growth of adrenergic nerves were described in I 4

The first fluorescence histochemical evidence for adrenergic reinnervation of intraocular transplants was reported by Owman (1964) using pineal grafts. This was later confirmed by Wragg, Machado Snyder and Axelrod (1967) A preliminary account of parts of the present results was given by Malmfors and Olson (1967)

2. Normal innervation.

The adrenergic innervation of the eye has been extensively studied in many species with the fluorescence technique (Falck 1962, Malmfors 1965 a, b Malmfors and Sachs 1965 a, b, Ehinger 1964 1966 a, b Laties and Jacobowitz 1966) All fluorescent intraocular nerves, apart from the retinal dopamin neurons (Malmfors 1963 Häggendal and Malmfors 1963 Ehinger 1966 c, Ehinger and Falck 1969) derive from the ipsilateral cervical sympathetic chain and certainly almost exclusively from the superior cervical ganglion. Varicose adrenergic nerve terminals are found in most parts of the rat eye. The corpus ciliare and iris contains a relatively dense autonomic ground plexus (for definition of ground plexus see Hillarp 1946 1959 1960, Norberg 1967) and the plexus has been shown to contain also numerous cholinergic nerves (Ehinger and Falck 1966, Laties and Jacobowitz 1966, Ehinger Sporrong and Stenevi 1968).

With the introduction of the potassium permanganate technique of Richardson (1966) and cholinesterase stainings combined with specific denervation experiments, the dual innervation of the iris has also been clearly demonstrated electronmicroscopically (see Csillik, Knyihár and Halász 1968 Hökfelt 1969 Roth and Richardson 1969) The cholinergic and adrenergic fibres run in small bundles close to each other commonly within the same Schwann cell envelope. The vast majority of the cholinergic supply derives from the ciliary ganglion (Richardson 1969) The knowledge of the autonomic inner

vation of the rat eye constitutes the necessary basis for the present experimental studies on growth of the intraocular adrenergic nerves.

The various sympathetic ganglia used for transplantation in the present study have earlier been thoroughly studied with the fluorescence method (Norberg and Hamberger 1964 Norberg 1965 Hamberger and Norberg 1965 Sjöstrand 1965) For a review of the adrenergic innervation of other tissues used in the present study see Norberg (1967)

3 Growth mechanisms of the adrenergic nerves.

Growth of the adrenergic neurons, like that of all other neurons, comprises several different forms which all lend themselves to experimental studies. Distinction should be made between the ontogenesis of the adrenergic neurons and the growth which take place in the adult individual Growth of mature neurons may be further subdivided according to whether the neuron grows in response to injury or extends its field of innervation because of other reasons. The various experimental techniques used (*e g* axotomy transplantation, explantation) provides another basis of division which is useful for clarification of the results hitherto reported in the literature

The fluorescence histochemical method has proved to be an excellent tool also for studying growth of the adrenergic nerves. The early appearance of NA in the sympathicoblasts (Enemar Falck and Håkansson 1965 Olson, de Champlain, Malmfors and Sachs 1970) as well as the ontogenesis of the adrenergic innervation apparatus (see Olson, de Champlain, Malmfors and Sachs 1970) have been described. Other ontogenetical aspects studied include the growth of fetal sympathetic ganglia in tissue culture (Sano, Odake and Yonezawa 1967) and the effect of the nerve growth factor (Olson 1967) and its antiserum (Hamberger Levi Montalcini, Norberg and Sjöq ist 1965) on the neuronal development.

Although growth during ontogenesis may differ in many respects from growth of mature neurons, as the latter was studied in the present investigation, certain fluorescence morphological characteristics are similar Thus, out growing axons look smoother and thicker than mature axons at rest and show a higher fluorescence intensity When the terminal nerve plexuses are being formed they are initially of a weaker fluorescence intensity and the varicosities of th nerves making up th strands of the plexuses less pronounced.

Regeneration of the non terminal adrenergic axons foll wing axotomy in the adult animal (Dahlström 1965 Blümcke and Niedorf 1965 Olson 1969) and of th terminal ramifications following 6-hydroxydopamine-induced

destruction (Jonsson and Sachs 1970) have proved to be comparingly rapid processes.

The present study using the anterior chamber transplantation technique, clearly demonstrates the ability of the adrenergic nerves to grow in the adult animal, not only after axotomy but also from intact nerve endings. The growing nerves do not seem to differ much from normal developing nerves and they reestablish adrenergic neurotransmission in an effector organ deprived of its original nerve supply. Some aspects of the growth mechanisms will now for the sake of clarity be discussed separately although they are intimately interrelated.

a) *Initiation of growth.* An important question is how the intact sympathetic neurons obtain the information to which they respond with growth when they reinnervate *e.g.* an iris transplant. The information originates in the transplant. It was shown by the control experiments that, in all probability it does not reach the sympathetic neurons by the systemic circulation since *this would presumably have caused a bilateral hyperinnervation of the* irides of the host following a unilateral homologous iris transplantation.

A nervous mechanism involving sensory afferents from the eye and efferents to the sympathetic cell bodies in the superior cervical ganglion is ruled out by the fact that the growth takes place in spite of decentralization of the ganglion. The systemic and nervous routes of information are thus improbable, making the local way via the aqueous humor and/or the direct contact between host iris and transplant highly probable.

The proliferation of sympathetic nerves is only one of a series of changes which includes also the rapid formation of connective tissue strands with vessels connecting the transplant to the host iris. It has not been determined in the present investigation whether the connective tissue bridges grow from the host, from the transplant or from both although it seems probable that the bridges are formed mainly from the vascularized host in response to *factors released from the transplant e.g.* by the initial ischemia.

The impetus to growth of the peripheral parts of a neuron is probably a local one, independent of the nerve cell body. This is supported *e.g.* by the fact that after severance of the axon of a motoneuron, *both* ends of the cut axon shows sprouting initially (see Bodian 1947) although after a few days the distal stump degenerates due to lack of axoplasmic flow. On the other hand the changes in the nerve cell bodies following axotomy is a classical feature described already by Nissl 1892 (chromatolysis, swelling of cell body eccentric nucleus) demonstrating that in this case "information" has also reached the cell body. It is now known that the nerve cell

body indeed has a period of increased protein turnover during the regenera tive cycle after axotomy (see Droz 1969)

In the case of reinnervation of transplanted irides from the intact sympa thetic neurons, the increased volume of the terminal parts of the neurons can probably not be due to a local protein synthesis in the axons during regeneration since axonal protein synthesis seems to be minimal (see Droz 1969) Information would then have to reach the nerve cell bodies if they were to respond with an increased protein synthesis. This could be obtained either by a retrograd flow of material (see Dahlström 1965) or simply by an altered hydrodynamic pressure in the cell body caused by the growth of the axons. However it is not necessary to postulate that information of some kind has to reach the cell bodies. An alternative explanation would be that the normal axonal flow of material is sufficiently high and that the terminal parts of the neuron now utilizes parts of it for growth instead of metabolizing it. — In fact, it almost seems to be more difficult to explain how the large amounts of axoplasm produced by the cell body and trans ported down to the terminals is handled under steady state conditions. If however one considers the large number of dichotomous divisions and the enormous increase in total cross section area of th axon ramifications, it is obvious that the flow rate in the most peripheral parts must be considerably slower than in the main axon

Growth of various types of neurons in tissue culture is now a well established technique, which shows that the presence of an effector tissue is not necessary for the proliferation of nerve fibres. However composite growth media, often containing extracts from embryonic sources are needed, and in the case of adrenergic nerves the absolute requirement for proper maintenance and growth has been found to be the nerve growth factor (NGF see Levi Montalcini and Angeletti 1968) A situation similar to tissue culture in the present work was the massive outgrowth of nerve fibres from transplanted ganglia during the first days when no vascularization was established the aqueous humor being the incubation medium. Growth stimulat ing factors may have been present in the aqueous humor or in the transplant. Even if the information for growth is derived from some extraneuronal source t seems that the initial growth is independent of the local surround ings. An exampl of this is the fanshaped arrangement of the outgrowing f bres from the ganglion transplant in the anterior chamber However after a few days, the outgrowing nerves have reached the original nerve trunks with Schwann cells, and the further growth seems to follow the old tracts.

b) *Direction of growth* Apart from th above mentioned mitial sprouting from transplanted ganglia, it seems as if the appearance of the newly formed

destruction (Jonsson and Sachs 1970) have proved to be comparingly rapid processes.

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presence of nerves inhibits the outgrowth and that the absence of nerves stimulates the growth. It seems unlikely that inhibition of stimulation of the adrenergic receptors controls the outgrowth One possibility of growth regulation would be that the nerves together with the receptor tissue form an inhibiting factor as the nerves will grow only into organs normally adrenergically innervated.

d) *Specificity of growth* Two general conclusions concerning the specificity of the growing adrenergic nerves vis-a-vis their effector organs can be drawn from the present experiments. Firstly the growth is specific in the sense that the new adrenergic nerves are largely confined to receptor tissues that are normally adrenergically innervated but have become denervated. The new nerves do not grow into areas where adrenergic nerves do not exist normally and they do not grow into already innervated areas. Thus, the adrenergic nerves obey to the same rules as *e g* motor nerves reinnervating muscles (see reviews by Guth 1956, 1968). This type of specificity has also been elegantly demonstrated in ganglion transplantation experiments in insects (Jaklet and Cohen 1967). Secondly the growth is unspecific in the sense that adrenergic neurons from any part of the adrenergic nervous system seem to be able to reinnervate any effector tissue. This unselectivity includes to a certain extent also heterologous transplantations, since a mouse iris became reinnervated in the rat eye and a mouse ganglion could reinnervate a rat iris.

There are indications in the literature that under certain circumstances not even a specificity between the sympathetic and the parasympathetic branches of the autonomic nervous system exists. The hypothesis that collateral sprouting of cholinergic fibres occurs after degeneration of the adjacent sympathetic neurons in the salivary glands was put forward by Nordenfeldt (see Nordenfeldt 1968) He found increases in glandular choline acetylase and cholinesterase following postganglionic sympathetic denervation. His mochemical studies on this subject are few However Roth and Richardson's (1969) interesting observation of what may be interpreted as sympathetic axonal sprouting in irides which had been parasympathetically denervated speaks in favour of such a lack of specificity Similar findings were reported by Taxi (1959) Garrett (1969) suggests that postganglionic parasympathetic sprouting in a retrograd direction from th cat submandibular gland refill the sympathetic trunk along the submandibular artery following sympathetic denervation.

Incidentally observed examples of non-adrenergic (*i.e* non-fluorescent) nerve cells, which apparantly survived transplantation in the present experiments, were the non fluorescent nerve cells in the superior cervical ganglion,

X SUMMARY

The anterior chamber transplantation technique offers unique possibilities for nerve growth studies. Transplants survive well and become rapidly vascularized from the iris of the host eye. The condition of the transplants can be followed in the living animal by microscopic inspection through the cornea. The iris of the host eye contains a thoroughly studied, dense and rich plexus of adrenergic nerves and the iris can easily be sympathetically denervated. All adrenergic nerves in the host irides and transplanted irides can be studied in every detail in stretch preparations.

Growing adrenergic nerves in the adult rat were studied qualitatively and quantitatively by a combination of the Falck Hillarp fluorescence method for cellular localization of monoamines with ^{3}H noradrenaline uptake measurements. Tissue transplantations to the anterior chamber of the eye were used in two principally different ways.

1) Transplantation of various adrenergic receptor tissues. Reinnervation of the transplants took place by collateral sprouting from the adrenergic ground plexus of the host iris. The time course of adrenergic reinnervation of the iris was followed in detail after autologous transplantation of one iris to the anterior chamber of the other eye. The iris transplant, which became sympathetically denervated because of the operation, was rapidly reinnervated by collateral sprouting from the intact adrenergic ground plexus of the host iris. The first adrenergic nerves were found in the transplant after 4 days, and reinnervation was complete within one month. Concomitantly to the reinnervation of the transplant, there was an adrenergic hyperinnervation of the host iris.

2) Transplantation of various sympathetic ganglia. When the host iris was simultaneously sympathetically denervated, the transplanted neurons reinnervated it.

The time course of adrenergic reinnervation was followed in detail after autologous transplantation of the superior cervical ganglion to the anterior chamber of the ipsilateral eye. The operation results in a complete denervation of the iris of the host eye. A large number of nerve cell bodies in the

ganglion transplant survived the close axotomy and immediately began to reinnervate the host iris. The first sprouting adrenergic nerves were found on the iris after one day and reinnervation was complete within one month.

Confirmatory experiments were performed in mouse and hamster. Transplantations of irides and ganglia to other locations than the anterior chamber were also described.

The adrenergic transmitter mechanisms of the newly formed nerves were similar to those of mature nerves at rest. Noradrenaline uptake and storage properties seemed to be well developed from the moment of formation of the new nerves. Release of the transmitter and restitution of adrenergic neuromuscular transmission in the new nerves could be demonstrated.

Sympathetic neurons from any part of the sympathetic nervous system seemed to be able to reinnervate any sympathetically denervated receptor tissue. The innervation pattern was always similar to that normally found in the receptor organ. Adrenergically not innervated tissues remained so after transplantation. Reinnervation could also be demonstrated after heterologous transplantations.

The presence of a normal adrenergic innervation almost completely inhibited the outgrowth of new nerves. On the other hand, the growth was very little affected by chronic treatment with drugs interfering with the adrenergic transmitter mechanisms, chronic adrenergic receptor blockade or chronic treatment with cortisone or testosterone. The nerve growth was likewise independent of a normal impulse traffic. Chronic sympathetic denervation of tissues to be transplanted did not essentially impair adrenergic reinnervation.

The adrenergic neurons of the adult rat have thus been shown to possess efficient growth mechanisms. Growth occurs both from intact neurons and following axotomy close to the nerve cell bodies. A more dynamic role of the peripheral adrenergic innervation apparatus is suggested, the nerves being in a labile equilibrium with the local surroundings. Many different disturbances of this equilibrium such as injury transplantation of denervated tissues and possibly also inflammation will initiate immediate proliferation of adrenergic nerves.

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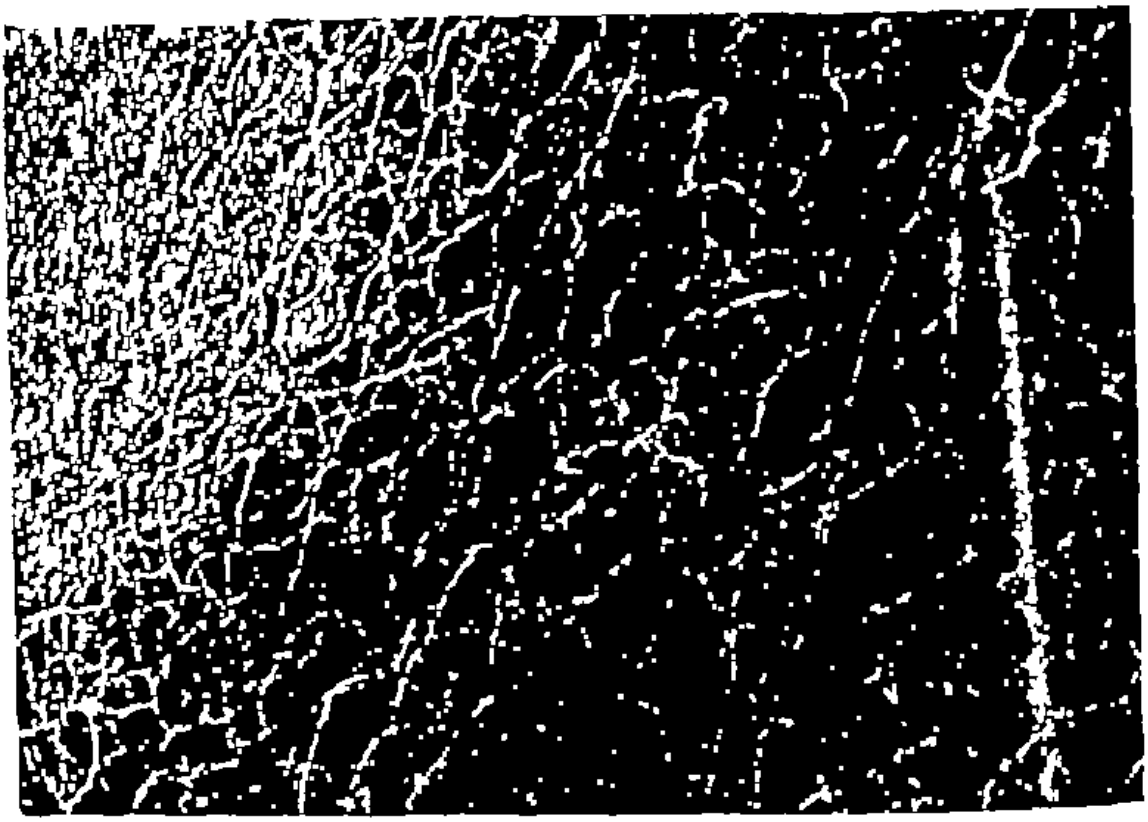
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FIGURES

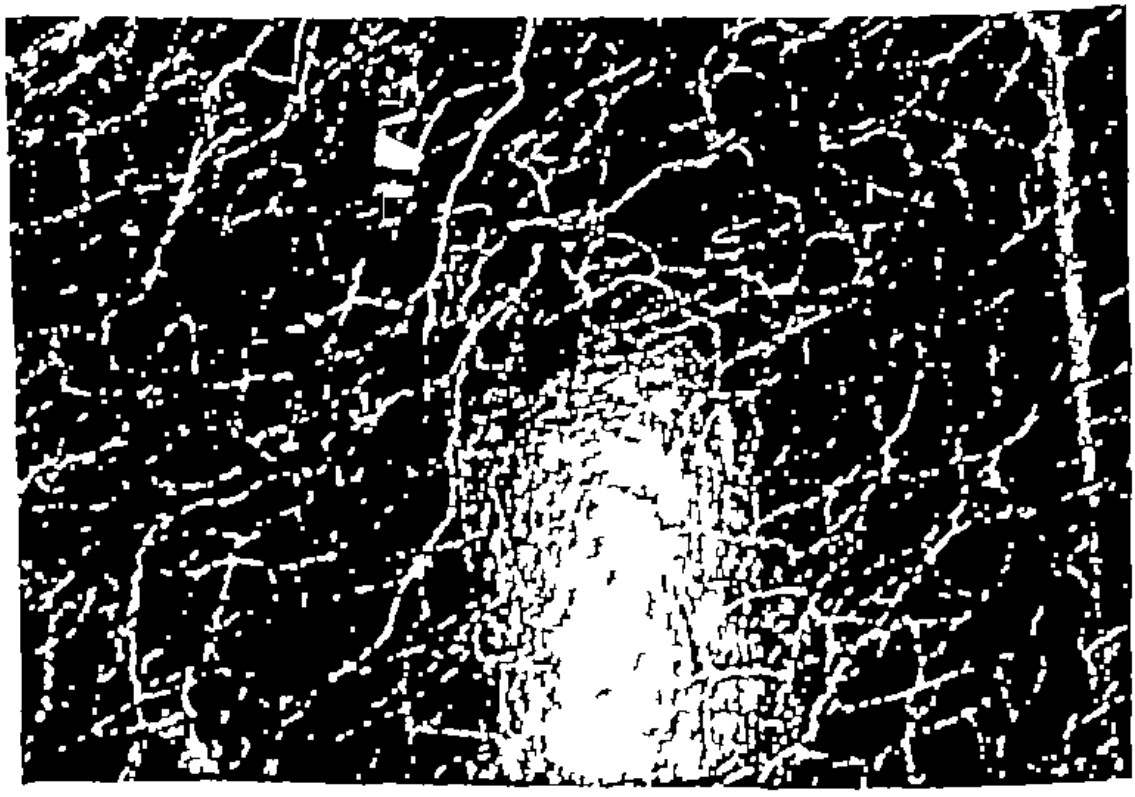
All figures, except Fig 38 are fluorescence microphotographs of stretch preparations (the irides) or 8 μ sections. When not otherwise stated the anterior chamber transplantation technique was used. All figures, except Figs 59—62, demonstrate rat tissues.



1 Partially sympathectomized denervated iris 2 days after the operation (see II.9). Most of the adrenergic ground plexus has disappeared thereby revealing the ramifications from single neurons of Malmfors and Sachs 1965). One, weakly fluorescent, beaded axon (lower left corner) branches twice to give rise to 3—4 separate systems of varicose strongly fluorescent, branching nerve terminals at different places on the iris. 120.



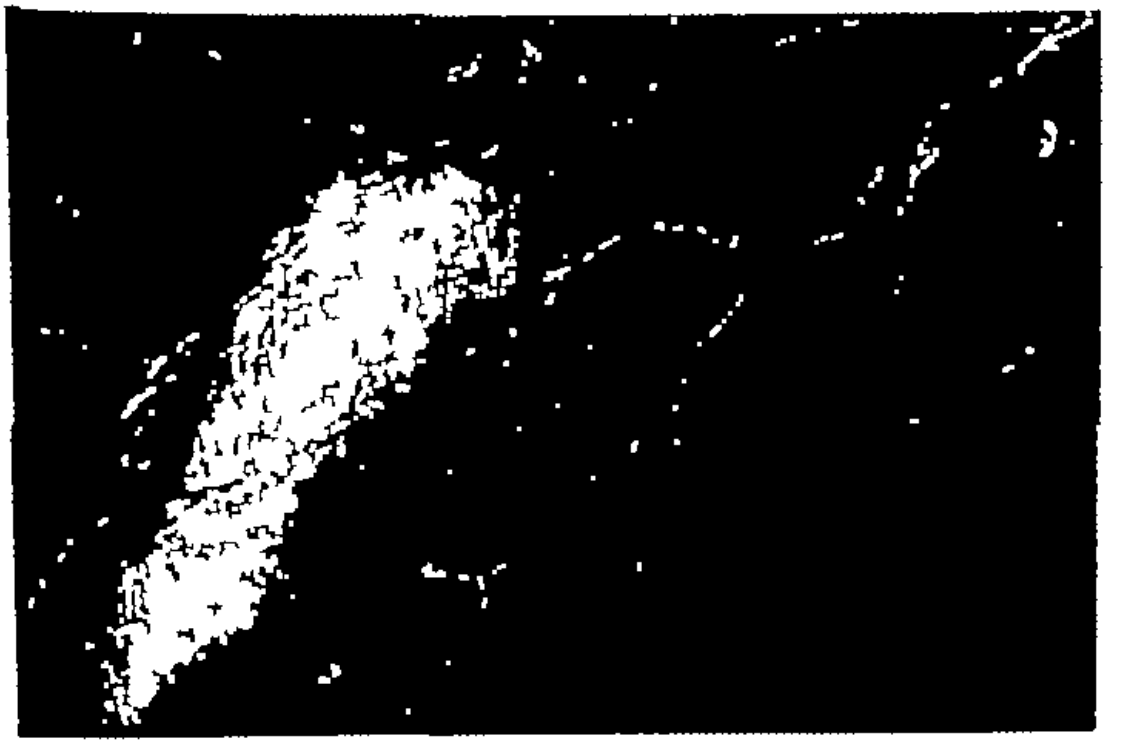
2. Iris from n rmal animal. The iris was incubated for 30 min in 10^{-7}M ^{3}H NA and rinsed in aminefree buffer for 10 min before the preparation. The adrenergic ground plexus, which consists of varicose nerv terminals and some smooth non-terminal axons, is seen. 140.



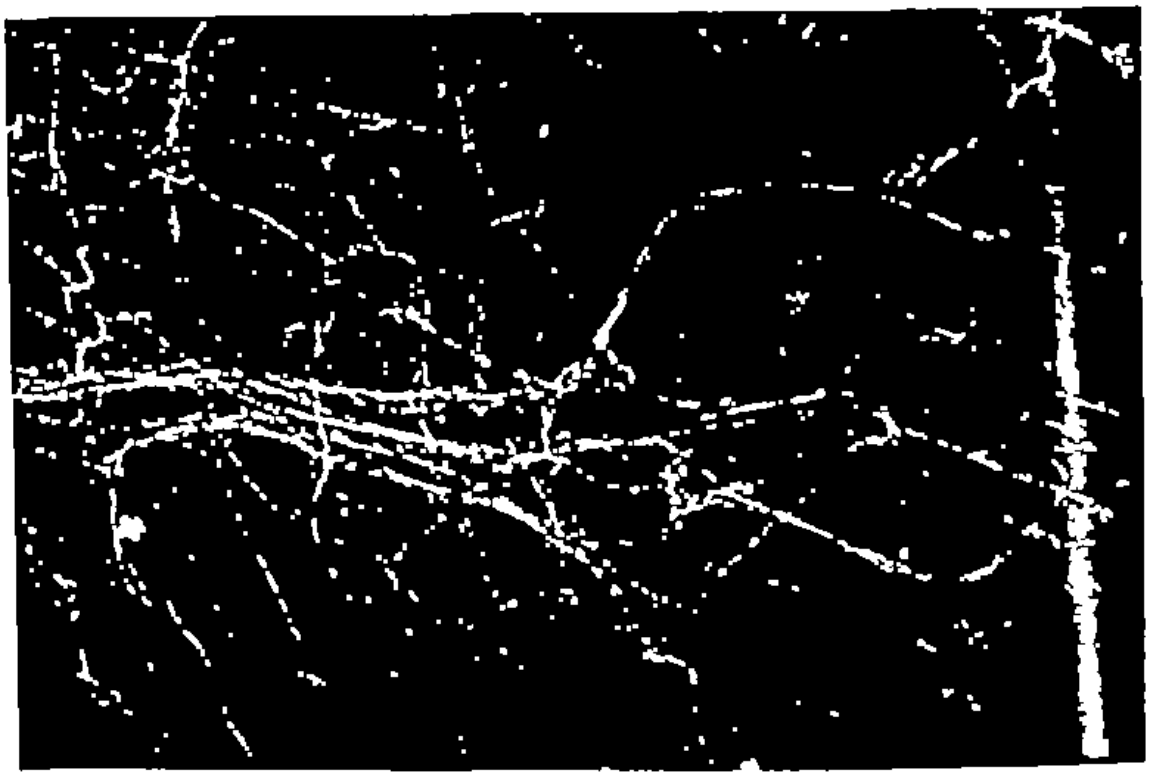
3 Iris from normal animal treated as in 1 y 2. ...5



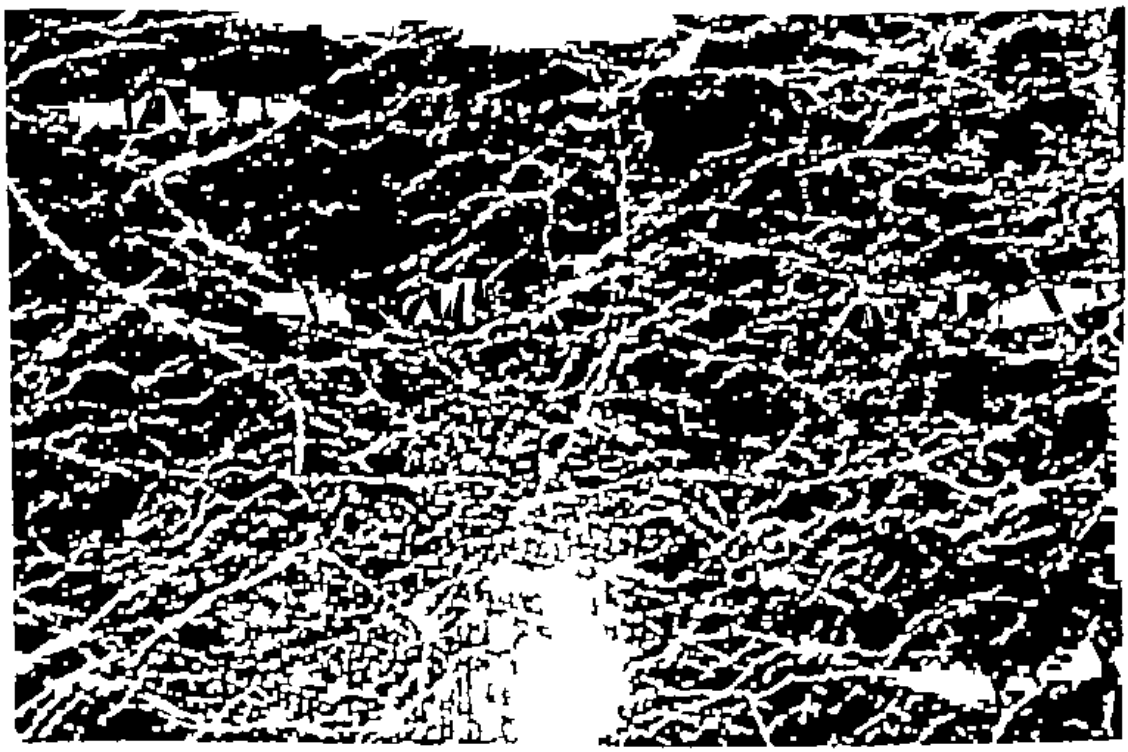
4. Connection between host iris and a transplant, 9 weeks. The new fluorescent axons which have a beaded appearance arise from the sphincter margin of the host iris (left) and bridge over towards the transplant (not demonstrated). In this case a duodenal muscle strip was transplanted. 140.



5 Iris transplant, 1 week. New nerves are beginning to form sparse plexus on the transplant. Note variable morphology and fluorescence intensity 225

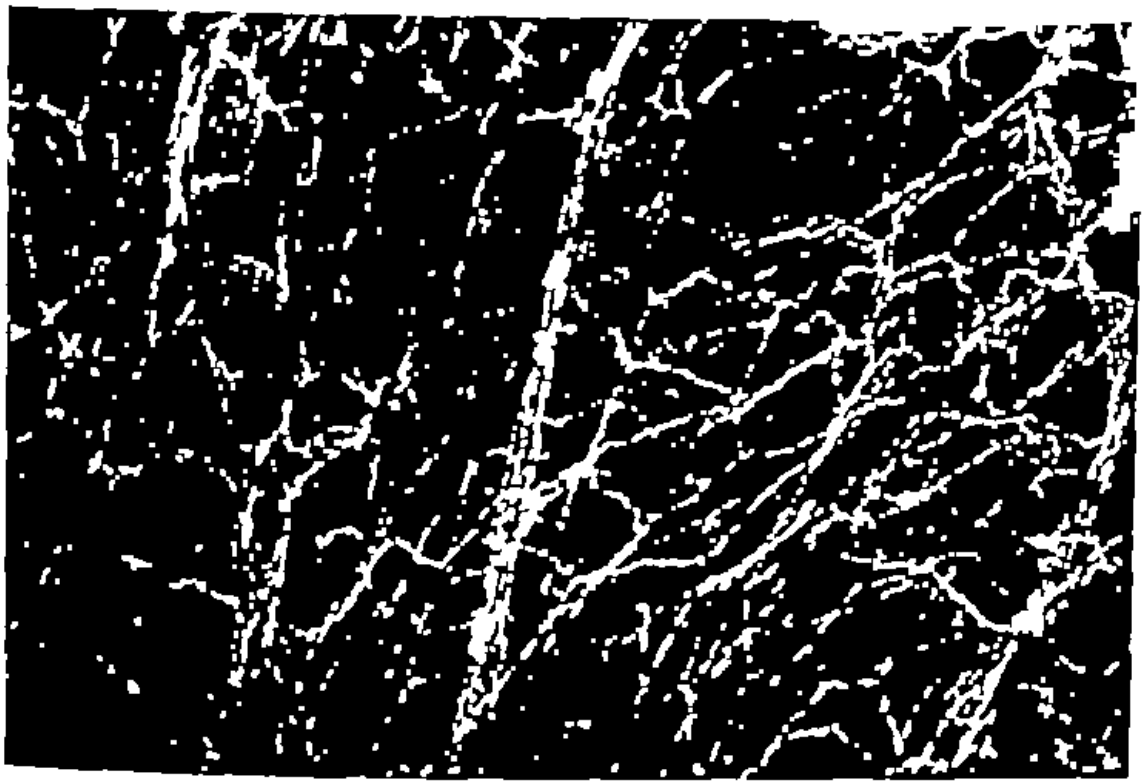


6. Iris transplant, 2 weeks. A relatively well developed plexus of varicose nerve terminals has been formed in the transplant. It is, however more irregularly arranged and less dense than the normal plexus (Fig 2) Several bundles of beaded axons ar seen 140.

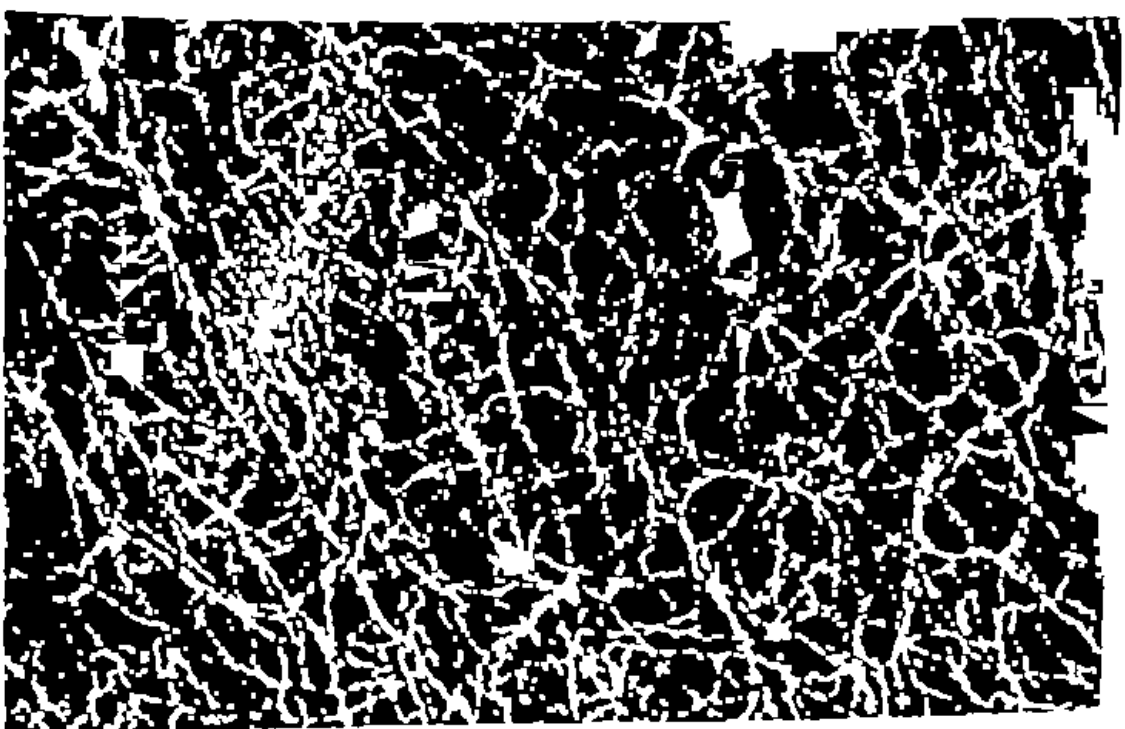


7 Host iris, 2 week
when compari g this in

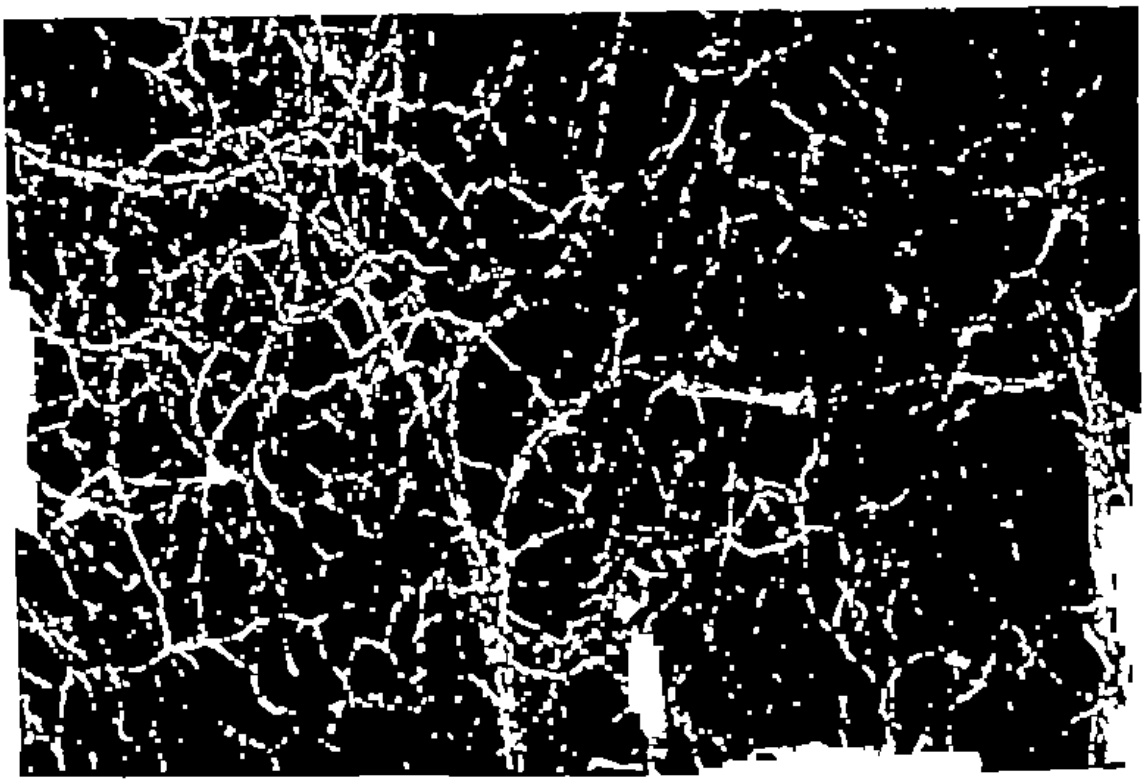
The hyperinnervation is evident



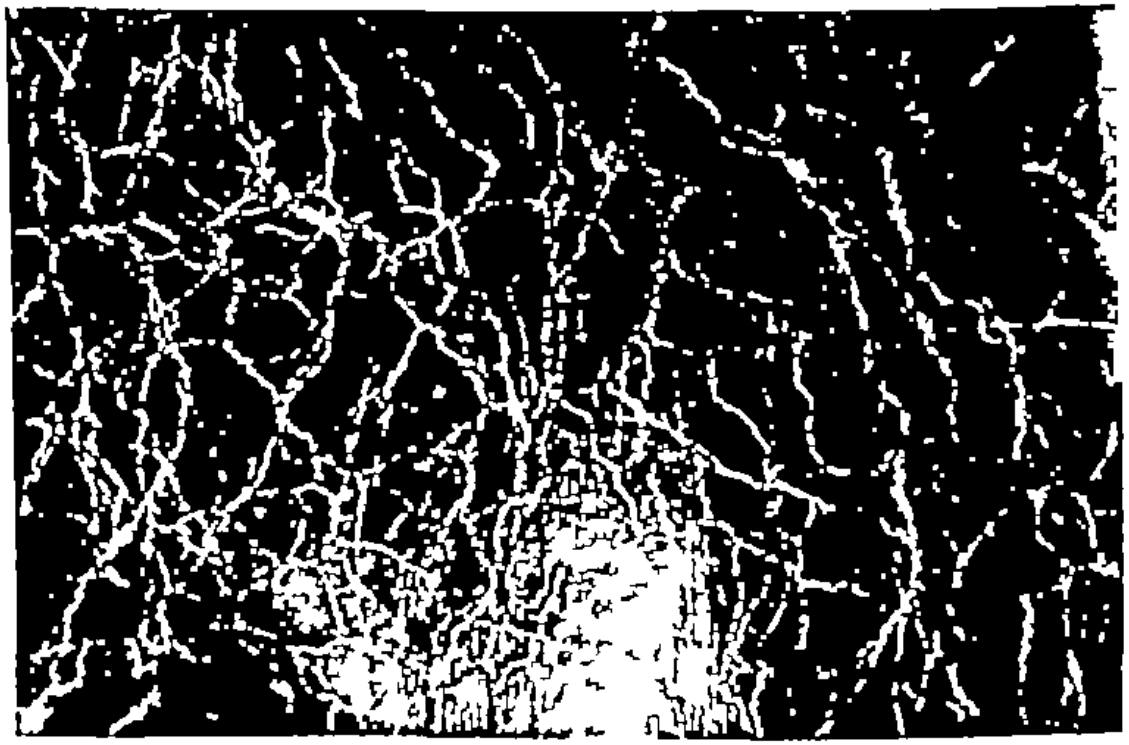
8. Iris transplant, 2 weeks. This particular transplant contained somewhat more nerves than the transplant depicted in Fig 6 and is therefore more similar to the corresponding normal iris (Fig 3). The ground plexus is, however more irregular and bundles of beaded axons can be found. ×25



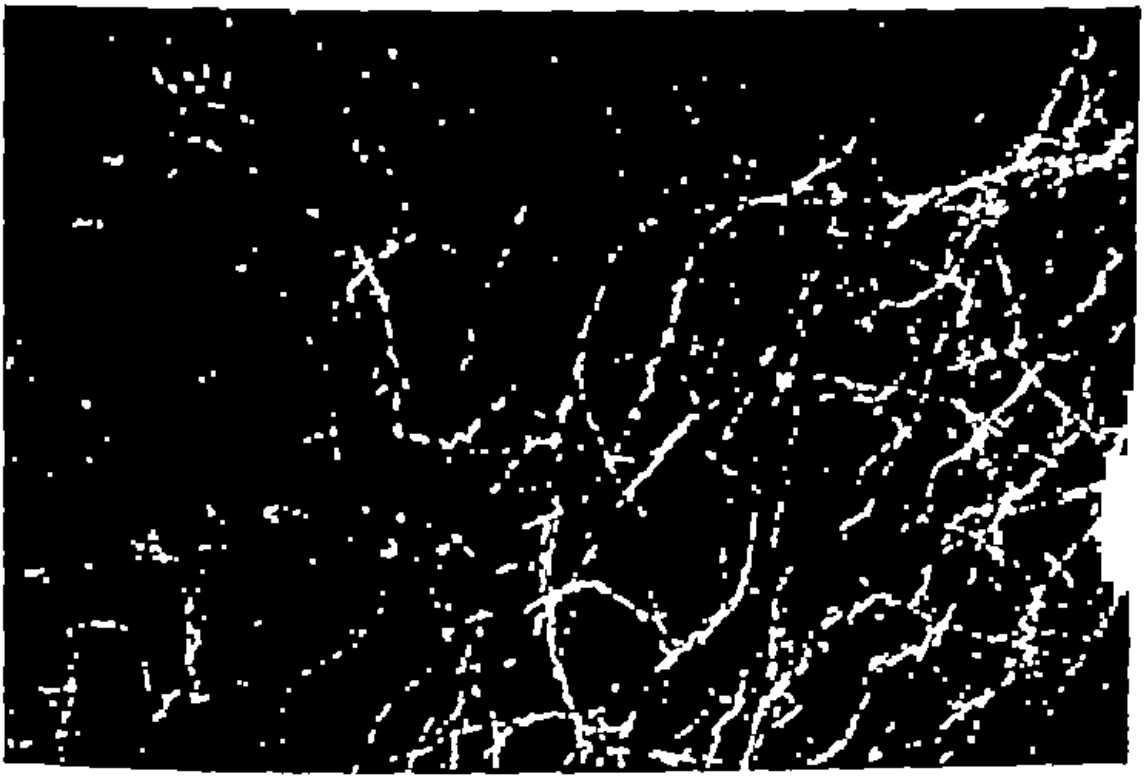
9 Host iris, 2 weeks after iris transplantation. Same magnification as normal Fig 3 and transplan Fig 8. The hyperinnervation is clearly demonstrated. ×25.



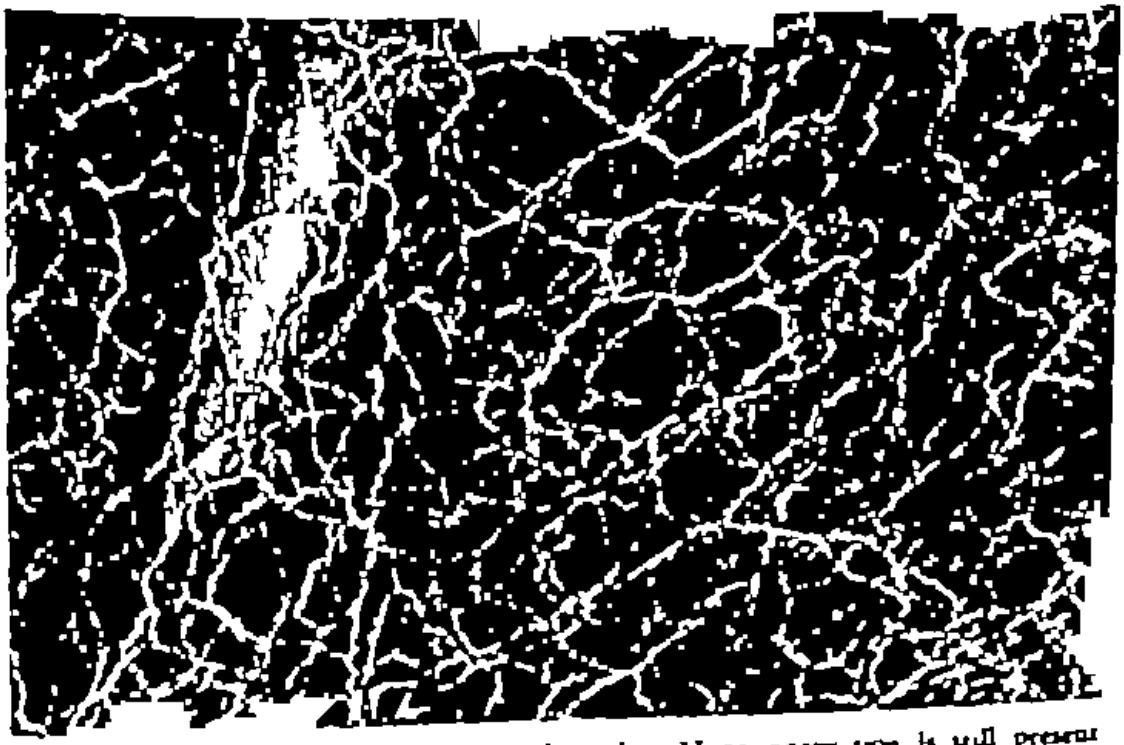
10. Iris transplant, 4 weeks. Well developed fairly regularly arranged ground plexus and larger dividing bundle of axons ar seen. 225.



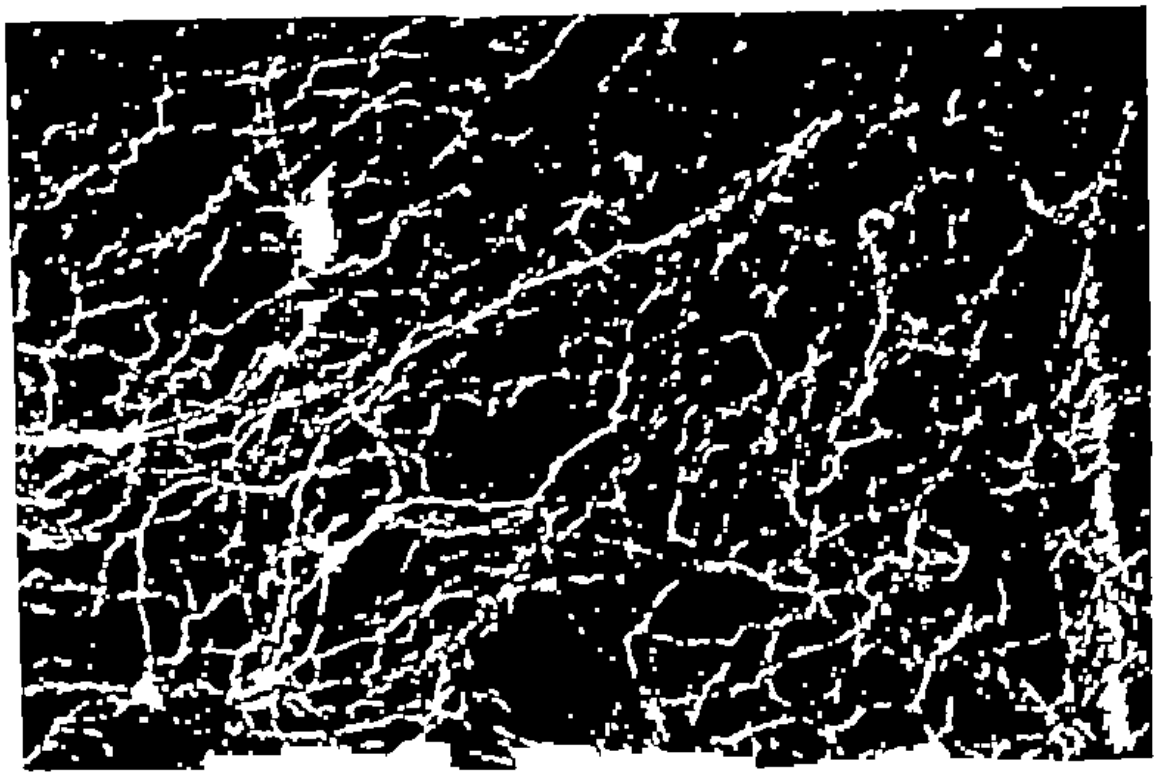
11 Iris transplant, 6 weeks. W ll d l ne gic ground plexus. 225



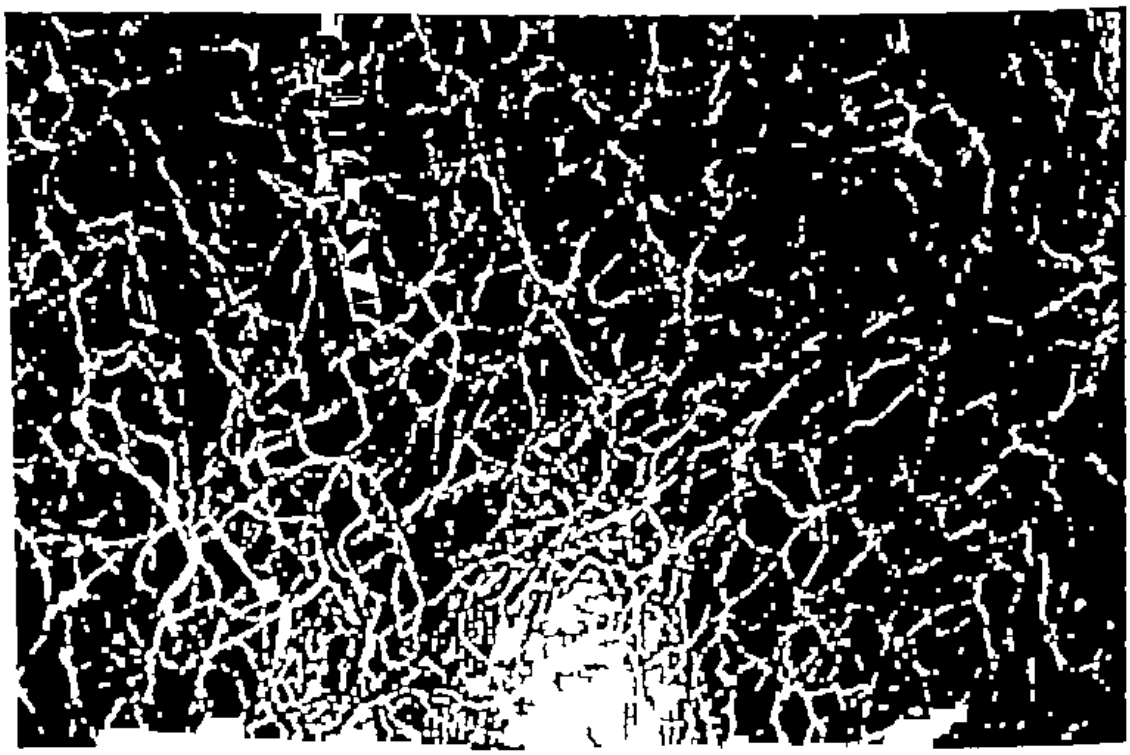
12. Iris transplant, 12 weeks. Well developed adrenergic ground plexus. 225



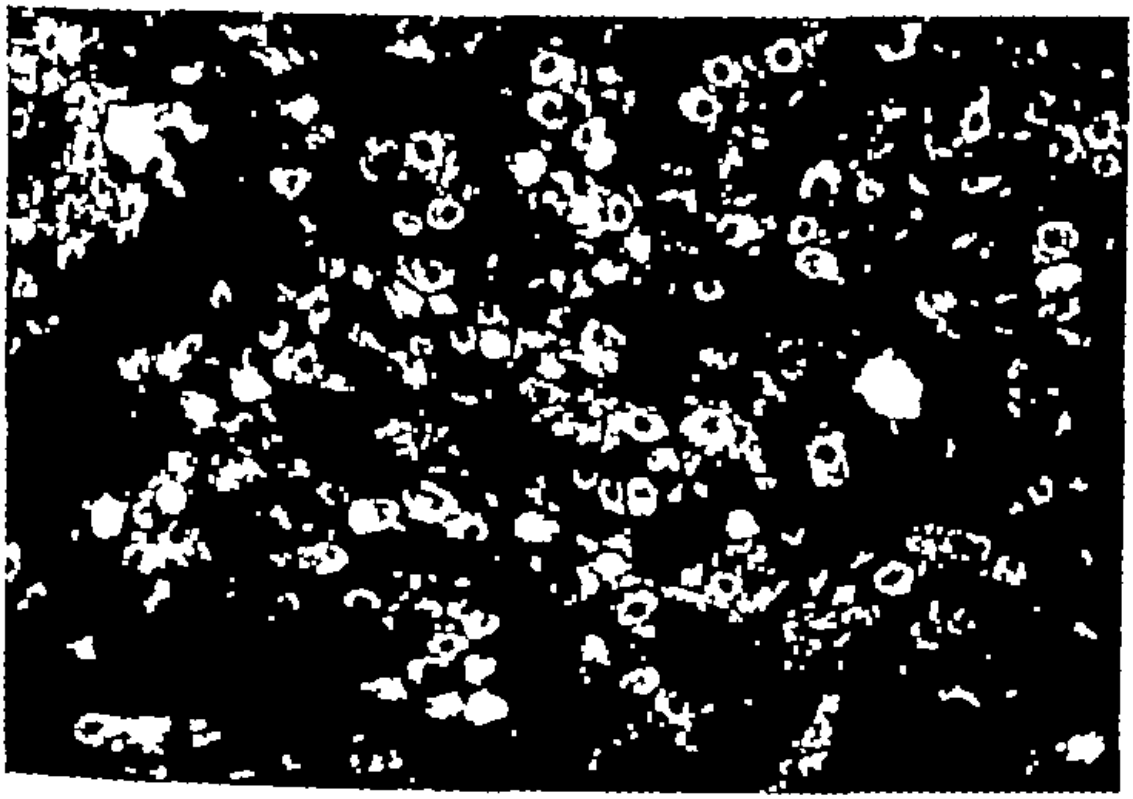
13. Host iris, 12 weeks after iris transplantation. Hypernnerv tion is still present but less conspicuous than after 2 weeks (Figs 7 and 9). 225.



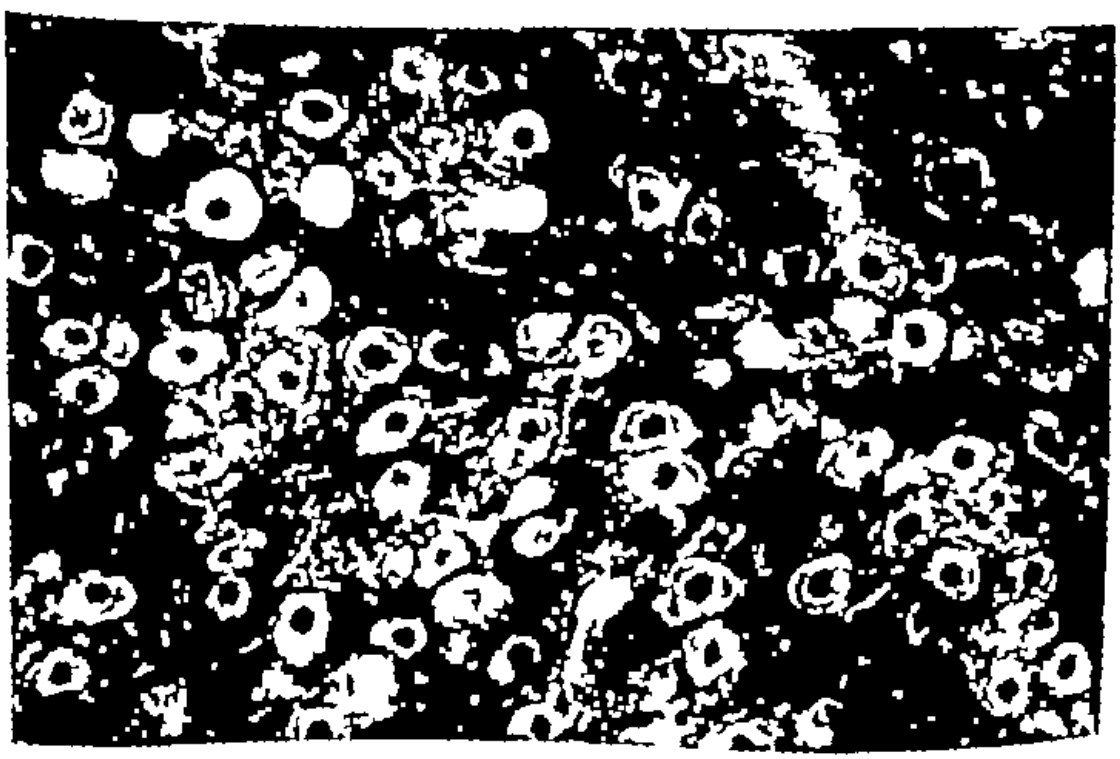
14 Iris transplant, 10 months. A well developed plexus of adrenergic nerves is found. Some of the irregularities in this transplant is probably an artefact due to difficulties in unfolding and stretching older transplants. 140.



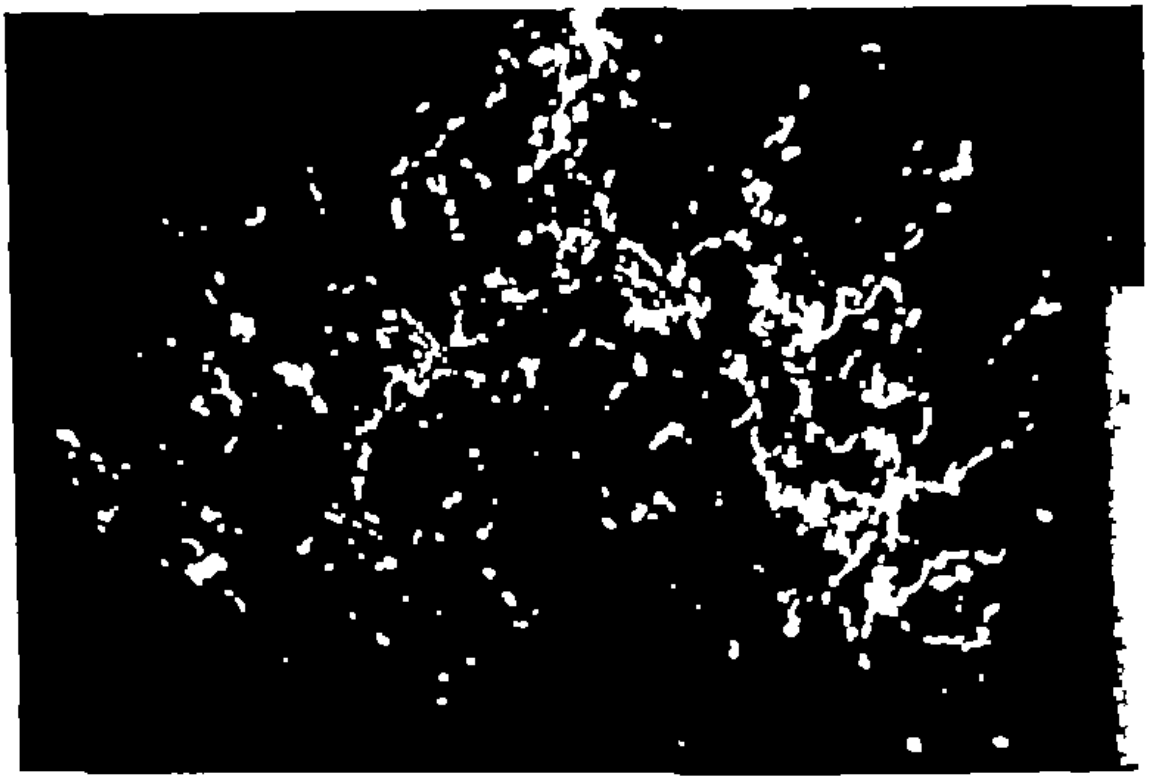
15 Host iris, 10 month ft tion. A slight hyperinnervation is present. 140.



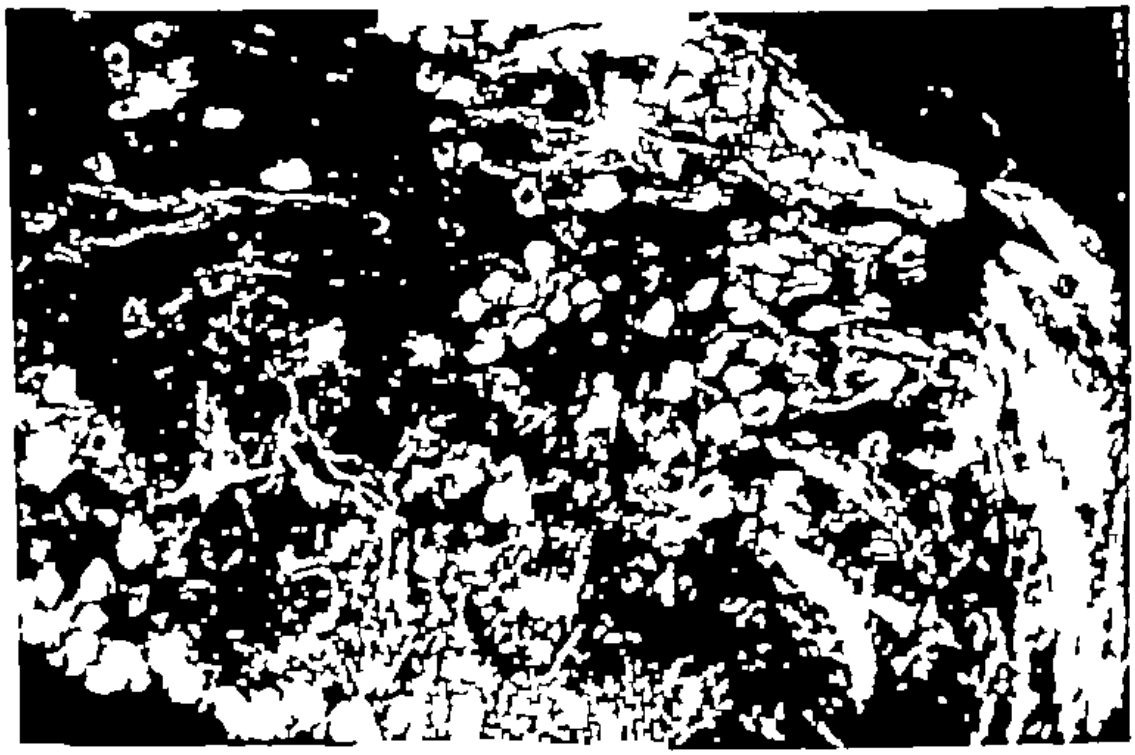
16. Normal superior cervical ganglion. Almost all nerve cell bodies show specific fluorescence although with varying intensities. The nerve cell bodies are closely packed together. A small group of strongly fluorescent small, so called SIF-cells are seen (upper left). 140.



1 Normal superior cervical ganglion. Densely packed cell bodies with varying fluorescence intensities and a few intraganglionic fluorescent axons are seen

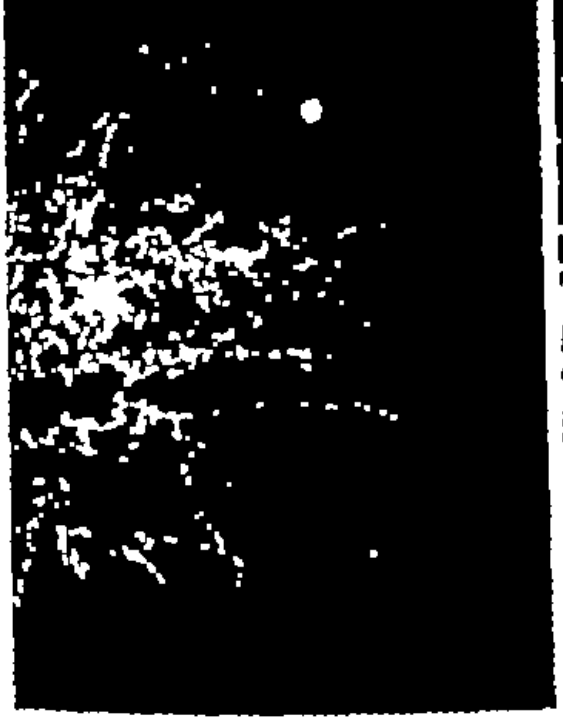


18. Host ris, 1 day fter ipsilateral transplantation of the superior cervical ganglion. The original adrenergic ground plexus has disappeared. The photograph show the site of attachment of a transplant which was peeled way before stretching. Thick, strongly fluorescent, distorted fibers and thinner fibres radiating out on the host iris can be found. 140.



19 Transplanted superior fluorescence intensities, some h fluorescent material re see ganglionic tructure is loos fibres. 140.

l d y The cell bodies have ryling ed tensities H vy accum lations f axon bundles. Note also that the in reased amount of intraganglionic



20. Host iris, 2 days after ipsilateral transplantation of the superior cervical ganglion. New fibres radiate out from the site of attachment of a transplant. A large blood vessel is passing through the innervated area. 60.



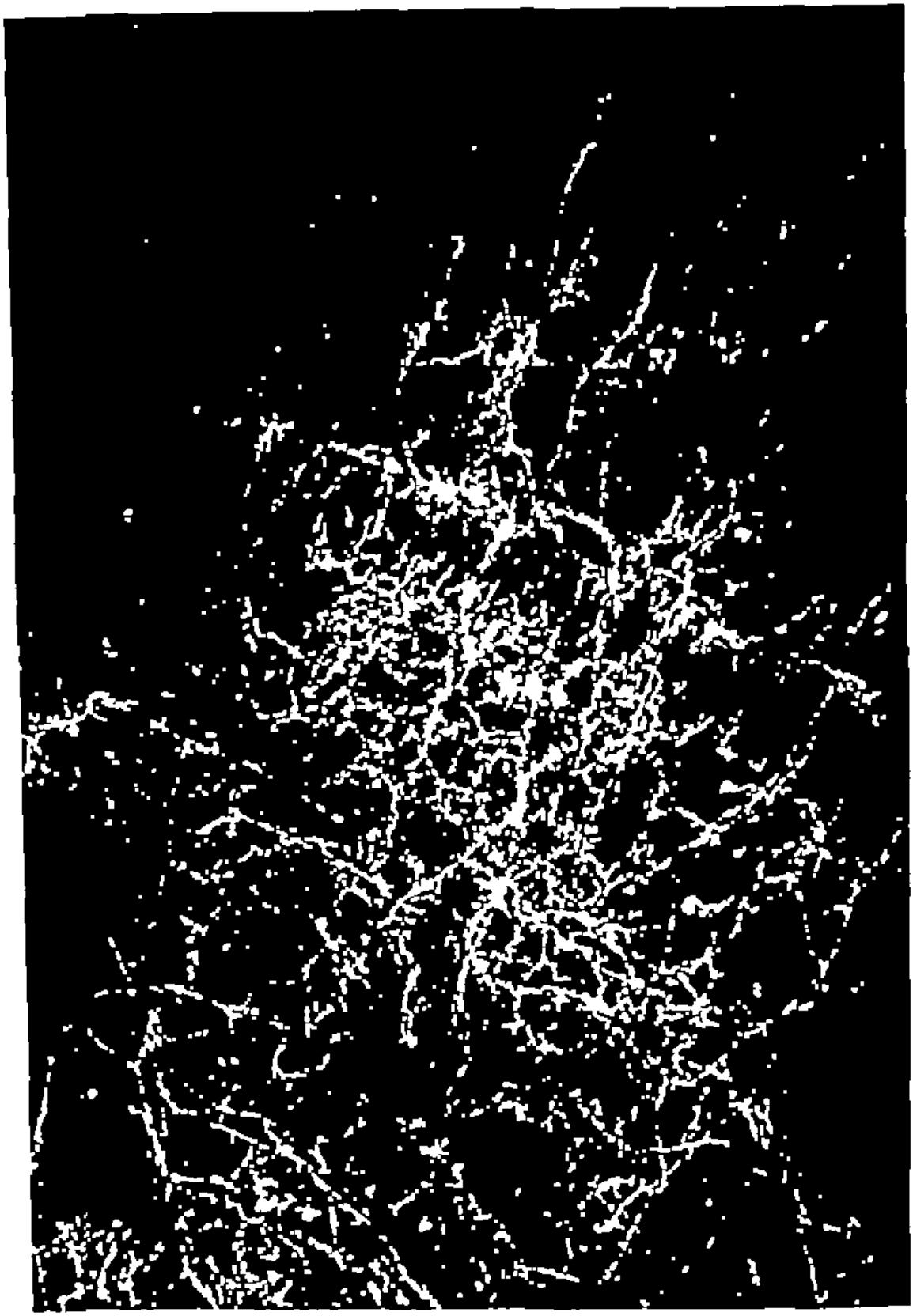
21. Host iris, as in Fig. 20. Distorted, swollen strongly fluorescent fibres and a formation of 4 slender axons with growth cones, leaving the site of attachment together as if they were following a preexisting pattern are found. 155



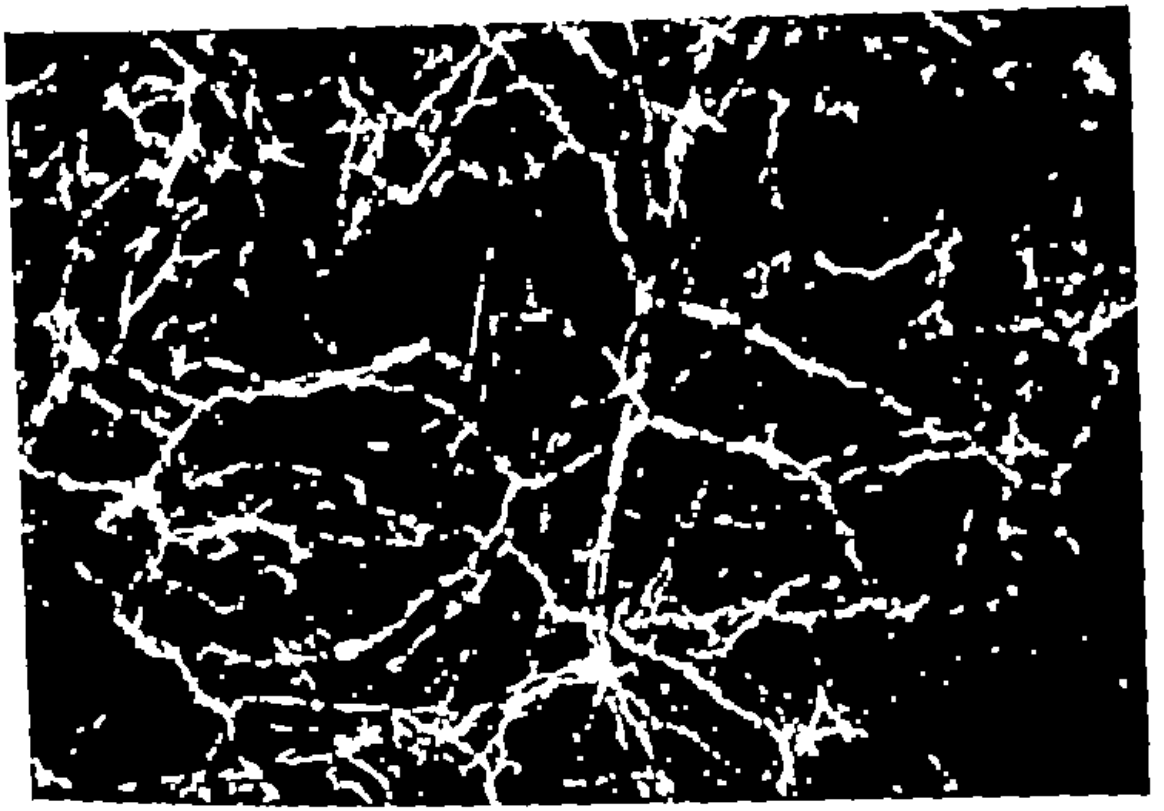
22. Transplanted superior cervical ganglion, 2 days. Numerous bundles of strongly fluorescent axons are seen the ganglion. 125



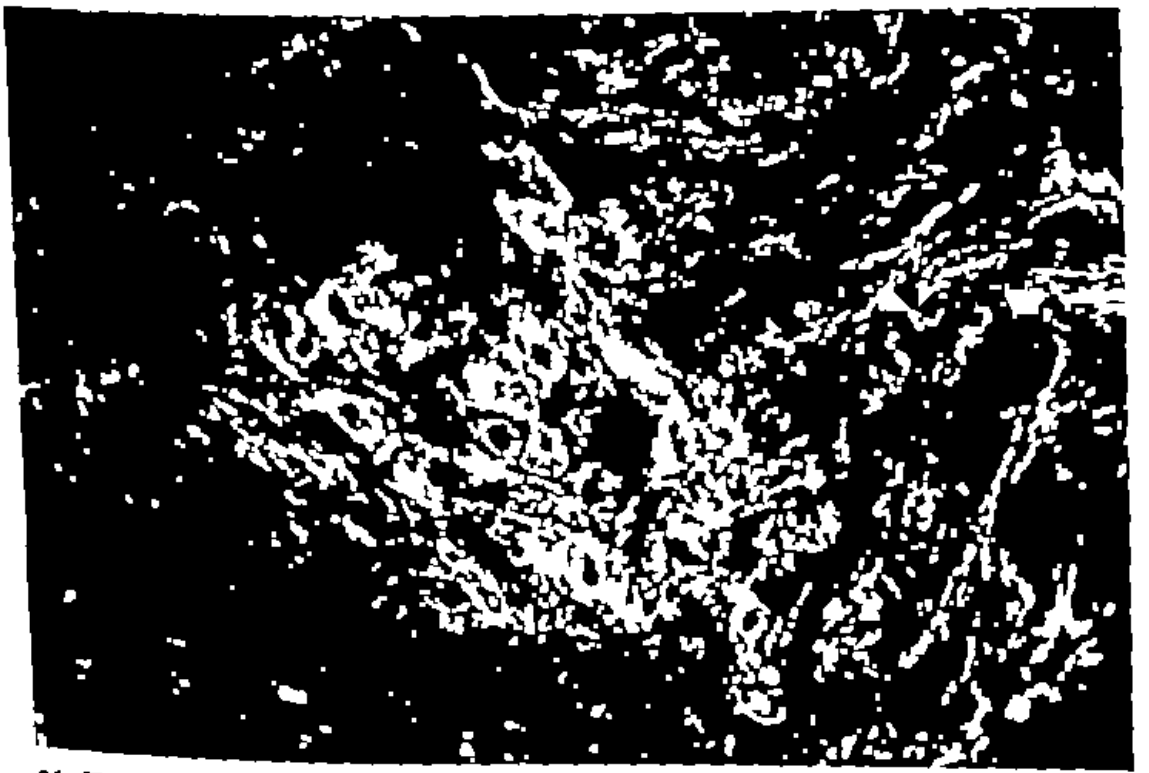
23. Transplanted superior cervical ganglion, 2 days. The cell bodies show strong fluorescence intensities and sometimes eccentric nuclei. Two large bundles of strongly fluorescent axons are cross-sectioned. x 310



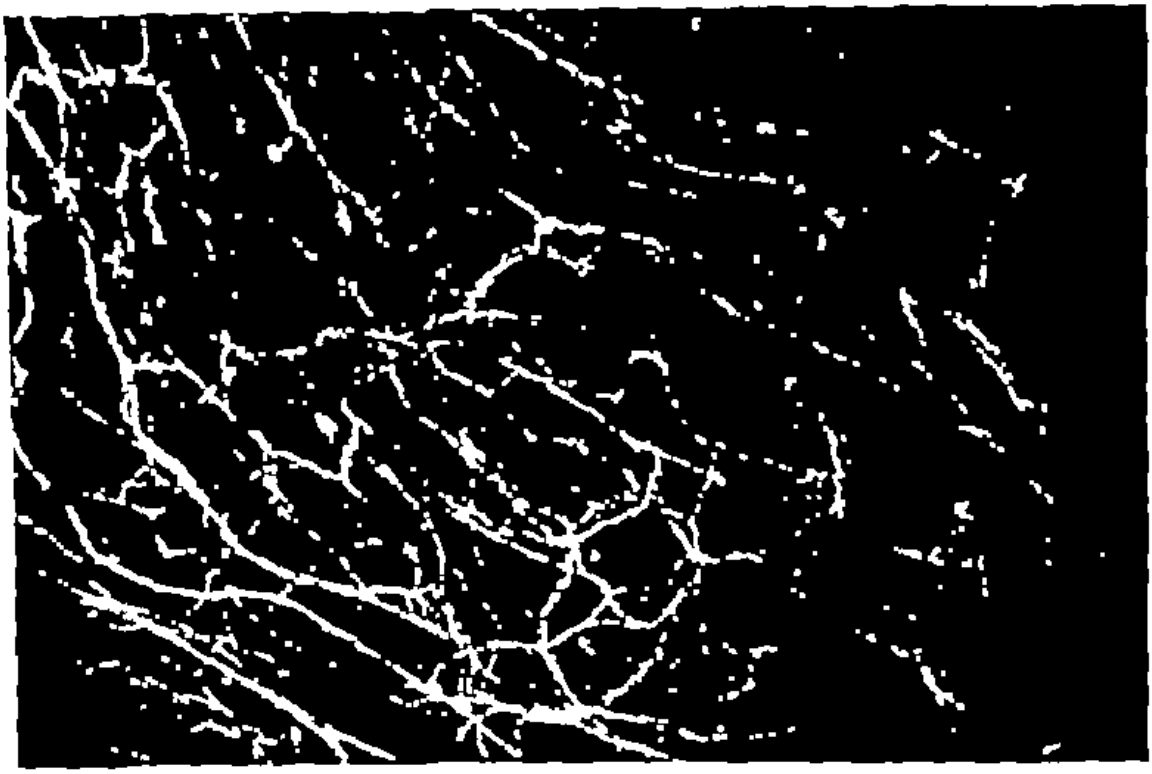
24 Host ris, 2 day fter psilateral transplantation f the superior cervical ganglion. Surv y f nnerv ted area. Thick, distorted and slender fibres are seen. Although several beaded f bres are seen, no typical aricose nerve terminals ha yet been formed. A la g blood sel tr erses the innervated area. 135



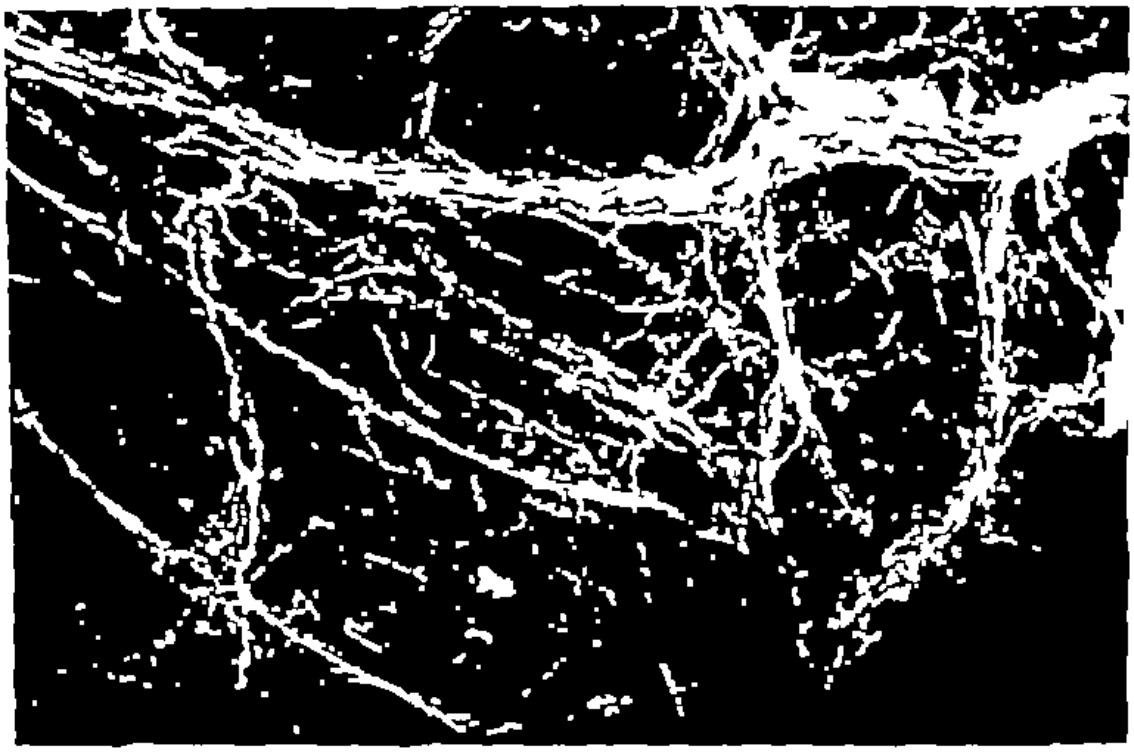
25. Host iris, 8 days after ipsilateral transplantation of the superior cervical ganglion. A richly innervated area is shown. The irregular plexus is composed of bundles of axons lacking the typical varicose appearance. 225.



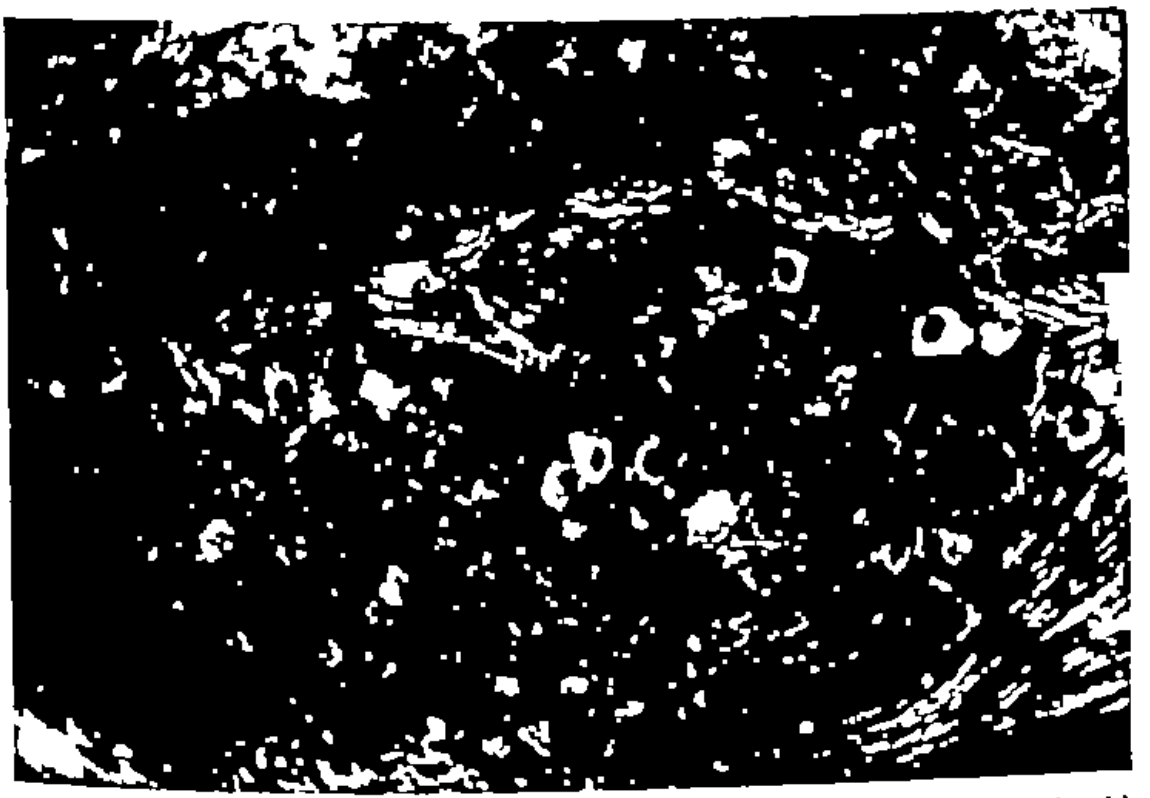
26. Transplanted superior cervical ganglion, 4 days. A group of cell bodies generally with relatively low fluorescence intensities and large, strongly fluorescent axon bundles are seen. 225



27 Host iris, 2 weeks after ipsilateral transplantation of the superior cervical ganglion. A more regular plexus of fluorescent nerves has been formed. 225



28 Host iris, 2 weeks after ipsilateral transplantation of the superior cervical ganglion. This figure gives the appearance of an host iris closer to the transplant than Fig 27. Thick, strongly fluorescent bundles of non-terminal axons are seen giving off branches over the iris. Such axon bundles are never found on normal irides, where the non-terminal adrenergic axons have a low fluorescence intensity and never form large bundles. 140.



29 Transplanted superior cervical ganglion, 17 days. This figure, which should be compared to Fig 30 below shows an area of a transplant with high nerve cell density The nerve cells ha an almost normal fluorescence morphology A few strongly fluorescent axon bundles are found. 225



30. As Fig 29 P rt of transplan where nerve cell bodies are few and axon bundles dominate. One large group and few solitary small intensively fluorescent c. SIF-cells are found. Figs 29 and 30 together demonstrates the variable nerve cell densities in the ganglion transplants. 225



35 Host iris, 6 week after unilateral transplantation of the superior cervical ganglion. The fan-shaped emergence of axon bundles from the site of attachment of the transplant is seen. 70.



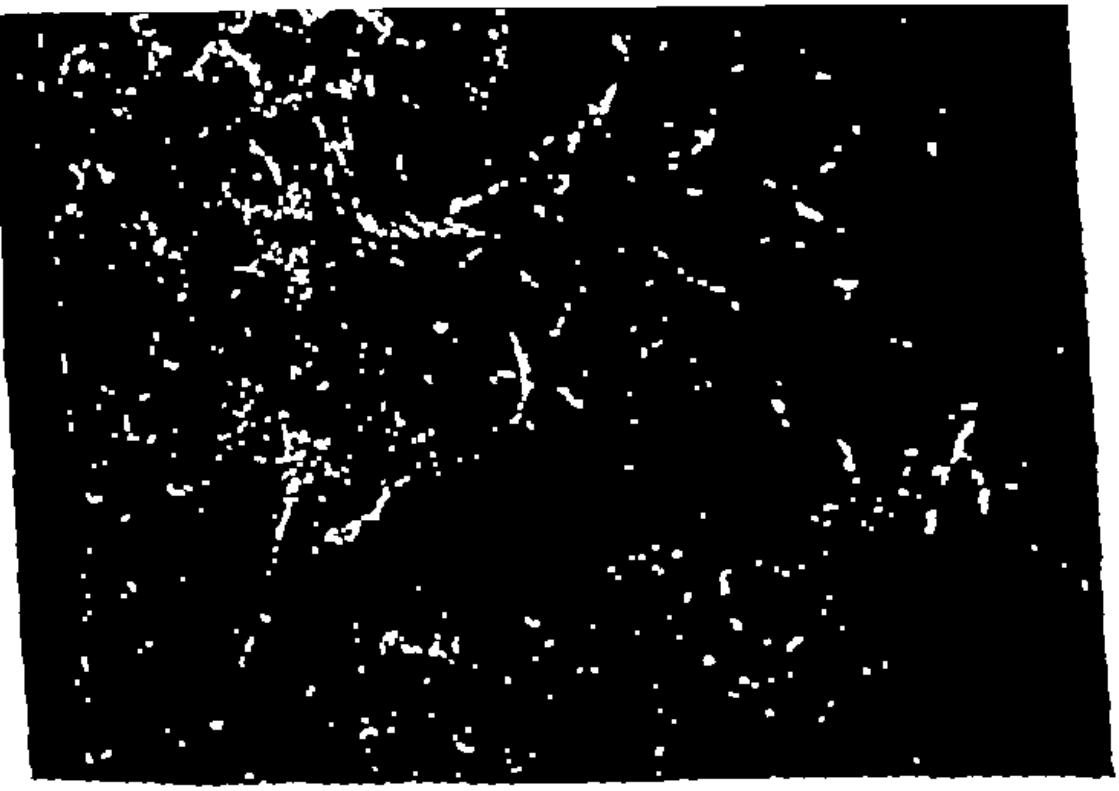
36. Host iris, as fig 35 The typical arrangement of fluorescent fibres at the border between the choroid membrane (left) and the ciliary body (right), as reformed on the host iris. 110.



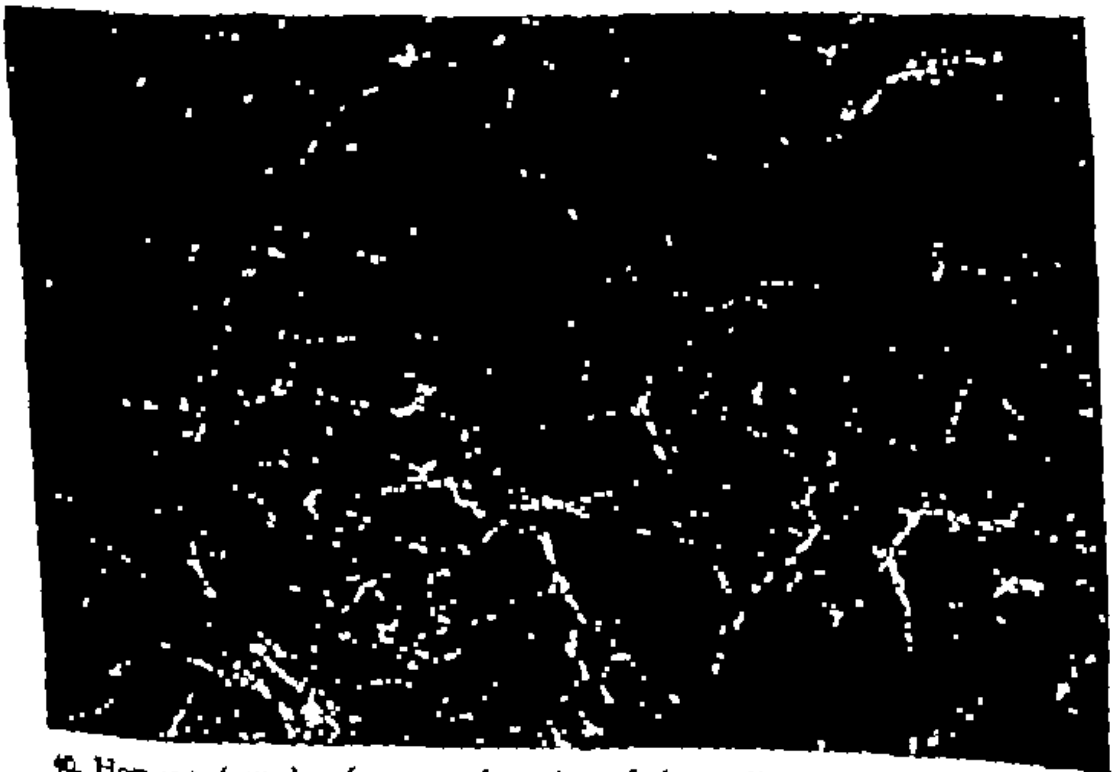
37 Iris transplant, 12 days. Detail of bundle f newly formed axons. Compare with I g 38 which shows the same ar a of the si -d ied Iris in incident light. 270.



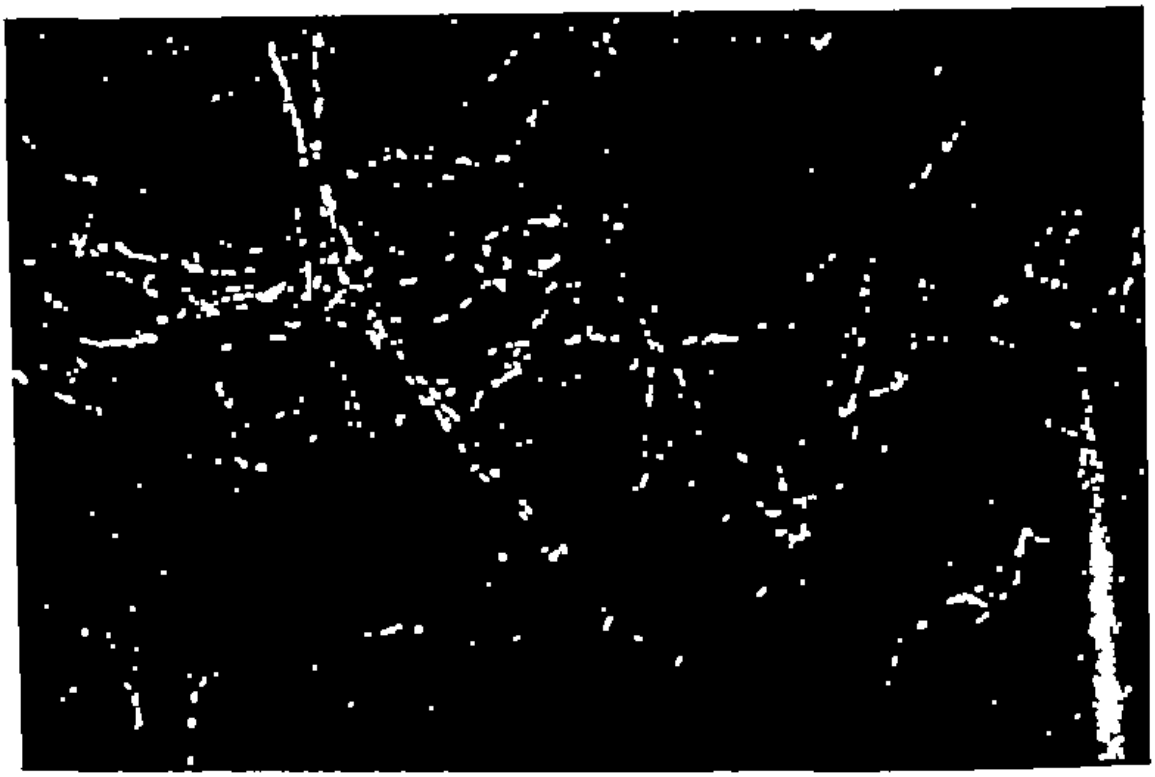
ris transplant, 12 days. Same area as g 37 but photographed in incident A large nerv tract is outlined as on the surface of the iris. The formed nerv bundles (Fig 37) the old nerv tracts. 270.



39 Host iris, 6 weeks after transplantation of a hypogastric ganglion. The transplant was situated immediately to the left of the cut edge of the iris. A plexus of sprouting adrenergic nerves extends from the site of attachment over a short distance towards the right part of the picture. The whole width of the inner zonal area is demonstrated. 140.



40. Host iris, 6 weeks after transplantation of the coeliac ganglion. A well developed, varicose adrenergic ground plexus has been reformed in the iris. Several axon bundles are also seen. 140.



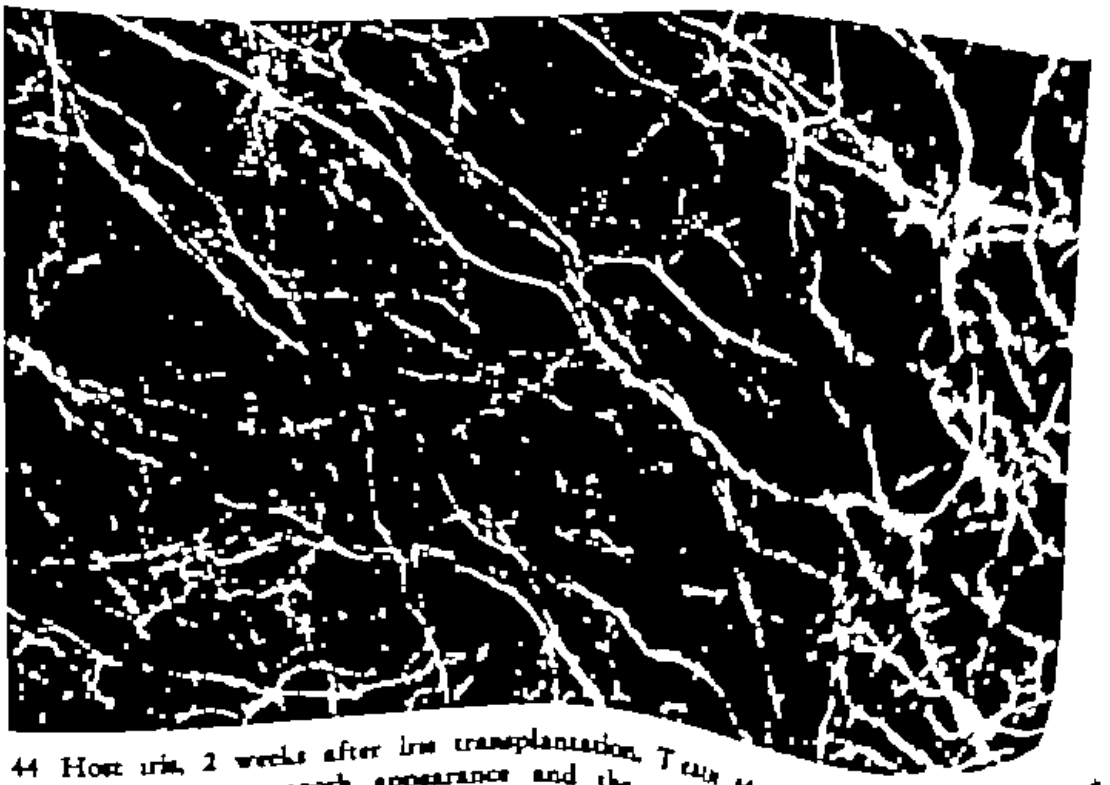
41 Iris transplant, 2 weeks. The animal was pretreated with the NA synthesis inhibitor *H44/68* (500 mg/kg i.p. 24 hrs) and received NA (0.2 mg/kg i.v.) 15 min before sacrifice The nerves in the transplant hav regained a fluorescence intensity and morphology comparable to that of a control ris treated in the same way 225.



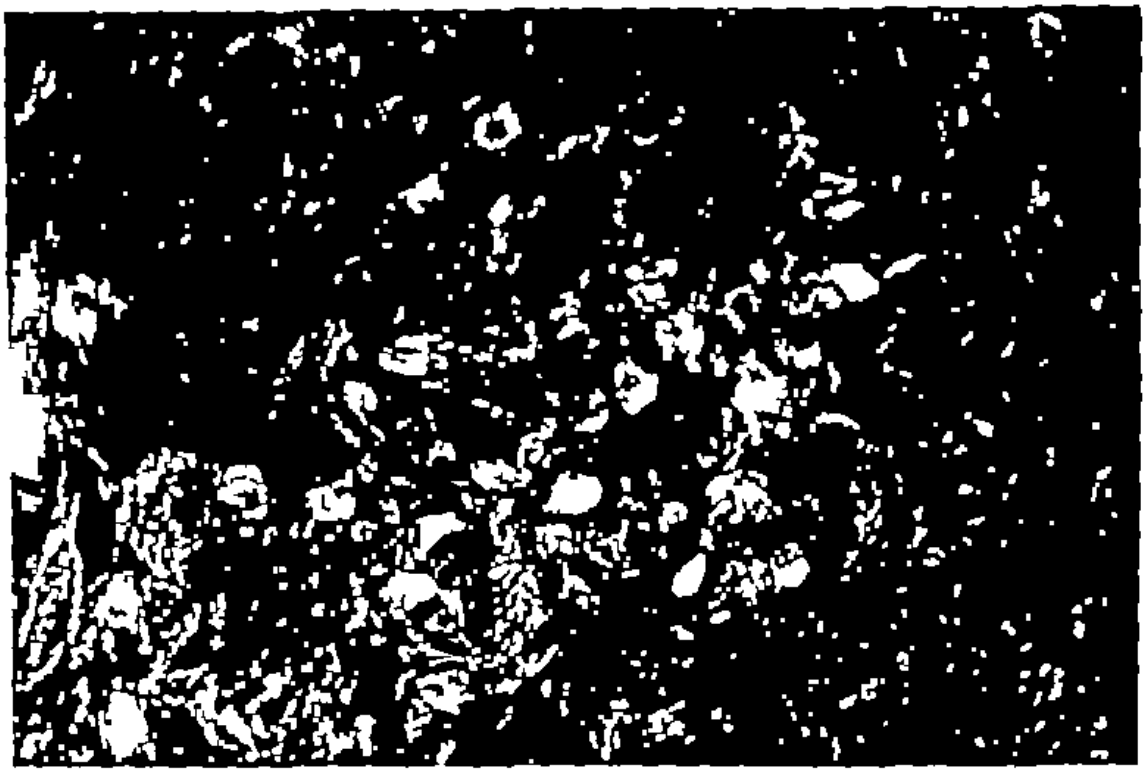
42. Host iris, 2 weeks f[illegible] Treatment as Fig 41. Also in the host iris the nerves h[illegible]d -nce intensity and morphology comparable to that of [illegible]on r[illegible] ame way 225



43. Iris transplant, 2 weeks. The animal was pretreated with reserpine (10 mg/kg i.p., 14 hrs) and nialamide (100 mg/kg i.p., 2 hrs) and received NA (0.2 mg/kg i.v.) 15 min before sacrifice. The nerve terminals are smoother than in an adult iris transplant but not as smooth as in the corresponding host iris. The nonterminal axons are beaded. 225



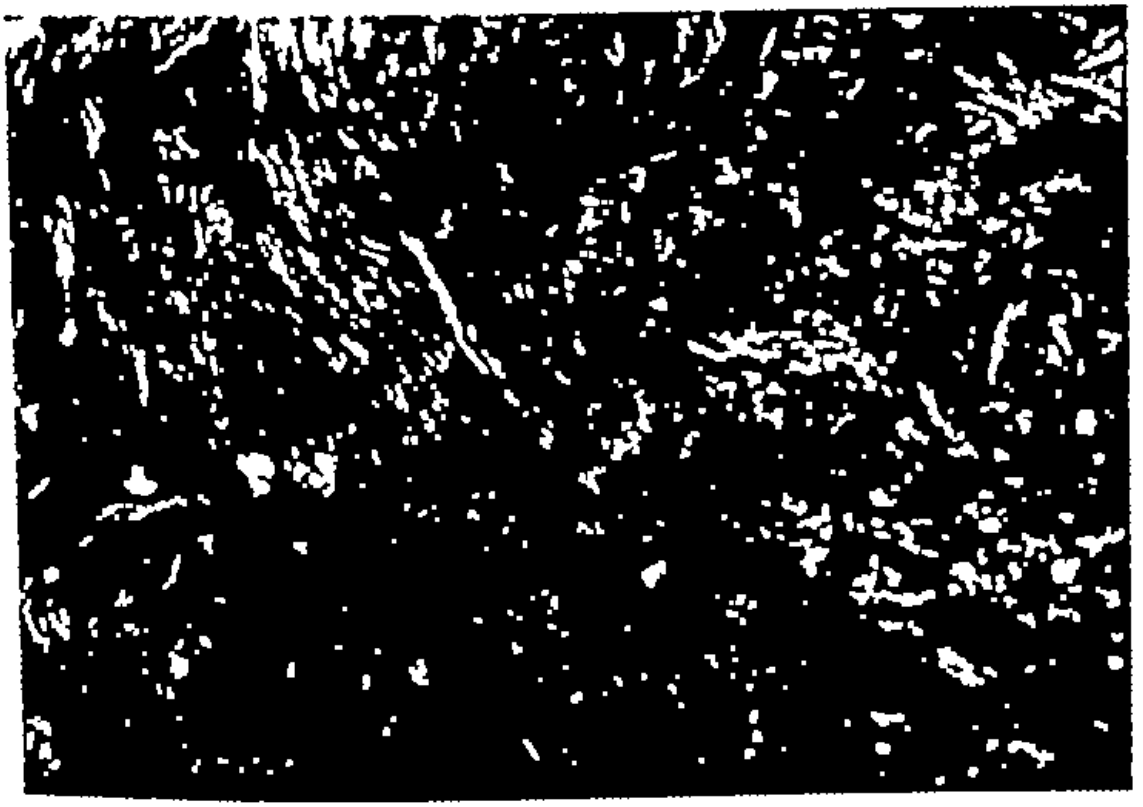
44. Host iris, 2 weeks after iris transplantation. Treatment as in Fig. 43. The terminals have a smooth appearance and the nonterminal axons are of fluorescence intensity. 225



45 Transplanted superior cervical ganglion, 1 week. Nerve cell bodies with varying fluorescence intensities and many relatively strongly fluorescent axon bundles. This figure serve as a control to Fig 46. 225



46. Transplanted superior cervical ganglion, 1 week. The animal was pretreated with reserpine (10 mg/kg, ip, 4 hrs) and nialamide (100 mg/kg, i.p., 2 hrs) and received NA (0.2 mg/kg,) 15 min before sacrifice. The axon bundles show strong fluorescence. 225

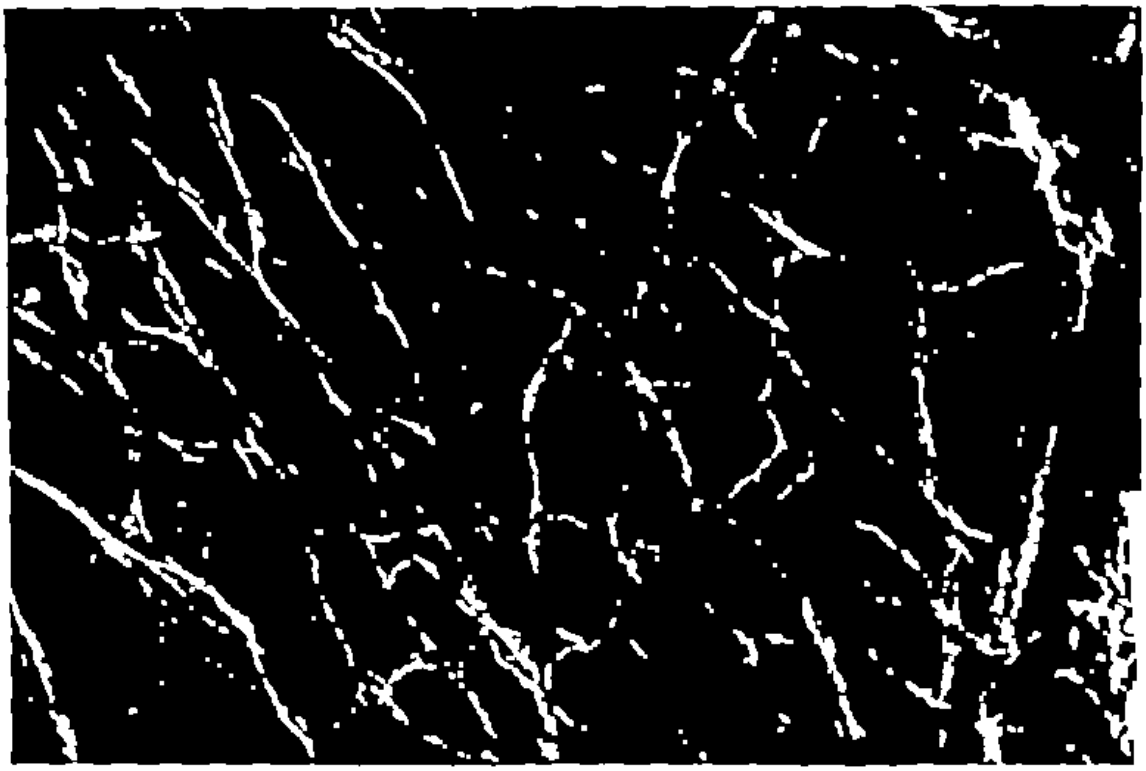


47 Transplanted superior cervical ganglion, 4 weeks. The animal was treated with the NA synthesis inhibitor H44/68 (500 mg/kg, i.p.) 24 hrs before sacrifice. The cell bodies have a low fluorescence intensity while the axon bundles show a rather strong fluorescence similar to that of an untreated transplant. 225.

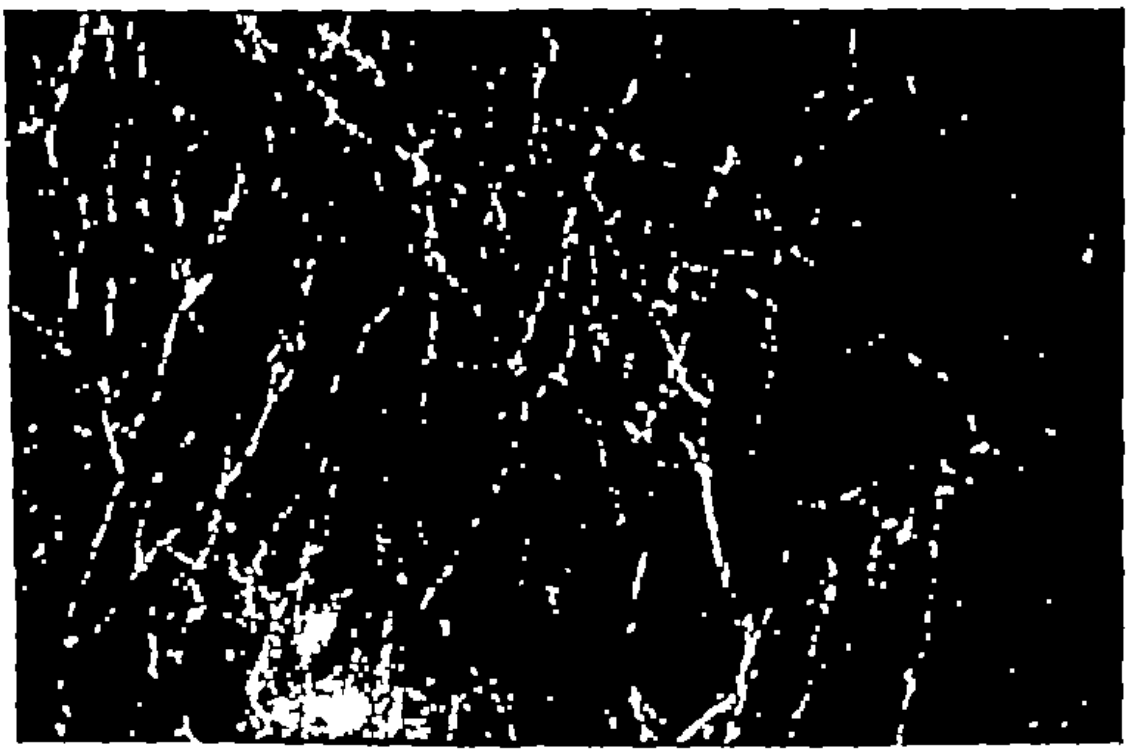


48. Transplanted supe
(10 mg/kg i p) 24 hrs
but stand out clearly

ganglion, 4 weeks. The animal was given reserpine
acrifice. The cell bodies show a weak fluorescence
the completely non-fluorescent axon bundles. 35.



49 Host iris, 1 week after ipsilateral transplantation of the superior cervical ganglion. The animal was given NA (0.2 mg/kg i.v.) 15 min before sacrifice. The newly formed nerves have a smooth appearance. 225



50 Host iris, 4 week[s] [after] ipsilateral transplantation of the superior cervical ganglion. Same trea[tment as] Fig. 49. The newly formed ground plexus has a varicose appearance in [contrast] to the smoother nerves found after 1 week. (Fig. 49). 225



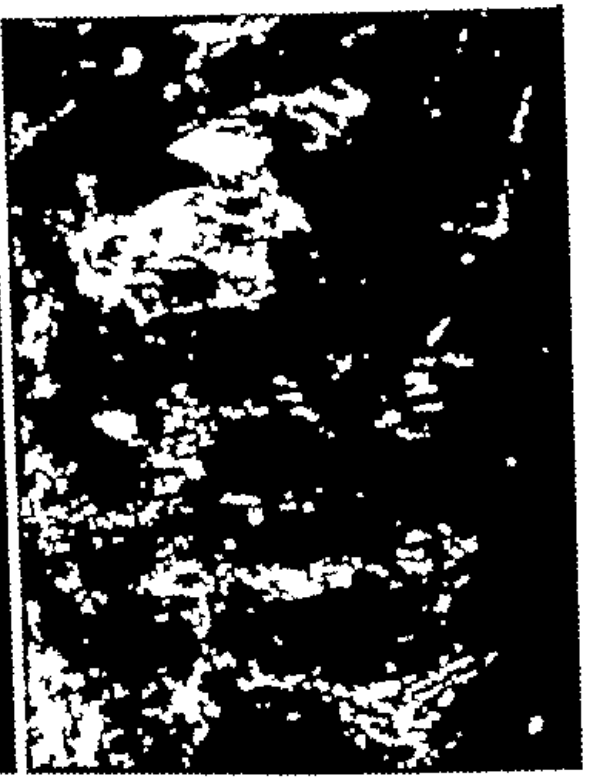
51 Connection between a host nrv and a transplanted duodenal muscle strip, 10 weeks. Detail of one slender connective tissue strand containing beaded, fluorescent non-terminal axons. 350



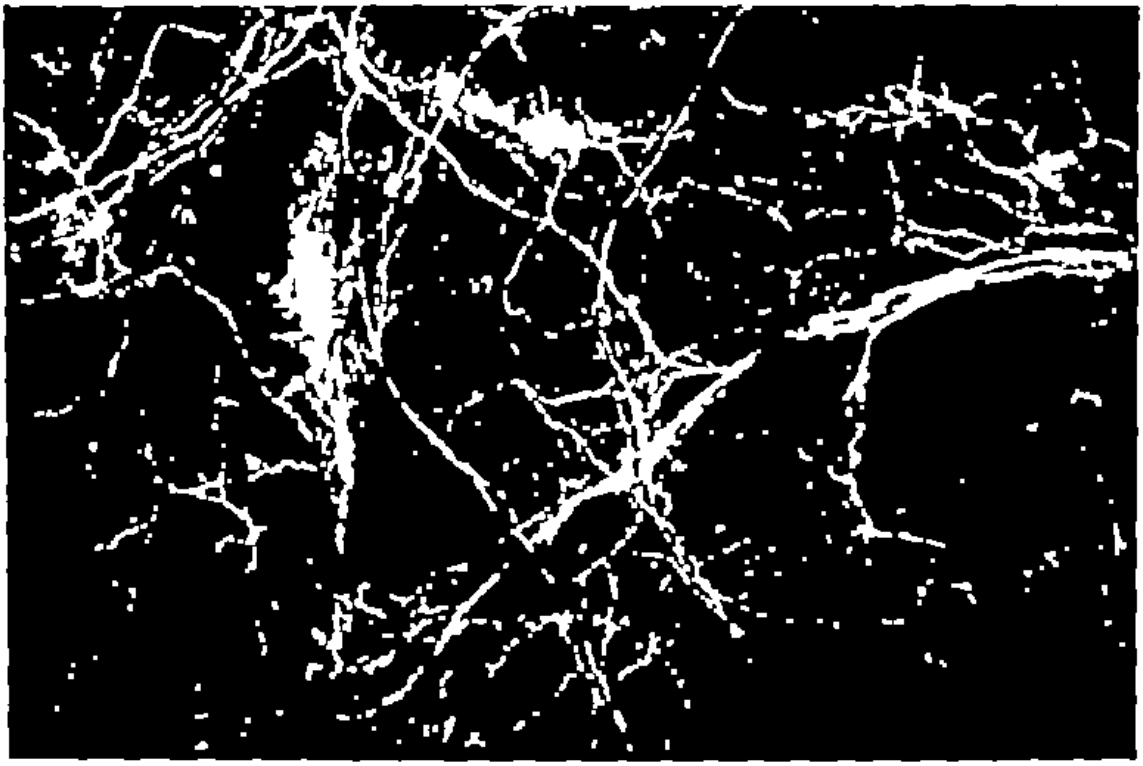
52. As Fig 51. Pretreatment with H44/68 (500 mg/kg, i.p., 140 min). The superior cervical trunk was stimulated during the last 10 min before sacrifice. The non terminal axons are essentially unaffected by the treatment. 350.



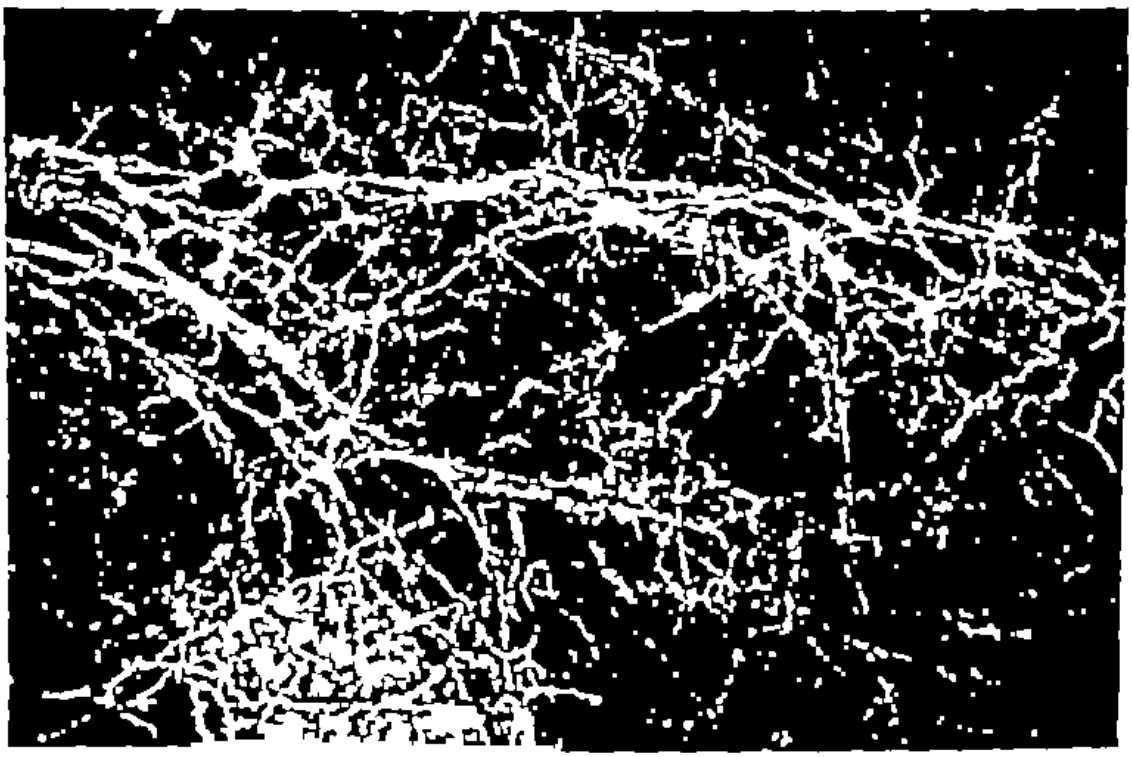
53 Transplanted vas deferens muscle strip 3 weeks. The smooth muscle bundles, here shown in cross-section, have become reinnervated by adrenergic nerves to an extent similar to that normally found in the vas deferens. 185



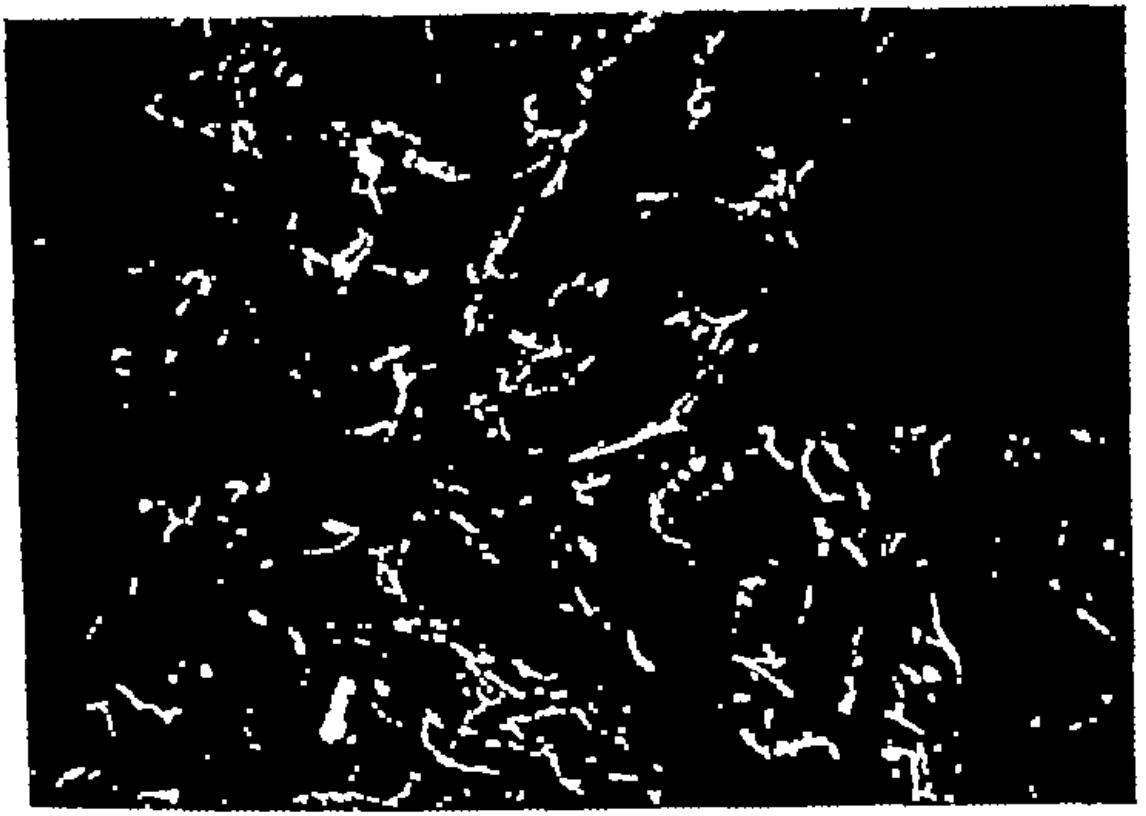
54 Transplanted duodenal muscle strip 9 weeks. Tangential section through the plexus of Auerbach between the two muscle layers. Several non fluorescent nerve cell bodies are surrounded by fine, varicose fluorescent nerves. 310.



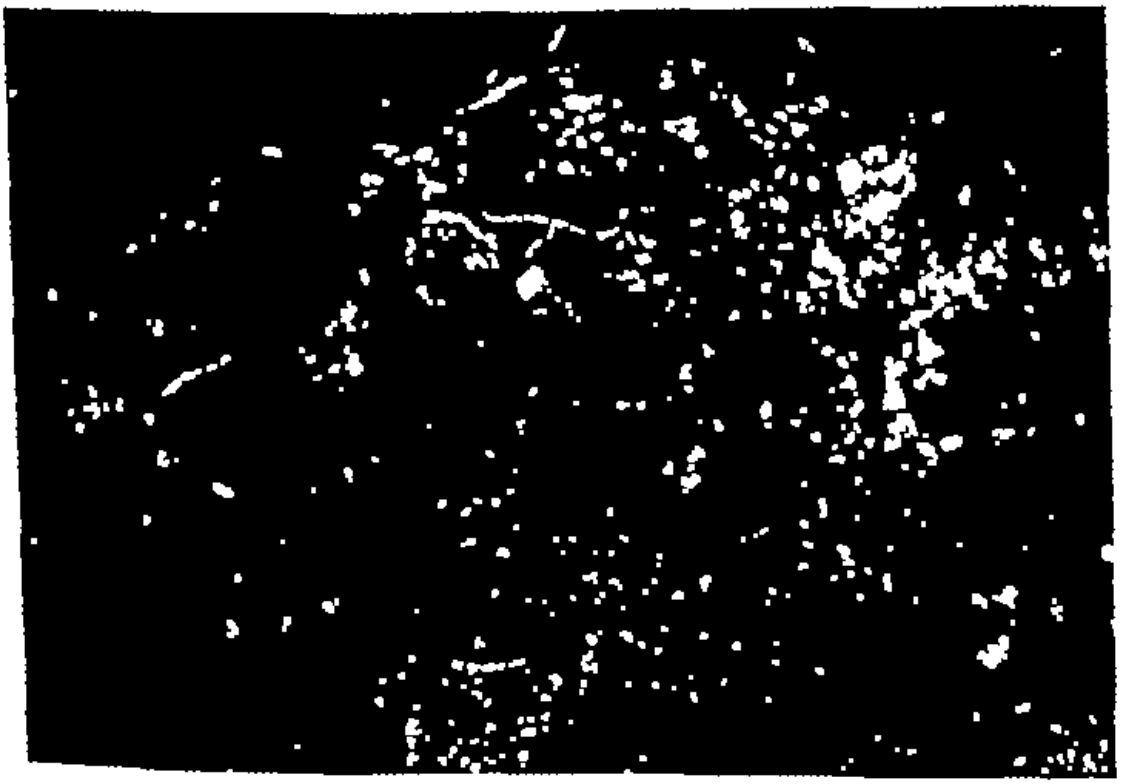
55 Host iris, 30 days after homologous transplantation of the superior cervical ganglion to an iris with intact adrenergic innervation. The original nerve supply of the iris was removed 2 days before sacrifice by extirpation of the normally situated ipsilateral ganglion, thus leaving only those nerves that had grown out from the transplant. A very scanty meshwork of newly formed nerves, mainly made up by axon bundles is seen. Varicose nerve terminals are few (cf Fig 56). 140



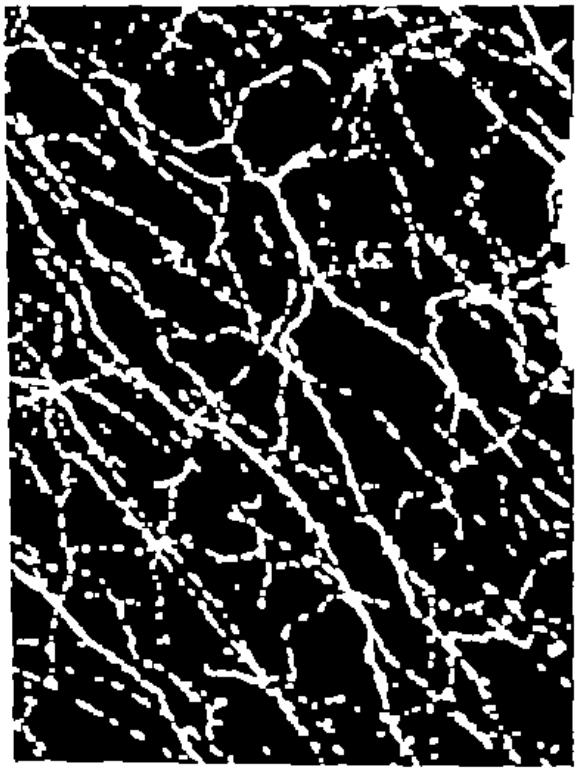
56. Host iris, 30 days after homologous transplantation of the superior cervical ganglion to an iris which was sympathetically denervated at the same time. Control to Fig 55. A well developed nerve ground plexus has been formed. 140.



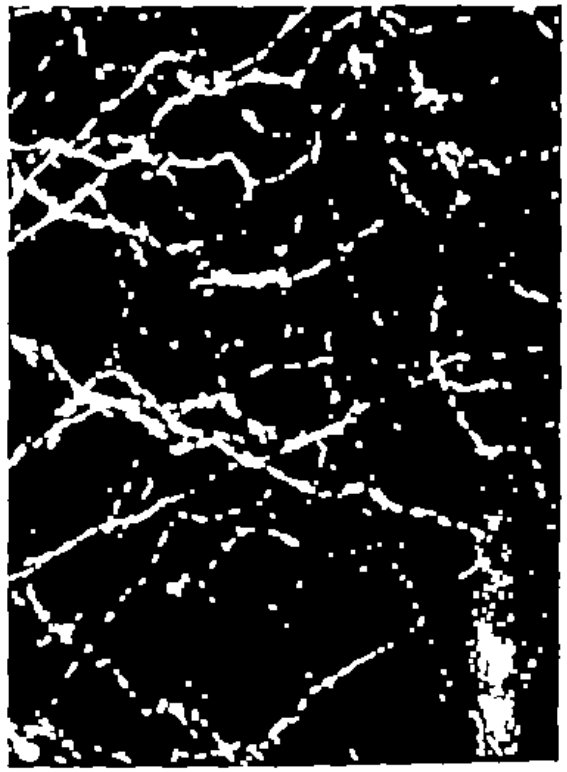
57 Transplanted piece of the submandibular gland, 4 weeks. The parenchyma of the transplant is richly supplied with new fluorescent nerves (cf Fig 58). Some tubular components are cystically dilated. 140.



58. Transplanted piece of the sublingual gland, 3 weeks. The parenchyma is almost completely devoid of fluorescent nerves (cf Fig 57). Cystic dilatation of some tubular components is seen. The many bright dots of varying sizes are brownish yellow non-specific fluorescence. 140.



59 Normal albino hamster iris. An adrenergic ground plexus similar to that of the rat iris is found. 225



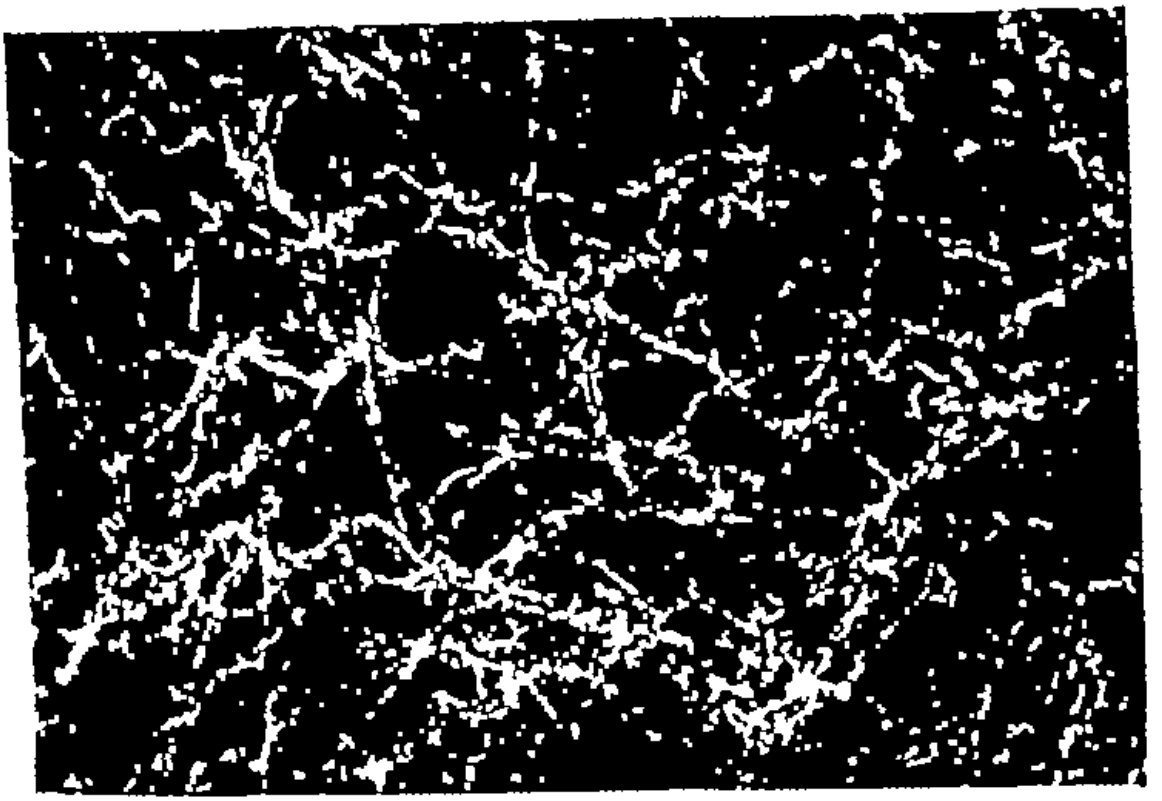
60. Autologous hamster iris transplant, 6 weeks. A new plexus of adrenergic nerves, similar to that normally found has been formed. 225



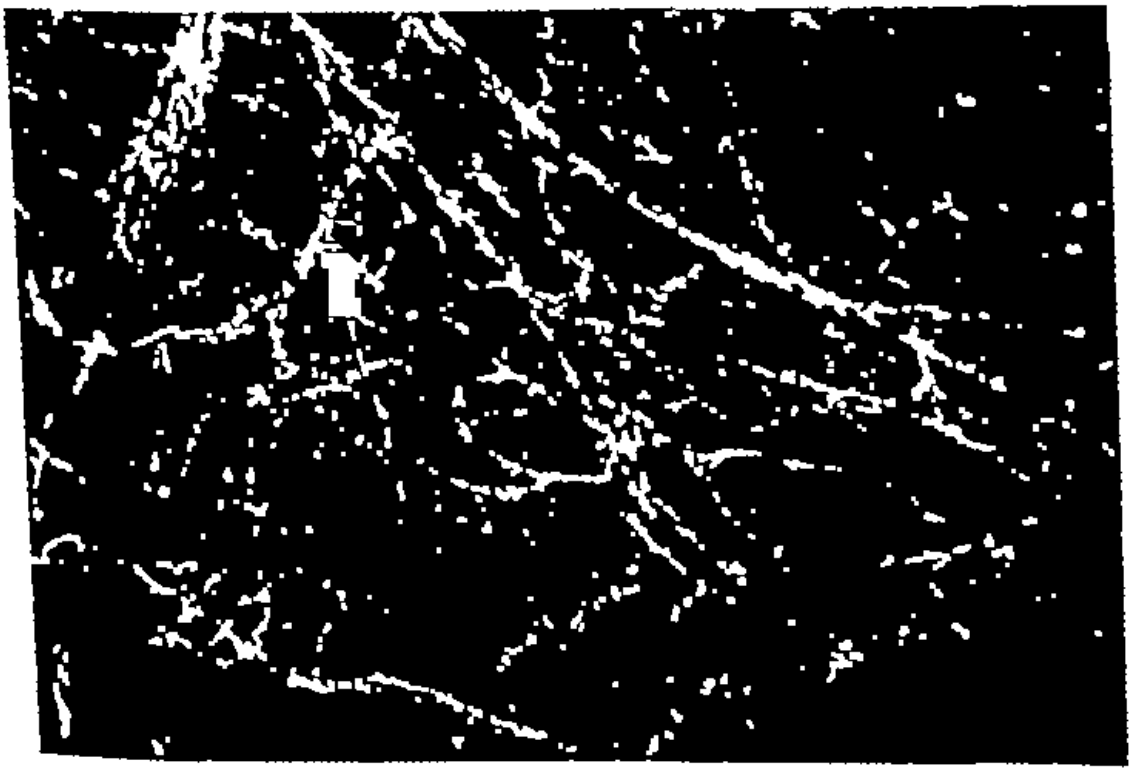
61 Heterologous transplantation of albino mouse iris to the rat eye 16 d Rat nerves have formed a sea ty w fluorescent plex on the mouse t plant. 350.



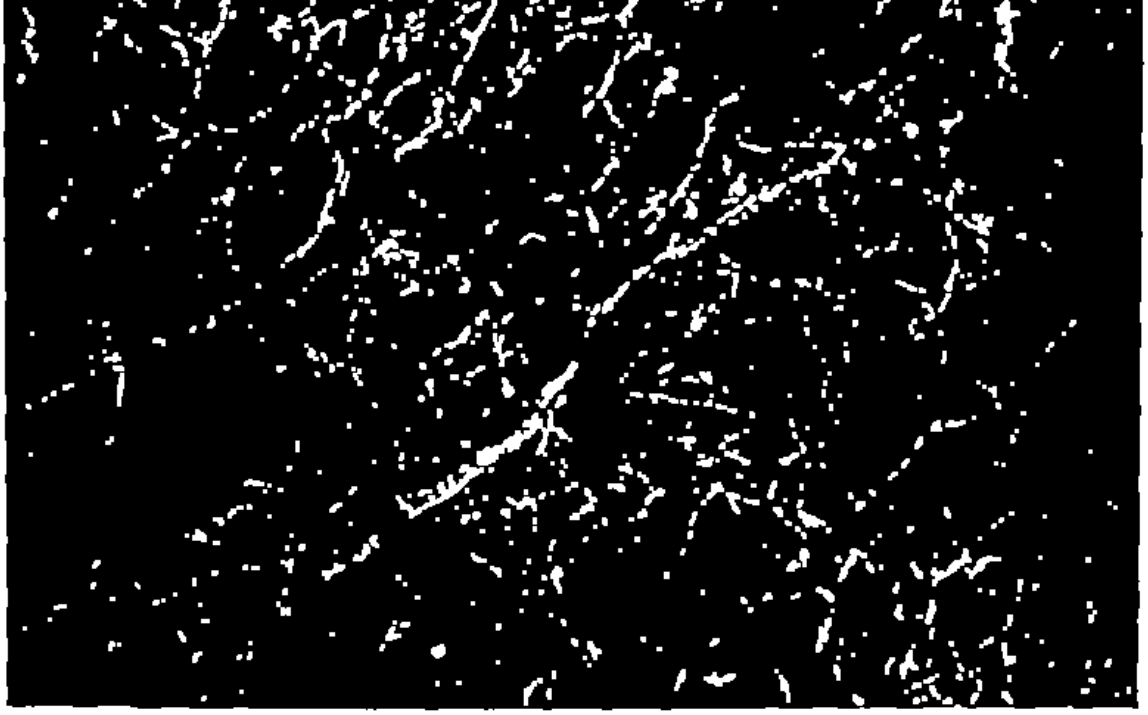
2 Heterologous transplantation of a ouse superior cervical ganglion to the mpathetically denervated rat ye. 10 A few mouse nerves are seen rning over the rat iris. 350.



63. Iris transplanted together with the anterior half of the eye to the submandibular gland, 24 days. The new adrenergic ground plexus is similar to normal and to that formed in irides transplanted to the anterior chamber 225.



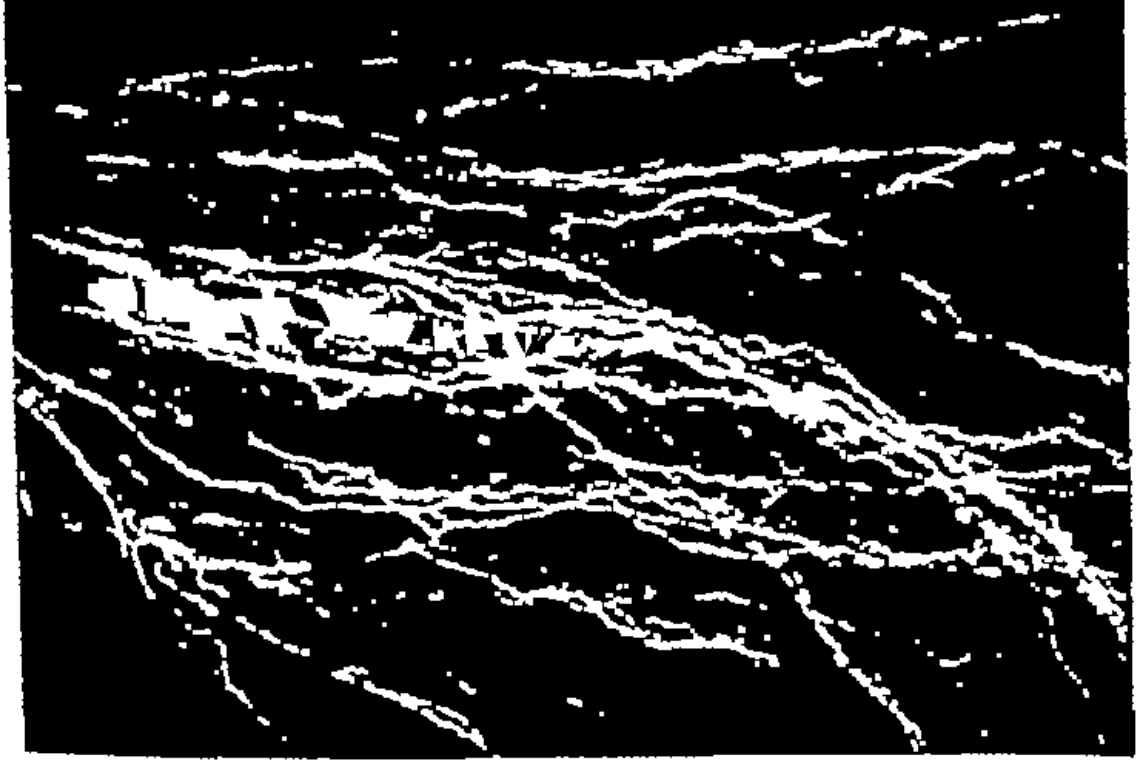
64. Iris transplanted together with the anterior half of the eye to the vas deferens, 25 days. The adrenergic ground plexus is similar to normal and to that formed in irides transplanted to the anterior chamber 225



65 Host iris, 25 weeks after homologous transplantation of the superior cervical ganglion from a newborn rat to an iris which was sympathetically denervated at the same time. The adrenergic ground plexus is similar to normal and to that formed from an autologously transplanted ipsilateral superior cervical ganglion (cf Figs 33 and 34) 140.



66. Superior cervical ganglion transplanted under the conjunctiva, 15 days. A decreased number of moderately fluorescent nerve cell bodies and an increased amount of strongly fluorescent axons in bundles as compared to normal can be seen. The appearance is the same as that of superior cervical ganglion transplanted to the anterior chamber (cf Figs 29 and 30). 2.5



67 Spread preparation of a sciatic nerve to which the superior cervical ganglion was transplanted close to the area shown in he figure The nerve was compressed proximal to the site of transplantation A great umber of fluorescent axons coming from the transplanted ganglion can be seen (f Olson 1969) 225





68 T ansplanted adr l ll d adrenal medullary cells
days. Strongly fluor 3 ll host ns, 8 weeks. Strong-
cells, more loosely rr d m longated cells with a
elongated thi in he o t be seen. Fluorescent
can be seen A few 3 d t ca be seen to e
processes 170 medullary lls. 110.



70. Iris transplant, 19 weeks. The whole width and about a quarter of the whole length of the transplant is shown. The different parts of the transplant, the sphincter (right) dilator and ciliary body (left) can be seen. The distribution of the new nerves in these parts is similar to that of normal nerves (cf. Olson et al. 1968). 50.

ACTA PHYSIOLOGICA SCANDINAVICA
SUPPLEMENTUM 330

On the relation between blood pressure and blood flow in the canine brain with particular regard to the mechanism responsible for cerebral blood flow autoregulation

BY

BARBRO EKSTRÖM-JODAL

GÖTEBORG 1970

ACTA PHYSIOLOGICA SCANDINAVICA

SUPPLEMENTUM 350

FROM THE DEPARTMENTS OF CLINICAL PHYSIOLOGY I AND II, UNIVERSITY OF GÖTEBORG, SWEDEN

On the relation between blood pressure and blood flow in the canine brain with particular regard to the mechanism responsible for cerebral blood flow autoregulation

BY

BARBRO EKSTRÖM-JODAL

GÖTEBORG 1970

This thesis is based on the following papers, which are published in this supplement

I. On the relation between blood pressure and blood flow in the cerebral cortex of dogs. By Barbro Ekström-Jodal Egil Häggendal and Nils Johan Nilsson 29–42.

II. The cerebral venous oxygen saturation during rapid changes in the arterial blood pressure An oximetric study in dogs. By Barbro Ekström-Jodal Egil Häggendal and Nils Johan Nilsson 43–50

III. Effect of increased venous pressure on cerebral blood flow in dogs. By Barbro Ekström-Jodal 51–61

These papers will be referred to in the text by the given Roman numerals.

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CONTENTS

Introduction 5

Aim of the Present Study 8

Methodological Considerations 9

- General procedure 9
- Measurement of cerebral blood flow 9
- Analysis of blood from the superior sagittal sinus 10

Part I Aspects on the Pressure-Flow Relation in the Brain (I and II) 12

- Description of the pressure-flow relation (I and II) 12
- Time relations in the autoregulatory response (II) 14
- Reactive hyperemia (I) 15
- The pressure-flow relation in arterial hypoxia (I) 15
- Reaction of the cerebral blood vessels to changes in th arterial carbon dioxide tension in hypotension (I) 18
- Discussion 18

Part II. Effect of Increased Venous Pressure on Cerebral Blood Flow (III) 20

- Discussion 1

General Summary and Conclusions 23

References 3

INTRODUCTION

Autoregulation of blood flow in the restricted sense that there is a tendency to keep the flow constant in spite of variations in perfusion pressure is a characteristic feature of the pressure flow relation in the brain, as well as in skeletal muscle, kidney intestine, liver and myocardium, as discussed at the symposium on autoregulation of blood flow in 1963 (Johnson 1964 a) The first evidence of such a regulation was presented in 1902 by Baylis, who studied the effects of changes in the arterial blood pressure on the volume of the hind limbs of dogs cats and rabbits. He found signs of an active regulation of blood flow in response to changes of the arterial blood pressure, acting in the direction to keep the flow constant This regulation also observed in the kidney and intestine, was independent of nervous influence

The mechanism by which the response is effectuated is, however still a matter of debate. Several different theories have been discussed, as reviewed by Johnson (1964 b) but the following three have been most emphasized.

In view of the contraction observed in other muscles in response to stretch Baylis postulated that the muscular coat of arterios reacts in a similar way to rises in the intravascular pressure He also suggested that there would exist a certain tone already in response to the normal blood pressure a lowering of the pressure implying a diminished stimulus, thus leading to dilatation. This hypothesis forms the basis for the *myogen c theory* which has been extensively described by Folkow (1964) It implies the existence of a basal tone, resulting from vasomotion An increased transmural pressure potentiating vasomotion would lead to a rise in vascular resistance and vice versa Th precapillary resistance vessels are considered to be the site of the changes in resistance and the important consequ nce would be the prevention of oedema formation when the arterial blood pressure is raised

The *metabolic theory* for flow autoregulation (Berne 1964) implies in principle that primarily the flow passively follows changes in the perfusion pressure leading to an altered chemical situation in the tissue Secondarily this hange in concentra tion of the metabolites induces changes of the calibre of the resistance vessels, which act to keep the flow constant Much interest has been focused on oxygen and on different products of metabolism uch as carbon di xide and lactic acid as being the possible trigger of the reaction, but so far no single substance has been hown to be responsible. Autoregulation according to this theory is thus a flow-dependent

phenomenon and the teleological view that it is designed to guarantee the chemical homeostasis of the tissue seems natural.

The *tissue pressure theory* does not include any active smooth muscle response in the vessel walls but ascribes autoregulation to purely mechanical events. Thus, when the arterial blood pressure is raised there is an increase in the outward filtration of fluid owing to the changed pre- to postcapillary pressure ratio Provided the organ is enclosed in a capsule that is stiff enough an increase in the tissue pressure will be produced The low pressure vessels with little rigidity in the wall, *i.e.* the veins should be the section of the vasculature most sensitive to such a pressure increase tending to collapse and thereby raise the postcapillary resistance The reverse course of events would take place, when the arterial blood pressure is lowered, resulting in a pressure-flow relation characteristic for flow autoregulation

Concerning the regulation of the cerebral blood flow there have been wide differences in opinions from time to time Already in 1783 Alexander Monro the younger as quoted by Bayliss and Hill (1895) presented the view that the quantity of blood within the skull must be almost invariable considering the brain substance enclosed in the rigid skull as practically incompressible This view did not seem to allow for the *dilatation of the pial vessels later observed by Donders (1851) in combination with* asphyxia, but it was vividly supported by Bayliss and Hill (1895) who especially criticized the report by Roy and Sherrington (1890) The latter authors recognized two factors of importance in the regulation of the cerebral blood flow Firstly changes in the arterial blood pressure were found to effect an alteration of the blood flow in the same direction, and secondly some intrinsic control probably independent of nervous influence was suggested This suggestion was partly based on observations made during asphyxia, of an expansion of the brain, which could not be ascribed to blood pressure changes Bayliss and Hill repeating these experiments, could not find evidence for any active regulation of cerebral blood flow and concluded that under physiological conditions the blood flow passively follows the arterial and venous blood pressures. This view further extended by Hill (1896) became dominant during the following decades and was upheld despite the later discovery by Bayliss (1902) of an active vascular response to blood pressure changes in other vascular areas

The first systematic study of the effect on the cerebral vessels of blood pressure changes *per se* in which clear signs of an autoregulatory response were found was presented by Fog (1934) using the pial window technique of Forbes (1928) Irrespective of how the arterial blood pressure changes were achieved (arterial bleeding or stimulation of the vagus or depressor nerves aortic compression or stimulation of the sympathetic nerves) the response to a pressure increase was vasoconstriction and to a pressure decrease vasodilatation In this work and later (1937 and 1938) Fog suggested that variations in the endovascular pressure induce the changes observed in the diameter of the pial vessels

The existence of flow autoregulation in response to changes of the arterial blood

pressure in the cerebral circulation has later been confirmed in a number of studies using different techniques (Forbes, Nason and Wortman 1937 Carlyle and Grayson 1955 Rapela and Green 1964 Hirsch and Körner 1964 Harper 1965 Häggendal and Johansson 1965 Bozzao *et al* 1968 for reviews see also Lassen 1959 and 1964) Moreover autoregulation has been shown to occur in response to increased intracranial pressure (Noell and Schneider 1948, Rapela and Green 1964 Shulman and Verdier 1967 Häggendal *et al.* 1970 a) Other investigators have reported a passive pressure-flow relation in isolated brains (Geiger and Magnes 1947 Sagava and Guyton 1961) The discrepancy of results may be attributable to the method used as cerebral autoregulation appears to be a vulnerable phenomenon, easily damaged by for instance traumatic preparation procedures (Fog 1934, Rapela and Green 1964)

The tissue pressure hypothesis at first seems to be a quite possible explanation for blood flow autoregulation in the brain with its very rigid enclosure. It is, however probably excluded by the fact, that autoregulation takes place to the same extent, where the perfusion pressure is reduced by intracranial pressure increases, as when the arterial blood pressure is lowered

In discussing the other two mechanisms mentioned above a commonly held opinion has been that both are actually engaged (*e.g* Lassen 1959 and 1964 see also Harper and Häggendal 1968) but some facts concerning the general regulation of cerebral blood flow seem to have favoured the concept of a mechanism governed by metabolic factors. Thus, it is well known from numerous reports, that the cerebral blood vessels react readily to changes in the arterial blood-gas tensions, so that hypoxia and hypercapnia induce vasodilatation, and hypocapnia vasoconstriction, for a review see Lassen (1959) Changes in the tensions of oxygen or carbon dioxide in the tissue have been considered likely to provide the sensitive mechanism required for the flow autoregulation to the extent observed in the brain (Rapela and Green 1964, see also Lassen 1964 and Harper 1965)

The myogenic theory discussed by Forbes, Nason and Wortman (1937) and Fog (1938) as a quite possible explanation of the reactions found, has received some support in more recent years. Häggendal and Johansson (1965) reported findings that appeared inconsistent with the metabolic theory but explainable in terms of the myogenic theory A preliminary study of the influence of changes in the transmural pressure, *as opposed to changes in the perfusion pressure*, on the cerebrovascular resistance was presented at the International CBF Symposium 1969 by Ekström-Jodal *et al* suggestive of a non-metabolic, probably myogenic mechanism. At the same symposium there was also presented a study by Held Gottstein and Niedermayer (1969) showing an autoregulatory response on a lower flow level when there was a pulsatile as compared to a non-pulsatile flow with the same mean pressure. This was interpreted as indicating a myogenic mechanism.

For a recent review of autoregulation of cerebral blood flow the reader is referred to Häggendal Nilsson and Norbäck (1970)

phenomenon and the teleological view that it is designed to guarantee the chemical homeostasis of the tissue seems natural.

The *tissue pressure theory* does not include any active smooth muscle response in the vessel walls but ascribes autoregulation to purely mechanical events. Thus, when the arterial blood pressure is raised there is an increase in the outward filtration of fluid owing to the changed pre- to postcapillary pressure ratio Provided the organ is enclosed in a capsule that is stiff enough an increase in the tissue pressure will be produced. The low pressure vessels with little rigidity in the wall i.e. the veins, should be the section of the vasculature most sensitive to such a pressure increase tending to collapse and thereby raise the postcapillary resistance The reverse course of events would take place when the arterial blood pressure is lowered resulting in a pressure-flow relation characteristic for flow autoregulation.

Concerning the regulation of the cerebral blood flow there have been wide differences in opinions from time to time. Already in 1783 Alexander Monro the younger as quoted by Bayliss and Hill (1895) presented the view that the quantity of blood within the skull must be almost invariable considering the brain substance, enclosed in the rigid skull as practically incompressible. This view did not seem to allow for the dilatation of the pial vessels later observed by Donders (1851) in combination with asphyxia but it was vividly supported by Bayliss and Hill (1895) who especially criticized the report by Roy and Sherrington (1890) The latter authors recognized two factors of importance in the regulation of the cerebral blood flow Firstly changes in the arterial blood pressure were found to effect an alteration of the blood flow in the same direction and, secondly some intrinsic control, probably independent of nervous influence was suggested This suggestion was partly based on observations, made during asphyxia, of an expansion of the brain, which could not be ascribed to blood pressure changes. Bayliss and Hill, repeating these experiments could not find evidence for any active regulation of cerebral blood flow and concluded that under physiological conditions the blood flow passively follows the arterial and venous blood pressures. This view further extended by Hill (1896) became dominant during the following decades and was upheld despite the later discovery by Bayliss (1902) of an active vascular response to blood pressure changes in other vascular areas

The first systematic study of the effect on the cerebral vessels of blood pressure changes *per se* in which clear signs of an autoregulatory response were found, was presented by Fog (1934) using the pial window technique of Forbes (1928) Irrespective of how the arterial blood pressure changes were achieved (arterial bleeding or stimulation of the vagus or depressor nerves; aortic compression or stimulation of the sympathetic nerves) the response to a pressure increase was vasoconstriction and to a pressure decrease vasodilatation. In this work and later (1937 and 1938) Fog suggested that variations in the endovascular pressure induce the changes observed in the diameter of the pial vessels

The existence of flow autoregulation in response to changes of the arterial blood

METHODOLOGICAL CONSIDERATIONS

General procedure

Unselected mongrel dogs, anaesthetized with pentobarbital were used The animals were artificially ventilated, and changes in the blood gas situation were achieved by administration of different gas mixtures. The arterial blood pressure was changed by bleeding or reinfusion of blood. Measurements of cerebral blood flow were not performed until 15 minutes after changes of ventilation and until 5 minutes after changes of blood pressure in order to assure a steady state

Measurement of cerebral blood flow

For the study of such a vulnerable process as the autoregulation of blood flow in the brain a leading principle in the choice of methods must be that as little trauma as possible is inflicted. In this respect the radioactive gas elimination technique of Lassen *et al* (1963) has definite advantages. No direct interference with the brain tissue is involved, because the elimination of intraarterially injected radioactive krypton (Kr^{85}) is recorded through the intact skull with a scintillation detector Another advantage with this method, in experiments where numerous flow measurements are desired, is that it includes no blood loss. In this study the injections were made via a catheter into the intact left vertebral artery close to the base of the skull, leading to a high concentration of the indicator in the brain with little extracerebral contamination The catheter left in an unwedged position hardly interferes with the blood supply of the brain Norbäck (1966) using the same technique reported that the difference between the blood pressure measured in the aorta and at the tip of the catheter in the vertebral artery was only 10 mm Hg

The elimination curve obtained was resolved into two main components, considered representative of the two major types of tissue in the brain, *i.e* grey and white matter (Lassen *et al* 1963) Experimental evidence for the correctness of this assumption was presented by Häggendal Nilsson and Norbäck (1965) and the reader is referred to this study for details of the curve analysis. A third slow component was often recognized probably due to such factors as administration of small amounts of indicator to extracerebral tissue and to recirculation. By the use of a careful collimation the background activity registered in the curves could be kept low and did not significantly affect the solution of the curves. Flow correspond

ing to the different components can be calculated with the equation for an exponential clearance function if the elimination rate of the indicator from the tissue and the partition coefficient of the indicator between tissue and blood are known. The value of the partition coefficient for Krypton is influenced by changes in the hematocrit of the blood (Lassen *et al* 1963 Glass and Harper 1962 Ingvar and Lassen 1962) but as only minor differences between the measurements were encountered no corrections were considered necessary and the value of 0 95 for cerebral grey matter was used throughout the study The flow in the grey matter *i e* the flow calculated from the fast component of the clearance curve is given in the results. One reason for this is that cerebral venous blood required for the calculation of the cerebral metabolic rate of oxygen can most conveniently be sampled in dogs from the superior sagittal sinus which has been found to drain predominantly cerebral grey matter (see below) Furthermore the component representative of grey matter is less influenced by possible errors in drawing the third slow component in the γ curve (Häggendal Nilsson and Norbäck 1965) Using the same method of flow determination and curve analysis as was used in this study Häggendal (1965) studied the error of the method in subsequent measurements, which was found to be around 10 % of the mean value.

Analysis of blood from the superior sagittal sinus

Cerebral venous blood was withdrawn via a needle bored through the skull bone down into the sinus. In combination with practically every measurement of cerebral blood flow simultaneous samples were taken from the sinus and a femoral artery for the determination of oxygen saturation and capacity pH and carbon dioxide tension the metabolic rate of oxygen in the grey matter could then be calculated. In the oximetric studies (II) the oxygen saturation of blood from the superior sagittal sinus was continuously followed during rapid changes in the arterial blood pressure changes in the oxygen saturation being taken as a relative measure of changes in the blood flow In these procedures, of course it is of critical importance that the sagittal sinus blood is representative of venous blood from the grey matter A substantial contribution of extracerebral blood could be expected to change the results, and in studies of cortical metabolism Gleichmann *et al.* (1962) used the blood from the superior sagittal sinus as representative of cortical venous blood only after elimination of the diploic veins. However earlier results from this laboratory by Häggendal Nilsson and Norbäck (1965) with registration of the β curve from sagittal sinus blood did not indicate any important extracerebral contribution and Häggendal and Norbäck (1966) did not find any changes in the venous oxygen saturation when blood was withdrawn with varying rates, which is in accordance with the findings in the present study (II) However care was taken to draw the samples relatively slowly (about 5 ml/min) in order to avoid a possible error from this source

The contribution of blood from white matter to the superior sagittal sinus is small enough to be almost negligible (Häggendal, Nilsson and Norbäck 1965) Moreover a contribution of a certain amount of blood from white matter should probably not invalidate the results as the relation between both flow and oxygen consumption in grey and white matter is about 5 to 1 (Homburger *et al* 1946 Häggendal Nilsson and Norbäck 1965 Gleichmann *et al.* 196–) giving the same arterio-venous oxygen difference for the two tissues. This relation between cerebral blood flow and oxygen consumption in grey and white matter seems to remain constant during the different conditions studied. Thus flow in white matter is autoregulated in the same pressure range as flow in grey matter (Bonnal *et al* 1968 James, Millar and Purves 1969) The latter authors found a similar percentual increase of flow in white as in grey matter when the arterial oxygen tension was lowered from normal to around 40 mm Hg and also when the arterial carbon dioxide tension was changed within the range from around 25 to 65 mm Hg

For the oxymetric studies it is of major interest that the cerebral oxygen consumption is constant during each registration. Available information indicates that this is the case Thus no changes in oxygen uptake were found by Hirsch *et al.* (1955) in combination with blood flow reductions down to around 50 % of the normal value. Häggendal and Norbäck (1966) demonstrated a maintained oxygen uptake in hypotension, even when this was so pronounced that the cerebral blood flow was reduced Moreover the cerebral metabolic rat of oxygen is unaffected by wide variations in the arterial carbon dioxide tension, as indicated by the studies of Kety and Schmidt (1948) and of James, Millar and Purves (1969)

be an all-or none phenomenon but rather a more less pronounced attenuation of the mechanism, as indicated by studies on the effect of artificial perfusion on the response (Rapela and Green 1964) Furthermore in the present study the perfect readjustment to the original venous oxygen saturation found in the oximetric study (II) when the arterial blood pressure was changed within the autoregulatory range supports the view that the autoregulatory mechanism in the brain is capable of maintaining constancy of flow

The pressure-flow relation in hypotension has not been extensively studied so far However Harper (1965) demonstrated a linear relationship in hypotension. Also in hypercapnia (Harper 1965) and hypoxia (Häggendal and Johansson 1965) linear relationships have been presented On the other hand Fog (1934) and Forbes Nason and Wortman (1937) reported contractions of the directly observed pial arteries in response to marked blood pressure reductions Furthermore, Rapela and Green (1964) found a pressure-flow relation in hypercapnia indicating a passive vascular bed *i e* curvilinear with the convexity towards the pressure axis The above-mentioned linearity implicates an ability of the vessel wall to react to some extent to pressure changes and/or metabolic factors.

In the present work the study of the pressure-flow relation in hypotension below the autoregulatory range (I) was facilitated by induction of a primary flow increase by hypercapnia or papaverine infusion before reducing the arterial blood pressure in small steps. A curvilinear relation was found with the convexity towards the pressure axis. No difference in the relation was observed whether hypercapnia or papaverine was used This appearance of the relation does not, however exclude an ability of an active response of some kind being retained in hypotension although not strong enough completely to counteract the effect of the pressure changes on the elastic vessel walls. In hypoxia profound enough to impair autoregulation metabolic factors, resulting from the hypoxia, might give rise to a vasodilatation when the pressure is reduced (see below) On the other hand the normal autoregulation in this case might be only impaired not abolished which would also modify the response (*cf p* 19)

Time relations in the autoregulatory response (II)

When the arterial blood pressure was changed within the autoregulatory pressure range by rapid withdrawal or infusion of blood, the cerebral venous oxygen saturation, continuously analysed oximetrically was found first to change in the same direction as the pressure, but then to return gradually towards the basal level (Fig 1 II) The time for this response to be complete *i e* from the pressure increase or decrease to the point where the control level of oxygen saturation was reestablished was about 45 and 10 seconds respectively These figures are in accordance with or somewhat shorter than those reported in other studies (for references see paper II)

Reactive hyperemia (I)

This term denotes an increased cerebral blood flow in normotension, combined with a passive pressure-flow relation, elicited by a period of reduced perfusion pressure. The phenomenon has been observed both when the reduction of the perfusion pressure has been accomplished by a rise in the intracranial pressure (Noell and Schneider 1948, Häggendal *et al.* 1969 and 1970 b) and after periods of pronounced arterial hypotension (Freeman and Ingvar 1968). Usually the period of hypotension preceding the reactive hyperemia has been combined with a substantial flow reduction. In some of the reported cases, however, no flow reduction had been observed (Häggendal *et al.* 1970 b), which is interesting with regard to the mechanism behind the phenomenon. In an attempt to a further evaluation of the role of hypoxia for the appearance of reactive hyperemia, the flow reaction to arterial blood pressure increases after a period of hypotension with reduction of flow was studied in animals, where a primary vasodilatation had been induced by hypercapnia or papaverine infusion. In spite of the fact that the blood flow had not been reduced to values below normal and that the venous oxygen tension had not been lower than about 40 mm Hg a marked flow increase was encountered (Fig. 7 I), which gradually subsided. These results confirm those obtained by Häggendal *et al.* (1970 b) and indicate that tissue hypoxia is not a prerequisite for reactive hyperemia. Nor does carbon dioxide accumulated in the tissue during the hypotension seem likely to uphold such a long lasting vasodilatation. It might be of interest to compare this derangement of the normal pressure-flow relation to the luxury perfusion syndrome. This term was recently introduced by Lassen (1966) and denotes a state characterized by "an over-abundant cerebral blood flow relative to the metabolic needs of th brain tissue". With normal arterial oxygen saturation "bright red venous blood" would be the cardinal feature of the syndrome. In cases where the high oxygen saturation in the venous blood is the result of the flow increase ordinarily seen when the normal autoregulation is impaired, the two terms, reactive hyperemia and luxury perfusion, are synonymous as far as the blood pressure and the arterial oxygen content are high enough. However it is clear that the combination of high flow values and "red veins" is compatible with a normal pressure-flow relation *e.g.* in hypercapnia. On the other hand a deranged pressure-flow relation may be associated with dark venous blood in hypotension and/or pronounced hypoxia.

The pressure-flow relation in arterial hypoxia (I)

In combination with an extreme arterial hypoxia Häggendal and Johansson (1963) found signs of an abolished autoregulation. In the present study however it was possible to demonstrate a well functioning regulation in some cases even when the arterial oxygen saturation was reduced down to 40 per cent and a certain degree of flow increase had occurred. From a consideration of the general relation between

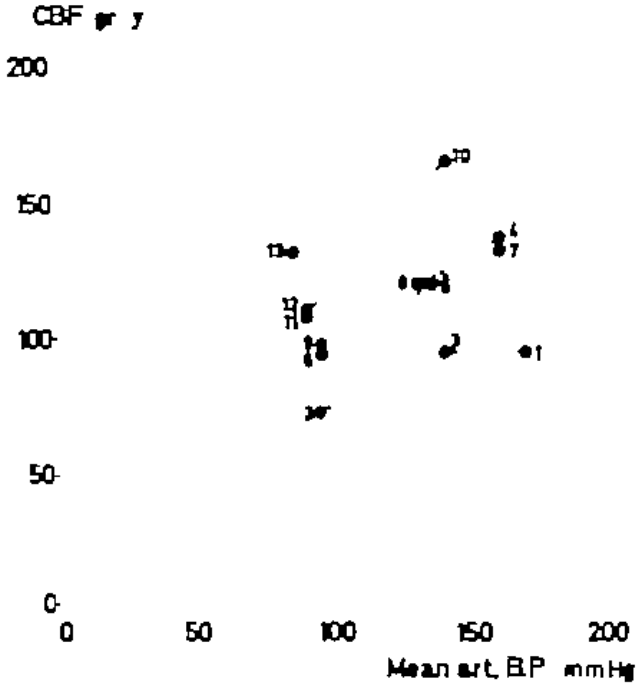


Fig 2. Relation between mean rterial blood pressure and cerebral blood flow in grey matt (CBF_{grey} ml/100 gmin) in one animal at different values of arterial oxygen saturation. Tho numbers mark the hronological order of the measurements and refer to the numbers in Table II Measurements performed at about the same arterial xygen saturation are connected by broken lines. An imperfect autoregulation is illustrated in hypoxia. In hypotension below the autoregulatory range there is a vascular dilatation in response to decreasing arterial oxygen saturation down to a certain limit (no 11) With maintained low arterial oxygen saturation and blood pressure a further vasodilatation beyond this limit is induced by intra venous administration of glucose (no. 13).

arterial oxygen saturation and cerebral blood flow (see Fig 2, I) it seems improbable that a complete abolishment of autoregulation could take place at a higher oxygen saturation than where the steep increase in blood flow begins, because such an abolishment would be expected to cause a comparatively large flow increase The vasodilatation encountered in pronounced hypoxia is probably related to tissue hypoxia (Noell and Schneider 1944 Häggendal Nilsson and Norbäck 1966) and in this range of arterial oxygen saturation optimal conditions for metabolic blood flow autoregulation should be provided. In Fig 2 and Table II the results are presented from an experiment where some points may be of interest in this connection Autoregulation in this animal was impaired but not completely abolished at an arterial oxygen saturation of about 55 per cent (nos 4 5 6) At an arterial pressure, where the blood flow in the normoxic situation was reduced due to low blood pressure the flow was higher in hypoxia. When the measurements were reposted after about 43 minutes (nos 7 8) no signs of reactive hyperemia were found With increasing arterial hypoxia a further vasodilatation was encountered in normotension (no 10) In hypotension there was a flow increase when the arterial oxygen saturation was lowered from 57 t 46 per cent (nos 6 11) and there seemed to be a tendency to

Table II. Data obtained from the experiment presented in Fig. —. The numbers refer to those in the figure.

Abbreviations

P_{aO_2} = Arterial oxygen tension.
P_{vO} = Venous oxygen tension in blood from the superior sagittal sinus.
CVR = Cerebrovascular resistance calculated from mean arterial blood pressure and CBF_{N2O}.
For the remaining abbreviations see legend to Table I.

No.	P_{aCO_2} mm Hg	S_{aO_2} %	P_{aO_2} mm Hg	P_{vO_2} mm Hg	Mean art. B.P mm Hg	CBF_{N2O} ml/100 gmin	CVR mm Hg ml/100 gmin	CMR_{O_2} ml/100 gmin
1	30	91	73	45	170	94	1.81	5.8
2	25	93	84	36	95	73	1.30	6.6
3	39	92	87	49	140	95	1.47	5.8
4	39	84	57	27	160	137	1.17	6.3
5	41	87	58	27	135	120	1.13	6.7
6	41	57	38	24	95	94	1.01	6.3
7	44	51	36	26	160	132	1.21	6.8
8	43	51	36	24	130	120	1.08	7.0
9	38	58	40	24	93	97	0.96	6.9
10	46	36	31	22	140	165	0.85	6.1
11	41	46	37	22	90	108	0.83	6.6
15	41	38	33	18	90	110	0.82	5.0
13	42	36	35	20	85	132	0.64	5.4

keep the venous oxygen tension constant (22–24 mm Hg nos. 6, 8–11) over a wide blood pressure range when the arterial oxygen saturation was varied between 57 and 36 per cent. Decreasing the arterial oxygen saturation in hypotension from 46 to 38 per cent did not induce any further vasodilatation and the venous oxygen tension and the oxygen uptake decreased, indicating an inability to respond to arterial hypoxia while the cerebrovascular resistance was still fairly high. This finding would be in line with earlier observations that maximal vasodilatation does not seem to take place even at very pronounced arterial hypoxia if the arterial carbon dioxide tension is not simultaneously increased (Häggendal and Johansson 1965). Against the background of a theory of hydrogen ions as the factor by which hypoxia acts on the vessels (Skinhoj 1966, Betz and Kozak 1967, Lassen 1968) a hypothetical explanation for the limited vasodilatatory response could be an insufficient supply of substrate required for the increasing anaerobic metabolism. Evidence of an interference with glycolysis in the brain due to substrate depletion in hypoxia was discussed by Cohen (1960). An indication that this might be a limiting factor for hypoxic vasodilatation is the flow increase encountered in hypotension after administration of glucose (no. 13), when no further dilatation had occurred with decreasing arterial oxygen saturation (nos. 11–12). In this animal 40 ml of a 10 per cent solution of glucose were given, which did not appreciably alter the oxygen capacity of the blood.

Reaction of the cerebral vessels to changes in the arterial carbon dioxide tension in hypotension (I)

Different results have been reported in the comparatively few studies published concerning the ability of the cerebral blood vessels to dilate in response to an increase of the arterial carbon dioxide tension, when autoregulation is exhausted. Thus Harper (1965) reported lack of response in hypotension. On the other hand Häggendal and Johansson (1965) studying the situation when autoregulation was impaired by extreme hypoxia found a scattering of the pressure-flow relation that could be attributed to differences in the arterial carbon dioxide tension and Häggendal *et al* (1969 and 1970a) also demonstrated a reactivity to carbon dioxide when the perfusion pressure was lowered beyond the autoregulatory range by induced increase in the intracranial pressure. The investigation of this reaction in hypotension meets with some difficulties in that the ensuing flow changes can be expected to be relatively small and the passive-elastic properties of the vessel wall imply changes in the vascular dimensions as a result of even small pressure changes.

In Fig. 6 I, a reaction to carbon dioxide in hypotension below the autoregulatory range is illustrated in terms of cerebral vascular resistance. There is a tendency to vasodilatation with increasing arterial carbon dioxide tension. With the oximetric technique a similar reaction was noticed during severe hypotension i.e. higher cerebral venous oxygen saturation with higher concentration of carbon dioxide in the inspired air.

Discussion

The compatibility of these results with the metabolic theory for the autoregulation of blood flow will be discussed. It is obvious that a metabolic mechanism being basically flow-dependent cannot keep the flow absolutely constant, some change is necessary to evoke a stimulus. Since the regulation, as discussed above is practically perfect it follows that an extremely sensitive mechanism would be required. The metabolic factors most discussed as eliciting the reaction have been oxygen, acting directly or via some metabolite resulting from tissue hypoxia, carbon dioxide and the hydrogen ion concentration in the tissue (Rapela and Green 1964, Lassen 1964). The latter factor has attracted further attention recently in view of the theory stating that changes in the hydrogen ion concentration may be the common link for the influence on the vessels by both oxygen and carbon dioxide (Skinhøj 1966, Betz and Kozak 1967, Zwetnow, Kjällquist and Siesjo 1968, Severinghaus 1968, Lassen 1968). In fact none of these factors seems likely to be able to establish a very sensitive regulation.

Thus the reaction of the vessels in response to changes in the arterial oxygen saturation is quite small around the physiological values and there is no marked vasodilatation until the arterial hypoxia has become pronounced (Fig. 2 I) (Häggendal and Johansson 1965). The constrictive effect of an elevation of the arterial oxygen tension is practically negligible (Lambertsen 1961, Reivich 1964). Further

more the presence of autoregulation at supernormal flow levels seems almost conclusively to rule out hypoxia as the regulatory factor

Concerning carbon dioxide the effect on vascular resistance by changes in its arterial tension is pronounced also around the normal value There is however evidence against carbon dioxide being a primarily important factor As discussed by Harper (Harper and Häggendal 1968) an increase in the hydrogen ion concentration in the extracellular fluid corresponding to a change in the arterial carbon dioxide tension from 40 to about 70 mm Hg would be required to reduce the vascular resistance to the extent observed, when the arterial blood pressure is lowered within the autoregulatory range

In addition when the lower limit for autoregulation is reached in hypotension the metabolic theory implies that the vessels are maximally dilated due to the regulatory factors. The fact that the response to both carbon dioxide and oxygen is retained below the autoregulatory pressure range adds to the improbability of *either being the main autoregulatory agent.*

A vasodilatatory influence of hypoxia in combination with a flow reduction secondary to a fall in blood pressure could be expected to be especially pronounced in severe hypoxia since here vasodilatation in response to a small decrease in oxygen availability is known to be very marked in normotension It might even be so large as to create a fairly effective autoregulatory response Such a regulation might be discussed in the case illustrated in Fig 2 where there are signs of an imperfect autoregulation Whether the autoregulatory tendency noted in the hypoxic state is a result of a hypoxic influence modifying a passive pressure-flow relation or is an expression of an impairment of the ordinary mechanism in the higher pressure range is not possible to judge In the hypotensive state however a metabolic influence would seem to be responsible for the cerebrovascular dilatation.

The relatively long time period required for flow adjustment has been assumed to favour a metabolic theory since a myogenic mechanism might be expected to induce a faster response (Harper 1960) In vitro studies on the reaction of vascular smooth muscle to quick stretch have shown, however that the response may require about 60 seconds to become fully developed (Sparks 1964), thereby differing from other smooth muscle *e.g* in the urinary bladder which exhibits a much faster response.

In regard to the mechanism responsible for the reactive hyperemia it should be pointed out that in paper I *the main problem studied was whether it was possible* to induce the phenomenon under conditions where metabolic factors could reasonably be excluded. No more detailed analysis of the duration and degree of hypotension was performed It seems likely that metabolic factors might contribute to the induction and maintenance of reactive hyperemia in some circumstances but they are not a prerequisite *for the appearance* of the phenomenon It may be discussed whether the reactive hyperemia demonstrated in paper I is of the same origin as the transitory passive flow response occurring in combination with pressure rises in the autoregulatory range as demonstrated in paper II using the oximetric technique.

PART II

EFFECT OF INCREASED VENOUS PRESSURE ON THE CEREBRAL BLOOD FLOW

An autoregulatory mechanism depending on vascular smooth muscle response secondary to changes in the chemical environment, should be effective when the perfusion pressure, and thus flow is altered irrespective of how this change is achieved A myogenic mechanism on the other hand, would in principle be independent of the perfusion pressure and respond to changes in the transmural pressure. Such considerations have been the background for an experimental approach previously used in different vascular beds employing increases in the venous pressure while the level of the arterial blood pressure is controlled preferably so that the venous and arterial blood pressures are raised to the same extent (see Folkow 1962) In this situation the perfusion pressure is maintained constant, while the transmural pressure is raised. In a passive vascular bed this would lead to an increased flow due to distension of the elastic vessel wall while in an actively responding vascular bed, governed by metabolic factors there would be a tendency to keep flow constant If the response were due to stretch of the smooth muscles, a contraction would ensue which if powerful enough would cause a flow reduction This latter kind of response has been found in skeletal muscle and intestine (Folkow 1949 Folkow and Öberg 1961 Johnson 1959 and 1964 c)

The brain cannot be directly subjected to this otherwise convenient procedure of differentiating, the effects of the two mechanisms as it is enclosed in the rigid skull and any change in the venous pressure consequently will lead to a change in the tissue pressure altering the transmural pressure in proportion In the present study this difficulty was overcome by the removal of large parts of the skull and dura over both hemispheres thereby providing conditions for expansion of the tissue The venous pressure was raised by inflation of a balloon attached to a catheter and placed in the right atrium of the heart No special measures were taken to maintain the arterial blood pressure during the increase in venous pressure since any additional surgical trauma was considered undesirable The inflation of the balloon tended to cause a moderate decrease of the arterial blood pressure presumably due to a reduction of the cardiac output. Such a decrease of the arterial pressure would curtail the possibility of revealing differences in the cerebrovascular response as the

accompanying decrease in transmural pressure would counteract the increase induced by the venous pressure rise. This difficulty was avoided by using a reference value for each animal (see paper *III*)

In Fig 2, III, was demonstrated a difference in the percentual changes of the cerebrovascular resistance if the perfusion pressure was reduced by venous pressure increase or by arterial pressure decrease. A reduced perfusion pressure induced by elevation of the venous pressure was accompanied by either unaltered or increased cerebrovascular resistance, while arterial pressure reductions were accompanied by proportionate lowering of the resistance When the arterial blood pressure was reduced by bleeding with the venous pressure maintained high and constant there was autoregulation on a lower flow level than with normal venous pressure (Fig 1 III)

Discussion

These results indicate a mechanism triggered by changes in the transmural pressure to be responsible for the cerebral blood flow autoregulation. No attempts to block possible nervous influence were made but available information from other vascular beds, where the response was shown to be independent of nervous factors make it unlikely that such an influence would be important in the case of the brain Furthermore the adrenergic innervation of the intracerebral vessels is sparse (Carlsson Falck and Hillarp 1962) On the basis of earlier studies it has been concluded that the nervous supply to the brain vessels is hardly of any functional significance (Sokoloff 1959) Although interest in this problem is now growing (Rapela, Green and Denison 1967 Langfitt and Kassell 1968 Mchedlishvili 1969 James, Millar and Purves 1969) there is no evidence to suggest that nervous vasomotor control should be of decisive importance for autoregulation in the brain

The myogenic hypothesis then remains to be considered This theory described in detail by Folkow in 1964, has recently been reviewed by Mellander and Johansson (1968) In short, parallels have been drawn to the function of the smooth muscles of the ureter and intestine which show spontaneous rhythmic contractions the frequency of which increases in response to stretch (Boxler 1948, Bülbring [illegible]) Evidence of vascular smooth muscle automaticity has been revealed by electrophysiological studies, beginning with the report of Funaki (1958) who recorded action potentials with microelectrodes in the muscle cells of the small vessels in the frog tongue. Furthermore rhythmic activity has been demonstrated in small pre- and postcapillary vessels studied microscopically *in situ* in the [illegible] (Nicoll and Webb 1955 Wiederman 1957 and 1968). This vasomotion, has been suggested to create the "basal tone" remaining in most vascular areas after elimination of all known extrinsic regulatory influence.

The observation that the basal tone is least pronounced in those vascular areas which show inefficient or no autoregulation (Folkow 1964) might suggest the regulation in response to changes in the transmural pressure is effective

same mechanism direct studies on small precapillary vessels have shown that vasomotion does become potentiated in response to an increase in the transmural pressure (Nicoll and Webb 1955 Wiedeman 1960) although the extent to which this increase in vasomotion can account for flow constancy has not been exactly established. Such a mechanism would, however explain one contradiction in the original theory namely that if the stimulus were distension it would seem to be abolished if the resistance to flow were upheld by a continuous contraction of the smooth muscle cells. With the concept of a changed frequency of contractions when the pressure is altered, a perfect constancy of flow although still remarkable implies no real contradiction Observations like those reported in paper I of well functioning autoregulation at high flow levels and of reactivity to oxygen and carbon dioxide in hypotension might possibly be explained in terms of a differentiated response to different stimuli in separate parts of the precapillary resistance section Some experimental evidence of such a response exists Thus studies in skeletal muscle during exercise have indicated that metabolic factors are comparatively more important than the transmural pressure as determiners of the tone of the resistance vessels and vice versa concerning the precapillary sphincter region (Lundvall Mellander and Sparks 1967)

GENERAL SUMMARY AND CONCLUSIONS

Different aspects on the pressure-flow relation in the brain have been studied and discussed with regard to the mechanism responsible for cerebral blood flow autoregulation.

Evidence of an ability of the autoregulatory mechanism to establish a perfect flow constancy in response to arterial blood pressure changes at normal and high flow levels was presented

The pressure-flow relation in hypotension below the autoregulatory pressure range in conditions with a primarily induced vasodilatation was found to be curvilinear with the convexity towards the pressure axis.

A cerebrovascular reaction to changes in the arterial tensions of both carbon dioxide and oxygen was demonstrated in hypotension, which indicates a possibility of this apparently passive pressure-flow relation being modified by metabolic factors.

With rapid changes of the arterial blood pressure there was initially a change of flow in terms of a change in the cerebral venous oxygen saturation in the same direction After that, within the autoregulatory pressure range, the flow gradually returned to th control level, this response being completed in about 40 seconds from the beginning of the alteration of the pressure

Reactive hyperemia could be elicited after a period of hypotension without signs of tissue hypoxia and it was suggested that this might be a prolongation of the normal transitory flow increase following pressure rises within the normotensive pressure range

During arterial hypoxia autoregulation was effective even when a hypoxic vasodilatation had occurred. In extreme arterial hypoxia an impairment of the autoregulatory function was noted, the pressure-flow relation however was not "purely passive In hypotension below th autoregulatory pressure range a vasodilatation was encountered with decreasing arterial oxygen saturation which might establish an important regulation when the normal autoregulation is impaired.

On account of the results obtained metabolic f ctors were considered unlikely to be responsible for the normal autoregulation of cerebral blood flow This was further stressed by the results from the following group of experiments. Here studies on the vascular response to increase of the venous pressure compared to the response to arterial blood pressure reductions were performed after removal of the skull coverings n an attempt to separate the effect on flow by changes in the perfusion pressure from

those by changes in the transmural pressure. A mechanism most likely of myogenic nature stimulated by changes in the transmural pressure was demonstrated to be responsible for the normal cerebral blood flow autoregulation.

In view of the increasing availability of methods to study regional cerebral blood flow and metabolism applicable to diseased states a detailed knowledge of the normal pressure-flow relation and the reaction of the blood vessels to different influences in different pressure ranges is important The present work might increase the possibilities for a sound clinical approach to the study of problems concerning cerebral blood flow

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ON THE RELATION BETWEEN BLOOD PRESSURE AND BLOOD FLOW IN THE CEREBRAL CORTEX OF DOGS

by

BARBRO EKSTRÖM-JODAL, EGIL HÄGGENDAL AND
NILS JOHAN NILSSON

ABSTRACT

Barbro Ekström-Jodal, Egil Häggendal and Nils Johan Nilsson. *On the relation between blood pressure and blood flow in the cerebral cortex of dogs* Acta physiol scand. 1970 Suppl 350 29–42 Some different aspect on the pressure-flow relation in the brain were studied in anaesthetized dogs with the radioactive gas elimination technique including external γ registration At high flow levels, primary vasodilatation being induced by hyperoxpnia or intravenous papaverine infusion, there was an effective autoregulation of flow in response to arterial blood pressure changes. In pronounced hypotension the pressure-flow relat on was found to be curvilinear with the convexity towards the pressure axis; an ability to react to changes in the arterial carbon dioxide tension was, however demonstrated At arterial hypoxia autoregulation functioned well, ven at very low values of oxygen saturation Reactive hyperemia could be elicited after a period of hypotension without preceding subnormal flow values. These results are discussed with respect to the metabolic theory for the mechanism responsible for cerebral blood flow autoregulation

INTRODUCTION

Mainly two theories for the mechanism behind the autoregulation of the cerebral blood flow in the sense that the flow is kept constant in spite of variations in the blood pressure are discussed at present The *metabolic theory* (e.g. Harper 1966) implies that the flow is autoregulated by the changes in chemical conditions in the

tissue, which arise when the blood flow passively follows increases or decreases in the perfusion pressure. The other theory the *myogenic* ascribes autoregulation to a myogenic response in the vascular wall induced by changes in the transmural pressure (see Folkow 1962 and 1964) So far neither of these theories has been definitively proved. In the nineteen sixties interest has been much concentrated on the role of metabolic factors in effecting cerebral blood flow autoregulation (Lassen 1964 Rapela and Green 1964 see also Harper and Häggendal 1968)

The present work is a study on the influence of some metabolic factors on the cerebral blood vessels during different blood gas and blood pressure conditions and deals with

Pressure-flow relations especially the problem of flow autoregulation when the blood vessels are dilated by different agents (carbon dioxide or papaverine)

Pressure-flow relations during graded arterial hypoxia.

Pressure flow relations during pronounced hypotension i e arterial pressure below the autoregulatory pressure range

The reactivity of the cerebral blood vessels to carbon dioxide during pronounced hypotension.

Reactive hyperemia elicited without preceding subnormal blood flow

METHODS

Thirty mongrel dogs with body weights of 12 to 20 kg were used The animals were anaesthetized with pentobarbital intravenously in a dose of 25–30 mg per kg body weight The operative procedure was completed within one to two hours with the animals breathing spontaneously After this the experiments were performed in muscle relaxation by suxamethonium chloride and the animals were ventilated with an adjustable pump through an endotracheal tube The body temperature controlled in the oesophagus was maintained at 37–38 C with the aid of an electrical heating pad Clotting was prevented by intravenous administration of heparin

The arterial tensions of carbon dioxide and oxygen were changed by ventilation with different gas mixtures No blood flow measurements were performed until 15 min after a change The arterial carbon dioxide tension was considered constant if there were no greater differences between measurements than ± 2 mm Hg at unchanged ventilation Results from measurements where the difference exceeded these limits were discarded Blood samples for analysis of oxygen saturation hemoglobin concentration, pH and carbon dioxide tension were drawn simultaneously from a femoral artery through a polyethylene catheter and from the superior sagittal sinus via a needle bored through the skull about three cm rostral to the occipital

protuberance. Oxygen saturation (Holmgren and Pernow 1950 Bjurs and Nilsson 1965) and hemoglobin concentration (Drabkin and Austin 1935) were determined spectrophotometrically concerning the latter with modifications according to the Decision of the Standardizing Committee of the European Society of Haematology concerning Haemoglobinometry 1963 and the oxygen capacity was calculated on the basis of the hemoglobin concentration. Determinations of pH and carbon dioxide tension were made according to the micro method of Siggaard Andersen *et al.* (1960) with corrections for low oxygen saturation. The arterial and venous oxygen tensions were calculated with the Blood Gas Calculator of Severinghaus (Radiometer Copenhagen) from the hemoglobin dissociation curve for dogs.

The arterial blood pressure was electromanometrically recorded from the catheter in the femoral artery Mean pressures were obtained by electrical integration, and the heart level was chosen as the reference point. The arterial blood pressure thus recorded, which was regarded as a measure of the cerebral perfusion pressure was changed by bleeding or retransfusion through a polyethylene catheter in the other femoral artery The blood was kept at 37–38 C in a reservoir before retransfusion Measurements of cerebral blood flow were not performed until 5 min after any change in blood pressure except in the studies on reactive hyperemia, where ~ min were allowed to pass.

The cerebral blood flow measurements were made with the radioactive gas elimination method of Lassen *et al* (1963) as described by Haggendal, Nilsson and Norbäck (1965) For intraarterial injections of the indicator the left vertebral artery was catheterized with a thin polyethylene catheter (PE 100) inserted through the external thoracic and subclavian arteries about 5–8 cm up into the intact vertebral artery Rapid injections (~ 3 sec) of radioactive krypton (Kr^{85}) dissolved in saline (1–2 ml) were given. The elimination was registered externally with a scintillation detector coupled to a ratemeter (Tracerlab Nuclear Instrumentation) with a time constant of 1.5 sec and recorded linearly on a potentiometer writer (Beckman Laboratory Potentiometric Recorder) The clearance curves were followed for 10–15 min Influence on the curves from extracerebral tissue was reduced by lead collimation of the detector After semilogarithmic plotting the curves were resolved according to the compartment analysis method as described by Häggendal Nilsson and Norbäck (1965) The values for the fast component considered representative of flow in the cerebral grey matter are given in the results

The studies of the passive pressure flow relation in pronounced hypotension and of the reactive hyperemia were facilitated by an initially high blood flow level, induced by hypercapnia or papaverine infusion The mean arterial blood pressure was lowered in steps of about 10 mm Hg Papaverine was infused intravenously at a rate of 0.4–0.5 mg per kg body weight and minute. Several measurements of cerebral blood flow were made before the first bleeding to control that the influence of papaverine on the cerebral blood vessels was constant

RESULTS

Pressure-flow relations at high levels of cerebral blood flow

Fig 1 demonstrates pressure-flow relations in four hypercapnic animals and in four animals where cerebral vasodilatation was induced by papaverine infusion. The actual flow values were very different in different animals. In spite of this there was in all cases perfect or almost perfect flow constancy with arterial blood pressure reductions over a wide range. It can be noted that even at the highest blood flow levels the ability to autoregulate was preserved down to a quite low arterial blood pressure In all of these animals the arterial oxygen saturation was normal and the venous oxygen saturation thus high on account of the increased cerebral blood flow

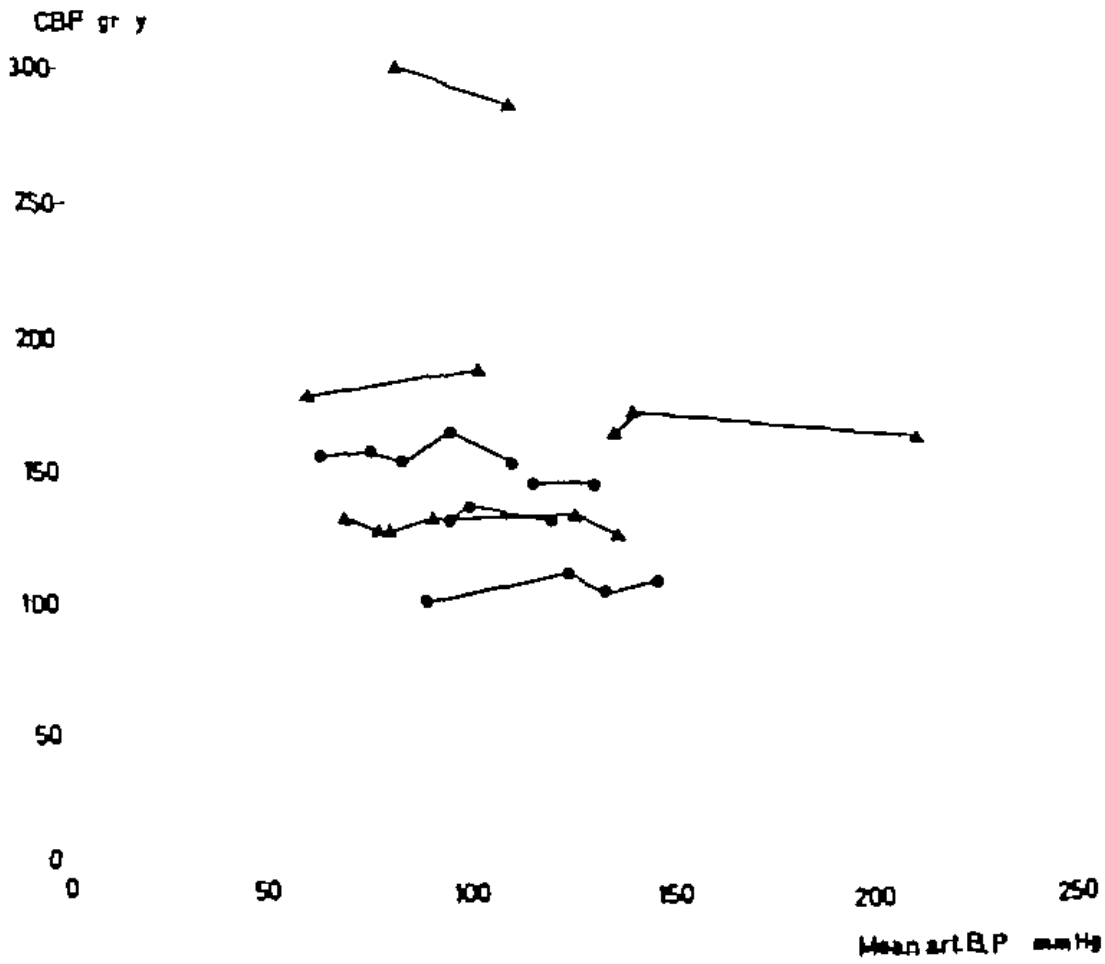


Fig. 1 The relation between mean arterial blood pressure and cerebral blood flow in grey matter (CBF_{grey} ml/l) gmin) in animals with a primary vasodilatation, induced by hypercapnia (filled circles) or papaverine infusion (filled triangles) Effective autoregulation is demonstrated within a wide arterial blood pressure range on different flow levels.

Pressure-flow relations during graded hypoxia

Fig. 2 shows the correlation between cerebral blood flow and arterial oxygen saturation (nine animals) with emphasis on preserved ability to autoregulate. Filled circles mean that with unaltered blood gas tensions the blood flow remained nearly constant in spite of blood pressure reductions of at least 45 mm Hg. Open circles imply that autoregulation was not tested. A typical experiment illustrating the ability of autoregulation even at pronounced arterial hypoxia is presented in Fig. 3. In this experiment the flow increased from about 50 to 85 ml/100 gmin when the arterial oxygen saturation was lowered from 95 to 40 per cent and the autoregulation was still functioning.

Pressure-flow relations during pronounced hypotension

The pressure-flow relation at blood pressures below the autoregulatory range was studied in one group of animals. These experiments were performed during cerebrovascular dilatation induced either by papaverine infusion (Fig. 4 and 5 a) in four animals or by carbon dioxide inhalation (Fig. 5 b) in another four animals. Auto-



Fig. 2. Correlation between arterial oxygen saturation ($S_{a_{O_2}}$) and blood flow in cerebral grey matter (CBF_{grey} ml/100 gmin). Filled circles indicate that autoregulation was tested to be well functioning when the arterial blood pressure was changed with the blood gases maintained constant. Open circles mean that autoregulation was not tested at that level of arterial oxygen saturation. Mean arterial blood pressure within the range of 128–140 mm Hg and arterial carbon dioxide tension 40–44 mm Hg.

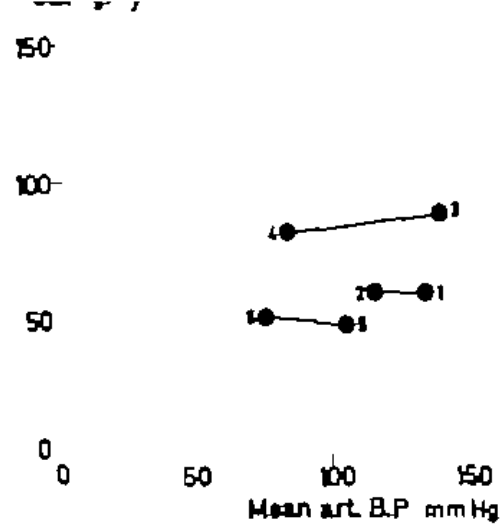


Fig 3. The relation between mean arterial blood pressure and blood flow in cerebral grey matter (CBF_{grey}, ml/100 gmin) in one animal studied at different levels of arterial oxygen saturation (S_{aO_2}) with a fairly constant arterial carbon dioxide tension (P_{aCO_2})
The numbers indicate the chronological order of measurements. CMR_O stands for cerebral metabolic rate of oxygen.

Measurement Number	P_{aCO_2} mm Hg	S_{aO_2} %	CMR_{O_2} ml/100 gmin
1	29	64	4.9
	29	61	4.7
3	31	40	4.6
4	27	40	4.9
5	31	95	4.9
6	31	95	5.5

Efficient autoregulation is illustrated at different levels of arterial oxygen saturation.



Fig 4 Relation between mean arterial blood pressure and flow in cerebral grey matter (CBF_{grey} ml/100 gmin) in an animal with intact autoregulation during intravenous infusion of papaverine. The relation is curvilinear with the inflection point at the pressure axis in hypotension

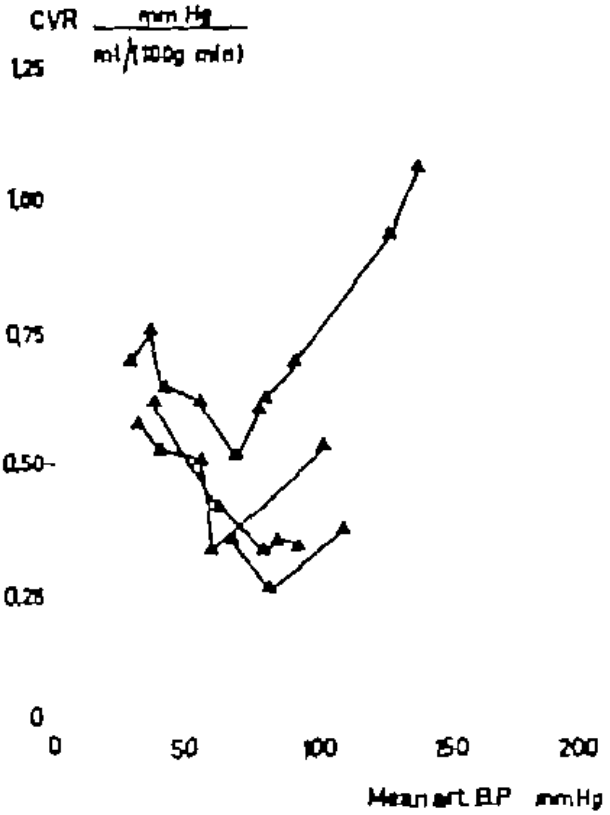


Fig. 5 a. Correlation between mean arterial blood pressure and cerebro vascular resistance (CVR) in dogs, where a primary vasodilatation was induced by intravenous papaverine infusion. From the lowest value obtained during autoregulation the CVR increased in hypotension. In two animals the increase was most pronounced initially and in one animal there was a practically constant increase in CVR thereby indicating a curvilinear pressure-flow relation as shown in Fig. 4. In another animal only one hypotensive flow measurement was obtained resulting in an increased CVR, which is indicative of either a curvilinear pressure-flow relation or a linear relation intersecting on the pressure axis.

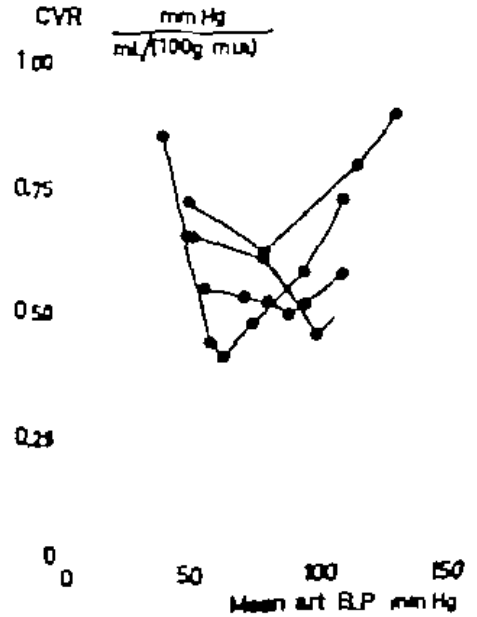


Fig. 5 b. The same correlation as in Fig. 3 a for hypercapnic animals.

regulation was first demonstrated at the supernormal flow level and when the flow had just begun to decrease at a certain blood pressure value the following pressure-flow relation was studied by further reducing the blood pressure in small steps. This part of the pressure-flow relation was curvilinear with the convexity towards the pressure axis as illustrated in Fig 4 In Fig 5 a and b the same phenomenon is expressed as the relation between the arterial blood pressure and the cerebrovascular resistance. Initially a successive decrease in the cerebrovascular resistance is observed when the arterial blood pressure was lowered, indicating normal autoregulation Then, in response to blood pressure reductions below the autoregulatory range there was a marked increase in the resistance.

Reactivity of the cerebral blood vessels to carbon dioxide in pronounced hypotension

Fig 6 shows the relation between the arterial carbon dioxide tension and the cerebrovascular resistance at an arterial blood pressure of 120 mm Hg which was demonstrated to be within the autoregulatory range for all the nine animals included (filled circles) The figure also illustrates the same relation for seven dogs in hypo-

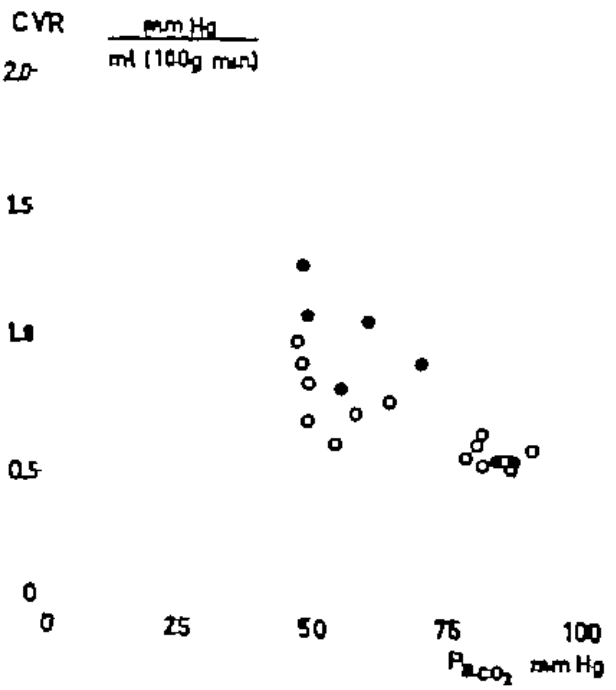


Fig 6. Correlation between arterial carbon dioxide tension (P_{aCO_2}) and cerebrovascular resistance (CVR) in two groups of dogs: one with mean arterial blood pressure 120 mm Hg (filled circles), which was within the autoregulatory range; the other at mean arterial blood pressure below the autoregulatory range (open circles) In both groups there was a decreasing CVR, when $P_{a.CO_2}$ was raised.

Table I. Values from one animal where the mean arterial blood pressure was kept 50 mm Hg, which was tested to be below the autoregulatory range when the carbon dioxide tension (P_{aCO_2}) was 49 and 78 mm Hg. A response to changes in P_{aCO_2} is seen at a fairly constant arterial oxygen saturation (S_{aO_2}). The cerebral metabolic rate for oxygen is practically unchanged between measurements.

CBF_{grey} ml/100 gmin	CMR_O ml/100 gmin	S_{aO} %	P_{aC} mm Hg
44	3.4	91	43
77	5.6	85	49
94	3.6	83	78

tension, tested to be below the autoregulatory range (open circles) although in this case the representation is not restricted to one single blood pressure value but different blood pressures are represented at most of the different values of arterial carbon dioxide tensions. The influence on these results of different hypotensive blood pressures will be discussed. There was a decrease in cerebrovascular resistance with increasing arterial carbon dioxide tension both in the hypotensive and the normotensive animals. The resistance however tended to be lower during hypotension especially when the hypercapnia was only moderate.

Table I is an example of the cerebrovascular reactivity to carbon dioxide in one animal, where the autoregulation was exhausted due to induced severe hypotension. The arterial blood pressure was kept constant (50 mm Hg) while the arterial carbon dioxide tension was changed in steps. It can be seen how the flow increased in response to the raised carbon dioxide tension. In this experiment loss of autoregulation was tested at the arterial carbon dioxide tension of 49 and 78 mm Hg. It cannot be excluded that in the hypocapnic state an ability of autoregulation was preserved.

Experiments on reactive hyperemia

Fig. 7 illustrates the pressure-flow relation, when the arterial blood pressure was reinstituted after a period of pronounced hypotension with failing autoregulation. Throughout these experiments the cerebral blood vessels were dilated by hypercapnia (4 dogs) or papaverine (3 dogs) as described earlier. The blood pressure was then lowered stepwise. The autoregulatory flow levels for the different animals are indicated in the figure. Reactive hyperemia ensued even in those experiments, where the blood flow during the hypotension had never decreased below normal values. The oxygen tension in the cerebral venous blood was never below 40 mm Hg.

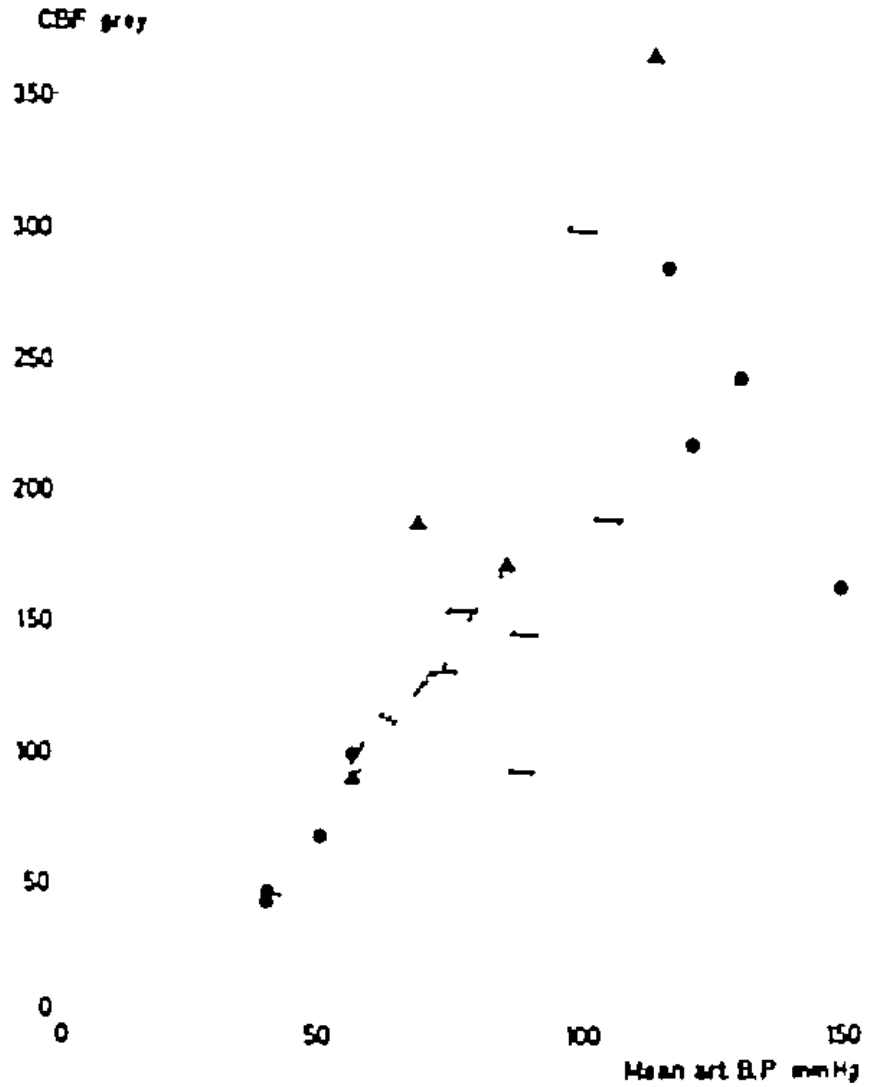


Fig. Illustration of reactivity to hypercapnia in two groups of dogs, one hypertensive group (filled circles) and one group during intravenous dopamine infusion (filled triangles). The lowest values of cortical blood flow in grey matter (CBF_{grey} ml/100 gram) obtained in hypotension and the highest values obtained in the post hypercapnic period are connected by broken lines. Solid lines indicate the autoregulatory flow levels.

DISCUSSION

Methodological considerations

The purpose of this investigation was to study some different aspects of the pressure-flow relationship in the cerebral circulation in an attempt to evaluate the role of mechanical factors in autoregulation. Such a study requires methods implying as little trauma as possible since the normal autoregulation seems to be a vulnerable process easily interfered with by surgical preparative procedures (Rapela and Green 1964). The method of investigation in the present study of Lassen *et al.* (1963) meets this

demand in that it includes γ registration through the intact skull. There is good experimental evidence that the fast component in the compartive γ curve is an expression of blood flow through the cerebral grey matter (Häggendal Nilsson and Norbäck 1965) The correct estimation of this component according to the compartment analysis method requires steady state conditions during a comparatively long period of time, depending on the blood flow With normal values for blood gases and blood pressure the time needed for a measurement is 10–15 min. This condition may not always be fulfilled in the studies of reactive hyperemia, this fact, however cannot alter the qualitative results even if the actual values are probably subject to a larger degree of uncertainty than is ordinarily inherent in the method. In some cases this uncertainty is indicated in the values for the cerebral metabolic rate of oxygen calculated from these values of blood flow and the arterio-venous oxygen difference obtained during the flow registrations 5–6 min after the pressure increase. These values being often somewhat higher than otherwise in the same animal.

The partition coefficient 0.95 for Kr^{85} between cortex and blood was used for the calculation of cerebral blood flow from the clearance curves throughout the experiments. The value of the partition coefficient is dependent upon the hematocrit value of the blood (Lassen *et al* 1963 Ingvar and Lassen 1962 Glass and Harper 1962) Since, however there were only moderate variations in this factor between measurements in the run of each experiment as well as between different animals, corrections were not considered necessary

Sagittal sinus blood was used as representative of venous blood from the cerebral grey matter Häggendal, Nilsson and Norbäck (1965) and Häggendal and Norbäck (1966) have demonstrated that the sagittal sinus blood emanates predominantly from the cerebral grey matter and contains no extracerebral contributions of importance.

In the experiments where papaverine was infused at a constant rate the cerebral blood flow was measured several times at the same blood pressure to check that an equilibrium was reached with respect to the papaverine effect. It was also tested that the flow gradually returned to the previous autoregulatory level after reactive hyperemia with constant papaverine infusion.

General discussion

The present results concerning autoregulation on high cerebral blood flow levels seem to exclude tissue hypoxia as the main stimulus to cerebral autoregulation, because the induced high flow with normal arterial oxygen content implies a rise in tissue and venous oxygen tension.

It should be stressed that the ability to autoregulate is really retained at decreased cerebrovascular resistance due to *e.g.* high carbon dioxide tension, since the opinion is often encountered that the autoregulation is lost at moderate to severe hyper

capnia (Harper 1965). The present results demonstrate a well functioning autoregulation in these circumstances even at pronounced blood pressure reductions.

The experiments on pressure flow relations during graded arterial hypoxia demonstrate that the hypoxic vasodilatation occurs at a higher arterial oxygen saturation than the inhibition of cerebral autoregulation (Figs 1 and 2). It was mentioned already by Ekelund in 1931 that such an inhibition may be elicited by hypoxia. As it has been shown that hypoxic cerebral vasodilatation is induced from the tissue (Newell and Schneider 1941, Häggendal, Nilsson and Norlbäck 1966), the present results of maintained autoregulation even at pronounced arterial hypoxia can at least theoretically be due to metabolic factors i.e. tissue hypoxia.

It can be noticed that not even during the most pronounced hypoxia were maximal flow values obtained (*Fig. 2*). This is in accordance with earlier suggestions that maximal vasodilatation does not occur due to arterial hypoxia (Häggendal and Johansson 1965). It might even be possible that a sudden abolishment of the normal autoregulation, elicited by the hypoxia, adds to the steep flow increase at low values of arterial oxygen saturation.

The pressure flow relation in hypotension below the autoregulatory range would be expected to be curvilinear with the convexity towards the pressure axis provided the vascular bed (Folkow and Lofving 1950). Green, Rapela and Conrad (1963) have described a transient curvilinear pressure-flow relation in the brain in combination with pressure changes within the autoregulatory range, but did not discuss the pressure flow relation in hypotension. When studying the pressure flow relation in pronounced hypercapnia, though curves characteristic of a passive pressure flow relation were found (Rapela and Green 1964), Harper (1965) in summing up results from different animals found a linear relation at high arterial carbon dioxide tensions. In hypoxic animals studied by Häggendal and Johansson (1965) the pressure flow relation was also practically linear. This might indicate some degree of active smooth muscle response under these circumstances. The finding of such linear pressure flow relations are interesting with regard to the mechanism of autoregulation as the *metabolic theory implies that the reactivity of the vessels* to chemical stimuli should be exhausted when the constancy of flow is no longer upheld in hypotension (Harper 1965). In the present study we invariably found a curvilinear relation as would be expected in a truly passive vascular bed. The *findings* however *must rule out the possibility that some active vascular* reaction still exists though not strong enough to counteract completely the effect of the lowered intravascular pressure.

Variability of the cerebral vessels to react to chemical stimuli is demonstrated in hypercapnia and hypoxia (Figs. 1 and 2, Table I). The figures obtained for cerebral vascular resistance obtained at different arterial blood pressure levels within the autoregulatory range. There was however no tendency for a higher blood pressure in the higher range of arterial oxygen saturations which would indicate any activity

owing to the curvilinear relation between pressure and flow demonstrated in Figs. 4 and 5 a and b. A quantitative analysis of the difference in reactivity to carbon dioxide in normotension and hypotension has not been made due to this curvilinear pressure-flow relation in hypotension.

The reactivity of the cerebral vessels to arterial hypoxia in hypotension has not been studied in detail, but in one experiment, where the arterial carbon dioxide tension was 47 mm Hg and the arterial oxygen saturation was decreased from 95 to 47 per cent with the blood pressure kept at 70 mm Hg, there was an increase in cerebral blood flow from 60 to 82 ml/100 g/min. The cerebral metabolic rate of oxygen was 4.8 and 5.2 ml/100 g/min respectively and the blood pressure 70 mm Hg, was tested to be below the autoregulatory range. An effect of hypoxic stimuli is indicated by this experiment.

Tissue hypoxia during the period of reduced perfusion pressure has been considered important for the occurrence of reactive hyperaemia (Zweifach, Kjällquist and Siesjö 1968). Our data indicate that reactive hyperaemia can be observed after blood pressure decreases with flow still above normal and with high venous oxygen tension. Thus tissue hypoxia does not seem to be necessary for eliciting this vascular response.

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FROM THE DEPARTMENTS OF CLINICAL PHYSIOLOGY I AND II,
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CEREBRAL VENOUS OXYGEN SATURATION DURING RAPID CHANGES IN THE ARTERIAL BLOOD PRESSURE AN OXIMETRIC STUDY IN DOGS

by

BARBRO EKSTRÖM-JODAL, EGIL HÄGGENDAL AND
NILS JOHAN NILSSON

ABSTRACT

Barbro Ekström-Jodal, Egil Häggendal and Nils Johan Nilsson. *Cerebral venous oxygen saturation during rapid changes in the arterial blood pressure. An oximetric study in dogs.* Acta physiol. scand. 1970 Suppl. 350 43–50 Time relations and efficiency of the autoregulatory response to arterial blood pressure changes were studied in anaesthetized dogs, alterations in the cerebral venous oxygen saturation being taken as a relative measure of changes in blood flow Blood withdrawn from the superior sagittal sinus was continuously analysed in a cuvette oximeter during rapid changes of the arterial blood pressure, brought about by withdrawal or infusion of blood The time period from a change of blood pressure to a fully developed response was around 40–45 sec Within the autoregulatory pressure range a complete flow adjustment took place sometimes with a transitory overcompensation. Below this pressure range no compensation appeared

INTRODUCTION

Previous works from this laboratory on the autoregulation of cerebral blood flow have concentrated on the investigation of the efficiency of autoregulation as well as on its limitations and the nature of the mechanism behind it (Häggendal and Johansson 1965, Ekström-Jodal *et al* 1969 Ekström-Jodal, Häggendal and Nilsson 1970 Ekstrom-Jodal 1970) In these studies a steady state has been desired during the actual flow measurements, and therefore a certain time has always been allowed to elapse after each change in the flow situation before a new determination was made.

The problem of the time required for the establishment of the autoregulatory flow adjustment has not been extensively investigated. Fog in his classical paper from 1934 reported reactive changes in the diameter of the pial arteries to appear within 1.5–2.0 min after a change of arterial blood pressure. In 1964 Rapela and Green diverting the outflow from the venous sinuses through an electromagnetic flowmeter probe could observe the time course of the autoregulation and found it to become effective within 0.5–1.0 min. Hirsch and Körner (1964) on the other hand measuring discontinuously the outflow from the superior sagittal sinus give values of several minutes. Betz (1968) noted a delay of 0.5–2.0 minutes for the blood flow to return to the orginal value. Kanzow (1969) reported a time of 30–60 sec. Thus there is still some uncertainty about the time value. The problem is difficult to study especially considering the delicacy of the autoregulatory mechanism, which can easily be damaged by traumatic surgical procedures.

In this work a cuvette oximeter has been used for continuous recording of the oxygen saturation of blood withdrawn from the superior sagittal sinus during rapid changes of the arterial blood pressure by bleeding or reinfusion of blood. The changes in oxygen saturation have been taken as an expression of changes in the cerebral blood flow. Apart from illustrating the time course of the transient events during pressure changes the method also provides an opportunity to evaluate the extent of the flow alterations.

METHODS

Experiments were performed on dogs weighing 12–30 kg. The animals were anaesthetized with pentobarbital in a dose of 25–30 mg per kg body weight. The experiments were performed during muscle relaxation with succamethonium chloride and ventilation with an adjustable pump through an endotracheal tube. The body temperature controlled in the oesophagus was maintained at 37–38° C by intermittent heating.

The arterial blood pressure was recorded from a polyethylene catheter inserted into the femoral artery with a transducer of the inductance type and recorded on one channel of a two-channel pen-recorder (Servogor II, Texas Instruments). The arterial mean pressure was obtained by electrical integration. The arterial blood pressure was changed rapidly as far as possible by withdrawal or reinfusion of blood through a wide bore (I.D. 2.50) polyethylene catheter inserted into the opposite femoral artery.

The oximetric analyses were performed on blood withdrawn from the superior sagittal sinus via a needle inserted through the intact skull of the heparinized animal about 3 cm rostral to the occipital protuberance. The photometric linearly responding cuvette instrument described by Nilsson (1963) was used equipped with interference filters having the wavelengths 525 and 805 mμ. The deflections were registered on a tangential Atlas mean value amplifier (Atlas-Werke, Bremen) the output

of which was recorded on the other channel of the potentiometer writer The arrangement of the oximeter was in principle the same as that earlier used by Nilsson (1956 1957). The cuvette was attached to the needle in the superior sagittal sinus by a thin polyethylene catheter (PE 160) kept as short as possible (about 3 cm) to minimize time lag in the recording system. The volume of the measuring system (puncture needle polyethylene tube and oximeter cuvette) amounted to about 0 3 ml. Blood was continuously drawn through the oximeter cuvette with a motor driven syringe at a constant rate of 5 ml/min. In some cases also the rates of 3.5 or 7.5 ml/min were used for comparison.

For calibration, samples of sagittal sinus blood drawn through the oximeter were analysed spectrophotometrically (Holmgren and Persow 1950 Bjure and Nilsson 1955) at several different levels of oxygen saturation.

RESULTS

Fig. 1 represents an original recording of mean arterial blood pressure and cerebral venous oxygen saturation, made during a period of about 20 min, when the animal exhibited irregular variations in mean arterial blood pressure. The exact nature of these variations was not analysed, they are probably at least partly due to a cardiac arrhythmia. It can be seen that the irregularities in the blood pressure, if they are rapid enough, are reflected in the oximeter tracing as corresponding variations with a time lag of about 5 seconds. The autoregulation is not effective against these sudden pressure changes so that a certain degree of pressure-passive flow variation appears.

At a the arterial pressure is reduced from about 125 to a little over 100 mm Hg, and the oxygen saturation displays a concomitant fall. After about 13 seconds the fall changes into a rise although the pressure remains low and even shows a slightly falling tendency Thus rise in venous oxygen saturation we interpret as the effect of an increase in cerebral blood flow the reduction in vascular resistance being brought about by the autoregulatory mechanism. The saturation reaches its original level about 40 seconds after the beginning of the fall in arterial blood pressure· then a transitory overshoot ensues. At "b the arterial blood pressure is raised by reinfusion of the withdrawn blood After th ordinary delay of about 5 seconds the saturation begins to rise only to fall again after another 10 seconds in spite of a continued rise of the blood pressure. This fall, again a result of autoregulation which now increases the vascular resistance to keep the flow at its previous value restores the saturation to the original level within about 35 seconds from the beginning of restitution of the pressure A certain tendency to over regulation is seen in this situation also.

Fig 2 illustrates a different type of reaction. In this animal arterial pressure was reduced from 80–90 to about 50 mm Hg the latter value can be presumed to be about the lower limit of autoregulation Initially th change in venous oxygen

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FROM THE DEPARTMENTS OF CLINICAL PHYSIOLOGY I AND II UNIVERSITY OF GÖTEBORG, SWEDEN

EFFECT OF INCREASED VENOUS PRESSURE ON CEREBRAL BLOOD FLOW IN DOGS

by

BARBRO EKSTRÖM-JODAL

ABSTRACT

Barbro Ekström-Jodal. *Effect of increased venous pressure on cerebral blood flow in dogs* Acta physiol. scand. 1970 Suppl. 350 51–61 The study performed on anaesthetized dogs, concerns the mechanism behind the cerebral blood flow autoregulation. The cerebral perfusion pressure was reduced by increasing the venous or decreasing the arterial blood pressure in order to separate the cerebrovascular response to changes in the transmural pressure from the response to changes in the perfusion pressure. The brain was provided with conditions for tissue expansion by removal of a large part of the skull and dura over both hemispheres The venous pressure was increased by obstructing the venous return to the heart. Cerebral blood flow was measured with the radioactive gas elimination technique using external γ registration. With proportionate reductions of the perfusion pressure the cerebrovascular resistance was consistently higher when the reduction was caused by a venous pressure increase as when it was caused by an arterial pressure decrease. When the venous pressure was maintained high while the arterial blood pressure was reduced, there was autoregulation on a lower flow level as when the venous pressure was normal. The results indicate that a change of transmural pressure is the adequate stimulus to cerebral blood flow autoregulation, probably acting via a basically myogenic response in the vessel wall

INTRODUCTION

In a previous paper (Ekström-Jodal, Häggendal and Nilsson 1970) evidence was presented against the theory which regards metabolic factors as the main cause of the autoregulation of cerebral blood flow in response to changes in blood pressure.

such evidence against the *metabolic* theory has focused interest on myogenic factors which are supposed to establish autoregulation in other vascular beds e.g. in skeletal muscle and intestine (for references see Mellander and Johansson 1968). The myogenic theory, based on Bayliss' hypothesis from 1902, has been comprehensively described by Folkow in 1964. It implies vascular smooth muscle automaticity, the adequate stimulus of which is the distension offered by the transmural pressure. The experimental approach in demonstrating the myogenic mechanism has often been based on primary increases of the venous blood pressure. Thus, in skeletal muscle of reserpine treated cats Folkow and Öberg (1961) found higher vascular resistance when a reduction of the perfusion pressure to the same value was achieved by increasing the venous pressure as when it was achieved by decreasing the arterial pressure. The difference in vascular resistance was explained by the higher transmural pressure in the former case potentiating vasomotion in precapillary resistance vessels.

Enclosed in the rigid skull however the brain offers special difficulties in this kind of experiment because any change in the venous pressure is followed by an equally large change in the tissue pressure. In the present study an attempt has been made to overcome this difficulty by removing most of the fronto-parieto-temporal part of the skull and opening the dura to give the tissue an opportunity to expand thus minimizing the increase in tissue pressure. A differentiation of the effects on vascular resistance by changes in the perfusion pressure and in the transmural pressure should then be possible yielding further information on the mechanism behind the autoregulation of cerebral blood flow. Preliminary results on this subject have been given at the International CBF Symposium in Mainz 1960 (Ekstrom Jodal Haggendal Nilsson and Norback 1960).

METHODS

The experiments were performed on ten mongrel dogs with body weights of 15–18 kg. The animals were anaesthetized with pentobarbital intravenously in a dose of about 30 mg per kg body weight. The operative procedure was completed with the animal breathing spontaneously. After that the animals were kept curarized with tubocurarine chloride and ventilated with an adjustable pump through an endotracheal tube. The body temperature measured in the oesophagus was maintained at 37–38°C by an electric heating.

The skull was removed over a large part of the hemispheres. A thin plastic catheter (PE 10) was inserted into the exposed superior sagittal sinus and left in an anterograde position. Clotting was prevented by flushing of the catheter with a heparin saline solution. The dura was carefully opened over both hemispheres leaving the middle part of each with the sagittal sinus intact. Desiccation of the brain surface was prevented by covering it with a skull preparation with a thin (15 μm) Mylar film

Arterial and cerebral venous blood pressures were electromanometrically recorded using a polyethylene catheter inserted into one femoral artery and the catheter in the superior sagittal sinus, respectively. Mean pressures were obtained by electrical integration, and the heart level was taken as the reference point. The perfusion pressure was calculated as the difference between the mean arterial and venous blood pressures. The arterial blood pressure was changed by withdrawal or reinfusion of blood through a polyethylene catheter in the other femoral artery. When stored outside the body the blood was kept in a reservoir at a temperature of 37–38 C.

The venous blood pressure was increased by means of a balloon catheter inserted through the right external jugular vein to a position with the balloon in the right atrium. The balloon was filled with maximally 30 ml of isotonic saline solution at 37–38°C, the exact volume being adjusted with respect to the effects observed on the venous pressure measured in the superior sagittal sinus and on the mean arterial pressure. With this technique the venous pressure in the superior sagittal sinus could be raised to maximally 96 mm Hg.

In combination with each measurement of blood flow the arterio-venous oxygen difference was calculated from oxygen determinations in blood samples drawn simultaneously from a femoral artery and the superior sagittal sinus. The oxygen saturation (Holmgren and Pernow 1969, Bjurø and Nilsson 1965) and the hemoglobin concentration (Drabkin and Austin 1935) were measured spectrophotometrically, the latter with modifications according to the Decision of the Standardizing Committee of the European Society of Haematology concerning Haemoglobinometry 1963. From these determinations oxygen capacity and content were calculated. Carbon dioxide tension and pH in the same blood samples were measured with the micro method of Siggaard Andersen *et al.* (1960).

The measurements of cerebral blood flow (CBF) were performed with the radioactive gas elimination method of Lassen *et al.* (1963), using intraarterial injections of radioactive krypton (Kr^{85}) and external registration. For methodological details see Ekström-Jodal, Haggendal and Nilsson (1970). The values given in the results are those calculated for cerebral grey matter (CBF_{grey}, ml/100 g·min).

PROCEDURE

The experiments included the following maneuvers:

Measurement of cerebral blood flow, blood pressure and blood gases with normal arterial and venous blood pressures;

Elevation of the venous pressure by inflation of the balloon in the right atrium followed by the same measurements;

Deflation of the balloon and control of autoregulation by arterial bleeding at normal venous pressure.

In three cases the cerebrovascular response to arterial blood pressure variations was studied at elevated venous pressure.

In some of the experiments the level of cerebral blood flow was elevated initially by adding five per cent carbon dioxide to the inspired air

A condition for accepting an experiment was that autoregulation could be demonstrated by arterial bleeding when the venous pressure was restored to normal after the measurements with an elevated venous pressure In six animals this condition was fulfilled and another animal (no VI in Table 1) was included in the table for reasons given below Thus three animals were discarded

RESULTS

The successful experiments are summarized in Table I. The lower case letters indicate the chronological order of the measurements. In experiment no I the blood gas situation changed spontaneously when autoregulation in response to arterial bleeding was tested in spite of constant artificial ventilation, probably due to pulmonary atelectasis Therefore the measurements at c and d were only used to prove autoregulation there was very little difference in arterial oxygen saturation and carbon dioxide tension between them. The comparison between cerebral blood flow at normal and increased venous pressures for this animal in Fig 2 refers to a and b

In experiment no. VI autoregulation was not tested by arterial bleeding because the arterial blood pressure decreased spontaneously This animal is nevertheless included in the table as it shows a considerably higher flow value at lower perfusion pressure when the venous pressure has its lowest value.

Fig 1 illustrates two examples (no. V and VII, open and filled symbols, respectively) where the cerebral blood flow was kept high by the administration of carbon dioxide to the inspired air There is a clear reduction of cerebral blood flow with elevation of the venous pressure (squares) that cannot be explained by the small fluctuations in the arterial carbon dioxide tension. Autoregulation in response to arterial bleeding is demonstrated with both normal (circles) and raised (squares) venous pressure on different levels of cerebral blood flow In Fig 2 all experiments in the table except no VI are illustrated. Percentual changes in the perfusion pressure from a reference value are given along the abscissa and the corresponding percentual changes in the cerebrovascular resistance (CVR) along the ordinate. The reference value (zero point) for perfusion pressure is the arterial pressure during the period of increased venous pressure (concerning experiment no. IV 140 V 110 and VII 133 mm Hg) minus the normal venous pressure. For cerebral vascular resistance the reference value is calculated from the measured cerebral blood flow at these pressures if such a measurement could be made However there was usually a moderate decrease in the arterial blood pressure when the balloon was filled, presumably due to the cardiac output being reduced by the obstruction of venous return to the heart. Thus in many cases no measurement of cerebral blood flow was performed at exactly the same arterial blood pressure, when the venous pressure was normal as when it was elevated. The blood flow used for the calculation of the

Table 1. Results from the 7 dogs included in the study. The series of measurements are indicated by lower case letters in chronological order for each animal.

Exp. animal	Mean art. B.P mm Hg	Mean ven B.P mm Hg	Perfusion pressure mm Hg	CBF_{grey} ml/100 gmin	CVR mm Hg / ml/100 gmin	CMR_O ml/100 gmin	Sa_{O_2} %	Pa_{CO_2} mm Hg
I a	120	7	113	60	1.88	6.5	93	43
b	110	20	90	53	1 70	6.8	91	44
c	85	9	76	137	0.56	7 1	77	51
d	65	7	58	146	0.40	7 0	74	53
II	118	20	89	67	1.33	7.3	88	51
b	95	7	88	78	1 13	7.2	96	54
	65	4	61	77	0 79	7 4	93	54
III a	95	15	80	41	1 95	8.5	94	40
b	150	6	144	47	3.06	6.0	91	—
	120	6	114	47	2.43	5.8	85	—
IV	120	30	110	39	2.83	6.1	93	19
b	140	26	114	39	2.93	5.9	96	23
c	150	6	144	44	3.27	6.2	98	32
d	105	11	94	44	2.14	6.2	97	34
V	95	4	91	132	0.69	6.6	89	68
b	110	3	105	134	0 78	5.6	88	71
	120	5	115	132	0.87	6.9	88	70
d	95	14	81	82	0.99	6.2	89	68
	110	15	95	77	1.23	6.3	90	69
f	80	15	65	73	0.89	6.3	91	71
g	80	1	79	110	0 72	6.6	95	73
VI	100	21	79	68	1.22	6.0	91	53
b	105	19	86	79	1 09	6.2	92	54
	73	1	61	93	0 66	6.2	89	53
VII a	123	10	123	106	1 16	4.3	90	39
b	123	17	116	82	1 41	4.8	96	55
	146	5	141	110	1.28	—	—	—
d	80	3	86	101	0.85	5.8	95	59
	124	4	120	112	1 07	4.7	91	—
f	124	17	107	82	1.30	4.8	94	35
g	102	16	86	88	0 98	5.2	90	5

Mean art. B.P = Mean arterial blood pressure.
Mean ven. B.P = Mean venous blood pressure
CBF_{grey} = Cerebral blood flow in grey matter
CVR = Cerebrovascular resistance.
CMR_{O_2} = Cerebral metabolic rate of oxygen.
Sa_{O_2} = Arterial oxygen saturation.
Pa_{CO_2} = Arterial carbon dioxide tension.

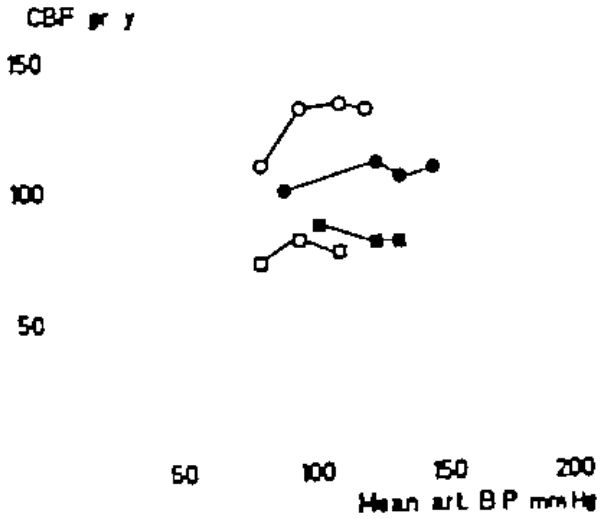


Fig. 1 Relation between mean arterial blood pressure and cerebral blood flow in grey matter (CBF_{grey} ml/100 gmin) i tx dogs (no. V open symbols; no. VII, filled symbols) at normal enous pressure (circles) and t increased enous pressure (squares) Aut regulation on d fferent flow levels is illustrated.

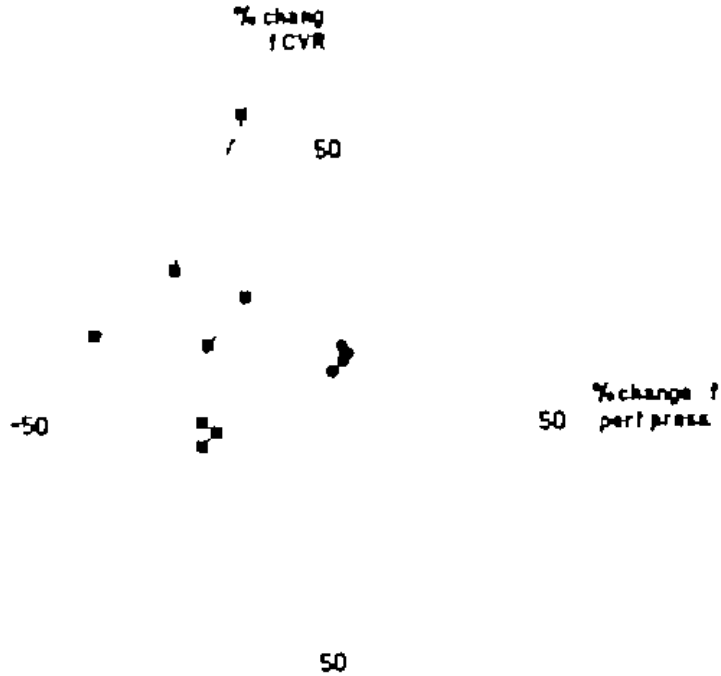


Fig. 2. Correlation between percentual hanges i the perfusion pressure and percentual changes in the cerebrovascular resistance (CVR) when the perfusion pressure was hanged by arterial bleeding (filled ircles) or by venous pressure increase (filled squares). With changes in the arterial blood pressure there are proportional changes in CVR, proving the existance of autoregulation. Lowering of the perfusion pressure by elevation of the enous pressure on the contrary leads to an unchanged or increased CVR. The autoregulatory response when the arterial blood pressure was lowered with the enous pressure maintained high, parallels the decline bserved with arterial bleeding at normal venous pressure. The values of CVR representing this response are connected by bi ken lines.

zero point for vascular resistance was then deduced from the connecting line between the blood flow measured during the autoregulation control measurements at arterial pressures higher and lower than the pressure in question. In animal no II autoregulation was only tested at lower arterial blood pressures and in no III only at higher but in both cases the arterial blood pressure during the venous pressure increase, 115 and 95 mm Hg respectively can be regarded as being within the so-called autoregulatory range With changes in the perfusion pressure brought about by changes in the arterial blood pressure a continuous, corresponding increase or decrease in the cerebrovascular resistance from the reference value can be observed as an expression of autoregulation. In one case (V) the decrease in the vascular resistance was broken, and even a slight increase was encountered, a finding probably explained by this value being obtained just below the pressure range of autoregulation

With elevation of the venous pressure there was a practically unchanged vascular resistance in some animals and an increase in others. Pronounced increases in the cerebral vascular resistance were found in two experiments where the cerebral blood flow was raised by hypercapnia In the cases where autoregulation was tested by bleeding with maintained high venous pressure the cerebral vascular resistance was observed to decline along a line practically parallel to the relation obtained when bleeding was performed with normal venous pressure

DISCUSSION

The present study was aimed at differentiating changes in the perfusion pressure from changes in the transmural pressure as possible stimuli to autoregulation of th cerebral blood flow With the skull and dura widely opened it was considered likely that the brain was provided with good possibilities for expansion so that the tissue pressure, i.e. the external pressure on the vessels, should not rise in proportion to the venous pressure increase. Therefore the rise in venous pressure can be expected to increase the transmural pressure. Relatively extensive surgery was required to achieve this, and since the autoregulation of the cerebral blood flow is known to be a vulnerable process which can be interfered with by for instance surgical trauma (Fog 1934 Rapela and Green 1964) a certain risk of failure had to be accepted In fact, three animals out of ten had to be excluded because they did not show a normal vascular response to arterial bleeding probably because of traumatic preparation. The method of increasing the venous pressure by obstructing the venous return to the heart was chosen, since it was considered the least traumatic way Also the flow method used the radioactive gas elimination method of Lassen *et al.* (1963) fulfills the requirement of being gentle to the tissue The value of 0.95 for the partition coefficient of Kr^{85} between cortex and blood was used throughout the experiments, although the value is known to be affected by the hematocrit (Lassen *et al.* 1963 Ingvar and Lassen 1961, Glass and Harper 1962), but a

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ACTA PHYSIOLOGICA SCANDINAVICA

SUPPLEMENTUM 349

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J HAGGENDAL, B. JOHANSSON J JONASON and B. LJUNG: Correlation between noradrenaline release and effector response to nerve stimulation in rat portal vein in vitro.

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INDEX

B Johansson B Ljung T Malmfors and L. Olson Prejunctional supersensitivity in the rat portal vein as related to its pattern of innervation 5

J Häggendal, B Johansson J Jonason and B Ljung Correlation between noradrenaline release and effector response to nerve stimulation in rat portal vein in vitro 17

B Ljung Nervous and myogenic mechanisms in the control of a vascular neuroeffector system 33

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Prejunctional Supersensitivity in the Rat Portal Vein as Related to its Pattern of Innervation

By

Börje Johansson, Bengt Ljung, Torbjörn Malmfors and Lars Olson

Abstract

Johansson B, B. Ljung, T. Malmfors and L. Olson. *Prejunctional supersensitivity in the rat portal vein as related to its pattern of innervation.* Acta physiol. scand. 1970. Suppl. 349. 5–16.

The smooth muscle of the portal-mesenteric vein of the rat is arranged in an inner circular layer (10 to 20μ thick) and an outer longitudinal layer (50 to 70μ thick). The latter is mainly found in the portal vein proper beginning a few mm caudal to the entrance of the gastro-splenic vein and extending to the liver hilus. Studies with the histochemical fluorescence technique of Falck and Hillarp showed a two-dimensional adrenergic network located between the two layers of muscle and some scattered adrenergic fibres at the mid entero-medial border. In view of this pattern of innervation it was of interest to investigate the degree of "supersensitivity" after denervation. Mechanical responses of isolated portal veins from control and ganglionectomized (coeliac and mesenteric) rats were recorded. Chronic denervation caused a parallel displacement of the noradrenaline (NA) dose-response curve to the left, corresponding to a change of ED_{50} from 1.2×10^{-6} M to 0.9×10^{-7} M. The ED_{50} values for innervated and denervated preparations treated with cocaine were 0.4×10^{-7} and 0.3×10^{-7} M respectively. The degree of the prejunctional supersensitivity to NA shows that the neuronal uptake effectively removes exogenous NA from the vicinity of the activated receptors, which therefore must be near the nerve endings. Consequently the smooth muscle cells which are lost to the nerves play a major role not only in the neural responses but also in the response to exogenous NA. Excitation then spreads by myogenic mechanisms through the muscle layers of the media.

Studies concerning the mechanisms behind the supersensitivity to sympathomimetic amines, which develops in smooth muscle after elimination of its adrenergic nerve supply, have contributed to the understanding of these neuroeffector systems. Many adrenergic agents, particularly the physiological transmitter noradrenaline

(NA) are taken up into the nerve terminals by an active membrane mechanism (see Iversen 1967). The concentration of NA at the effector cells is thereby reduced. After nerve degeneration or pharmacological blockade of the uptake an apparent increase in sensitivity to exogenous NA results, the prejunctional supersensitivity (*cf.* Trendelenburg 1966). This phenomenon has been studied most extensively in tissues like the nictitating membrane where the adrenergic innervation is dense and appears to reach most of the individual muscle cells (*cf.* Malmfors 1965). Chronic denervation of sufficient duration can also lead to non-specific postjunctional supersensitivity (Trendelenburg 1966).

The adrenergic nerve supply in vascular smooth muscle is in most cases arranged in a two-dimensional way so that only a limited number of the muscle cells is close to the nerve terminals and hence directly innervated (Norberg and Hamberger 1964, Ehinger, Falck and Sporrong 1967, Norberg 1967). Studies on supersensitivity after denervation in blood vessels have for the main part been performed on isolated strips of large elastic arteries and on perfused vessels of different types. The results have been quite variable, which may reflect differences in the functional organization of the neuroeffector systems of different sections of the vascular bed which are as yet not satisfactorily understood.

The isolated rat portal vein has been used in a series of recent studies aimed at an integrated understanding of the nervous control of propagating vascular smooth muscle (see Ljung 1970). In the present study morphological aspects on the adrenergic innervation, as revealed by the histochemical fluorescence method of Falck and Hillarp, have been correlated to the development of supersensitivity after denervation in the portal vein. The results obtained seem to elucidate certain principles in the neuroeffector organization of vascular smooth muscle.

A preliminary report of the present results was presented at the XIII Scandinavian Congress of Physiology (Johansson *et al.* 1969).

Methods

I. Operative procedures

Rats of the Sprague-Dawley strain of about 250 g body weight and of either sex were used. The animals were given atropine sulphate (1 mg/kg, s.c.) preoperatively and were anesthetized with ether. The abdomen was opened in the midline and under a dissection microscope the coeliac and mesenteric ganglia on both sides were removed together with the bundles of postganglionic fibres running towards the portal vein. The abdominal wall was sutured in layers. Sham operations were performed at the same time, involving only laparotomy and suture. All animals recovered satisfactorily.

II. Organ bath experiments

On the day of the experiment one sham operated and one ganglionectomized rat were killed by a blow on the neck. The portal veins were dissected out, cut open longitudinally and mounted for isometric recording in a 20 ml organ bath as described previously (e.g. Arnason *et al.* 1967).

The composition of the Krebs solution was in mM: NaCl 12.; KCl 4.73; $NaHCO_3$ 18.5; KH_2PO 1 19; $MgCl_2$ 1.19; $CaCl_2$ 2.49; glucose 11.5 and $CaNa_2$ ersenate 0 026. It was continuously bubbled with 4% CO in O_2. Temperature was kept at 38°C.

A pair of platinum electrodes, 5 mm apart, were placed in the bath on either side of each preparation. These were connected to a Grass stimulator for field stimulation with square wave impulses of 15 V amplitude between the electrodes and 0.8 msec duration. Control experiments showed that responses of isolated portal veins to such stimulation at 4 to 16 imp/sec were completely abolished by adrenergic blocking drugs (guanethidine, phenoxybenzamine). This indicates that the sympathetic nerves in the vascular wall but not the smooth muscle cells themselves, were stimulated (*cf* Paton 1955, Paterson 1965). Field stimulation was used in the present experiments as one indicator of the completeness of denervation.

The preparations were allowed to accomodate in the bath for 1 hr before the actual experiment started. Following the test with electrical field stimulation a dose-response curve to NA was determined by sequential administration of increasing concentrations of the amine in the range of 10 to 10^{-4} M. The exposure periods were of 3 min duration and the intervals between them 15 min or more, allowing complet recovery of the normal spontaneous activity In order to void β-adrenergic influence on the dose-response relationship β-receptor blockade was maintained throughout the experiments by giving propranolol, 10 M, for 5 min every 60 min. In one group of experiments the preparations were continuously exposed to cocaine, 10 M, during the determination of the NA dose-response curve.

The excitatory effects of graded concentrations of acetylcholine on denervated and innervated preparations were studied in experiments of similar design as with NA.

Contractile activity was recorded on a Grass polygraph and continuously quantified by means f an electronic integrating device. Excitatory drug responses were calculated as the difference between the mean tension development (dyn/min) during the 3 min exposure period and the preceding 3 min of spontaneous activity The data from each preparation were expressed as a per cent of its maximal response to the agent. Mean dose-response curves and mean ED_{50} values (concentration required for 50 per cent of maximal response) were calculated for the different sets of experiments. These calculations were performed by means of computer program which determined the least squares fit of all data points from each group for a hyperbolic dose-response function.

After each experiment the portal veins were mounted as stretch preparations on glass slides and allowed to dry for histochemical studies.

III Fluorescence Histochemistry

The histochemical fluorescence method of Falck and Hillarp (Falck, Hillarp, Thieme and Torp 1962, see also Corrodi and Jonsson 1967 Norberg 1967) was used for studies of the adrenergic innervation of the portal-mesenteric vein. In addition to the examination of the portal veins used in the organ bath studies, more detailed analysis was performed on preparations of the portal vein and the adjacent 10 mm of the anterior mesenteric vein, especially dissected for this purpose. Some vessels were opened longitudinally stretched and dried as whole mounts on glass slides (Malmfors 1965). These as well as those used for the functional studies were dried for more than 1 hr in desiccator containing P_2O_5. They were then treated with paraformal lehyde gas for 1 hr at 80 C. In some cases the portal-mesenteric vein was quenched at different degrees of stretch in propane cooled by liquid nitrogen, freeze-dried (Olson and Ungerstedt 1970), treated with formaldehyde gas, embedded and sectioned. The stretch preparations and the sections were examined in fluorescence microscope with a 400 mμ activation filter and 500 mμ secondary filter

We want xpress our sincere gratitude to professor Arne Brändström and mr Lars Palmer Alf Hassle, Goteborg, for applying their computer program to our data.

11 Drugs

The following drugs were used: l-noradrenaline bitartrate (Noradrenaline conc. Astra), Cocaine hydrochloride, acetylcholine bromide and propranolol hydrochloride. Fresh stock solutions, 10 M, were prepared on the day of the experiment. These were diluted with saline immediately prior to injection to the bath.

Results

I Fluorescence histochemistry

The media of the portal mesenteric vein is composed of an inner muscle layer in which the cells show predominantly circular orientation and an outer longitudinal layer. The circular muscle is 10–20μ thick and about equally well developed along the entire length of the vessel. The outher longitudinal muscle coat 50–70μ thick, is found mainly within a part of the vein extending from about 5 mm caudal to the entrance of the gastro-splenic vein to the liver hilus. The longitudinal muscle thus forms a relatively thick and well defined "cuff" surrounding the circular layer.

Transverse sections of the caudal part of the portal mesenteric preparation showed the fluorescent nerve fibres to be located at the outer border of the thin circular muscle (Fig. 1). In sections of the portal vein proper which included the longitudinal muscle fluorescent fibres were found between, but not within, the two muscle layers and further on the outside of the longitudinal layer (Fig. 2 and 3). The former system of fibres was a rarefied continuation of the terminal plexus surrounding the circular muscle in those parts of the vein where the longitudinal sheath was lacking and seemed in some tangential sections to be close to the circular muscle layer (Fig. 4). The nerve fibres on the outside of the longitudinal muscle were few and irregularly arranged; only a small fraction of them were of the varicose terminal type.

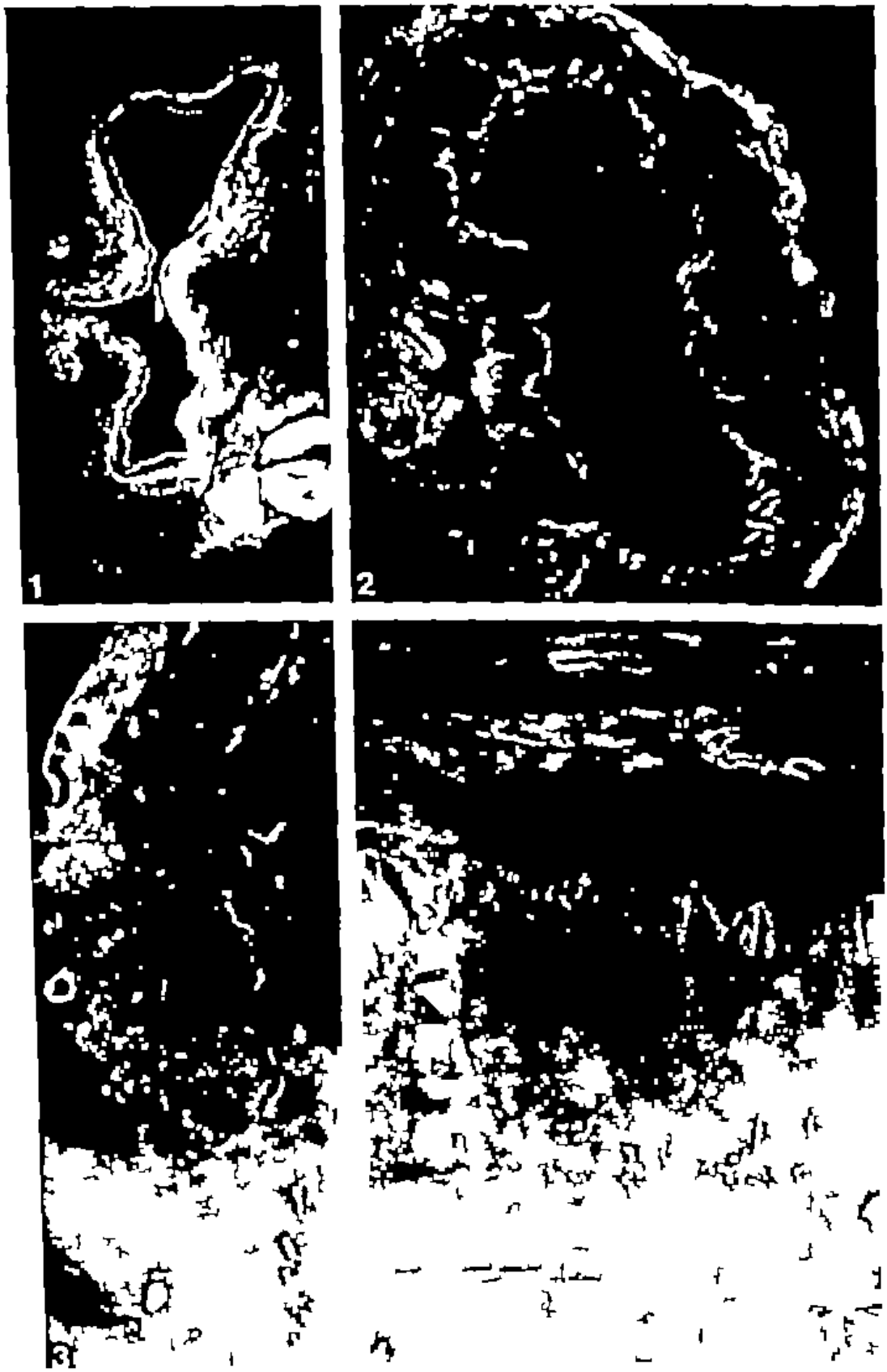
Stretch preparations of the portal mesenteric vein showed a system of adrenergic nerve fibres consisting of smooth non terminal axons with weak fluorescence and more intensely fluorescent varicose terminals. The adrenergic ground plexus was quite dense within the caudal part of the mesenteric vein and the nerve fibres had

Fig. 1. Transverse section (10μ) of the mesenteric vein. The thin media consisting of a circular muscle layer is limited towards the lumen by the supravitally fluorescent internal elastic membrane and towards the adventitia by the specifically fluorescent adrenergic nerves. Three bundles of non terminal adrenergic axons can be seen in the connective tissue surrounding the vessel. 80×

Fig. 2. Transverse section (10μ) of the portal vein. A thin inner circular muscle layer and thick outer longitudinal muscle layer can be seen. The fluorescent adrenergic nerves are located between these two muscle layers. On the surface of the longitudinal layer few fluorescent nerve fibres are detected. Nerve fibres penetrate into the muscle layer. 80×.

Fig. 3. Same as Fig. 2. The adrenergic nerve plexus is clearly seen between the two muscle layers. A few fluorescent mast cells can be seen in the adventitia (left). 120×.

Fig. 4. Tangential longitudinal section (10μ) of the portal vein. The longitudinal muscle layers can be seen on both sides of the tangentially sectioned circular layer. The nerves located between the muscle layers (see Fig. 3) have a tendency to accompany the circular layer. A few nerve fibres can be seen on the outside of the longitudinal layer. 120×.


1
2
3

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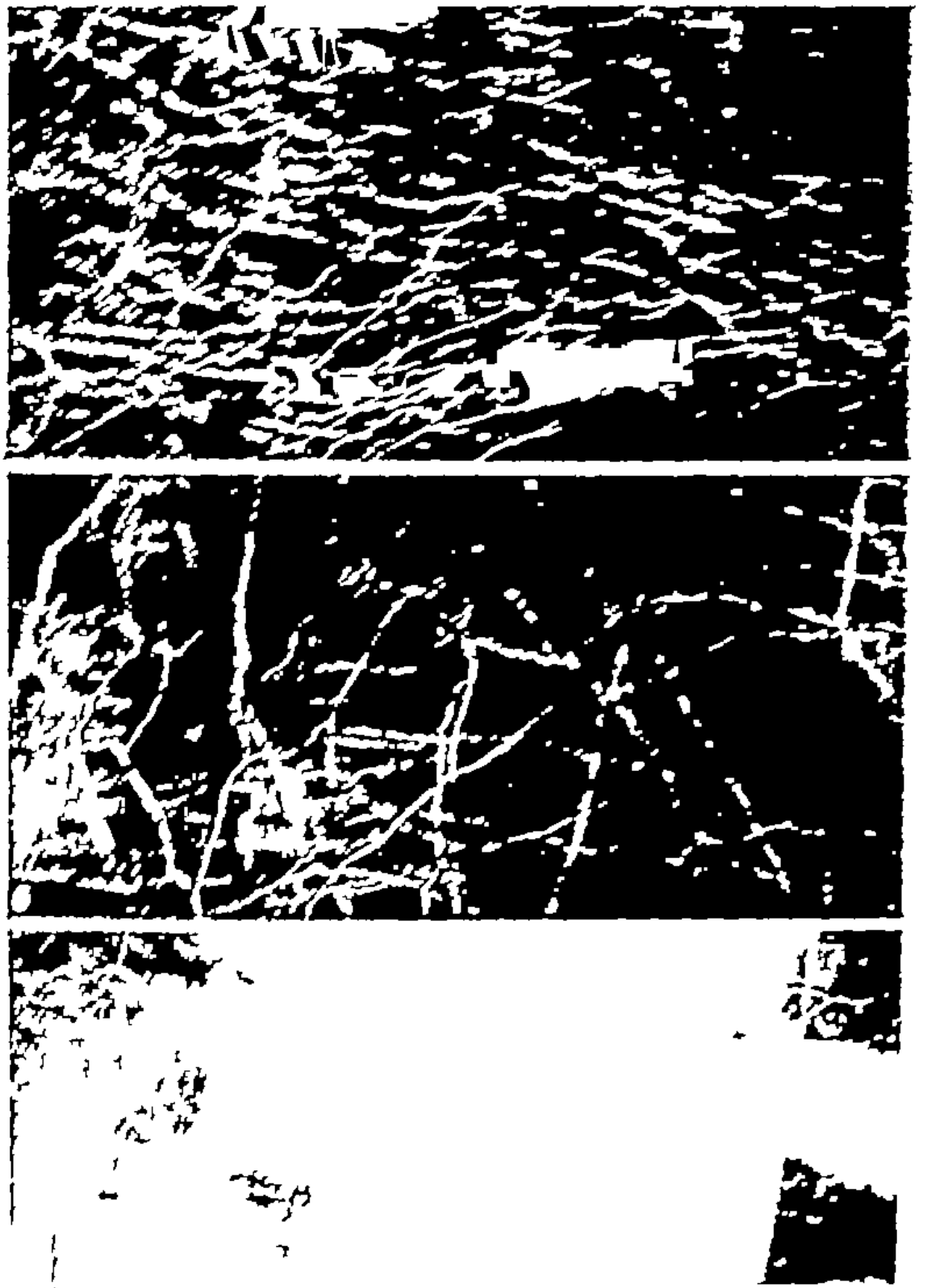
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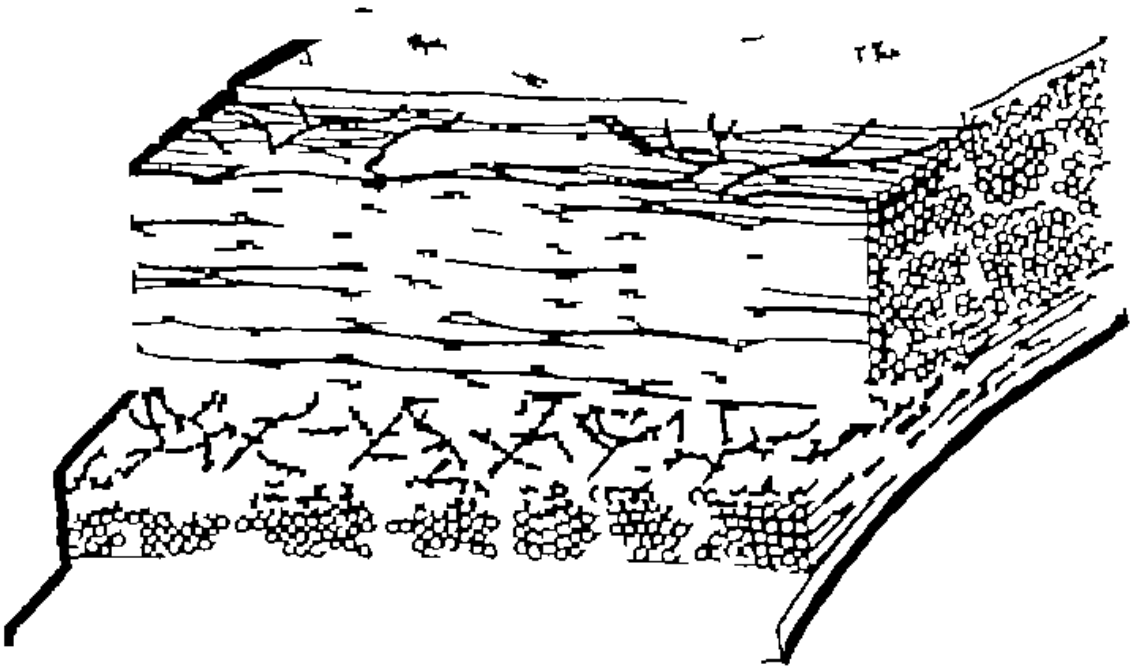


Fig. 8. Schematic drawing of the wall of the portal vein. Outer longitudinal muscle layer is 50–70μ thick, inner circular layer 10–20μ. Note main adrenergic plexus between the muscle layers and the scatt red nerves at adventitio-medial border

here a predominantly transverse orientation (Fig 5) In the region of the longitudinal muscle a more sparse and irregular adrenergic network was usually present (Fig. 6) which however again became somewhat denser near the liver (Fig 7) Mast cells and groups of small cells with intense green fluorescence were occasionally seen in the stretch preparations.

A schematic illustration of the morphological characteristics of the rat portal vein is shown in Fig 8

II Organ bath experiments

Portal vein preparations from sham operated and ganglionectomized rats were examined with regard to the effects of graded concentrations of [illegible] after varying time intervals following operation. Successful [illegible] indicated by the absence of response to field stimulation at [illegible]

Fig. 5. Stretch preparation of the mesenteric vein. A dense plexus of [illegible] can be seen mainly circularly orientated. Longitudinal direction [illegible] short side of the fig. 50 ×

Fig. 6. Stretch preparation of the portal mesenteric vein. The [illegible] close to the longitudinal layer On the surface of the longitudinal [illegible] non-terminal axons can be seen and few irregularly [illegible] parts of the longitudinal layer the more regular plexus [illegible] is seen Longitudinal direction of the vessel parallel to the [illegible]

Fig 7 Stretch preparation of the portal vein close to the liver [illegible] terminals associated with the circular muscle layer can be [illegible] axons are present Longitudinal direction of the [illegible]

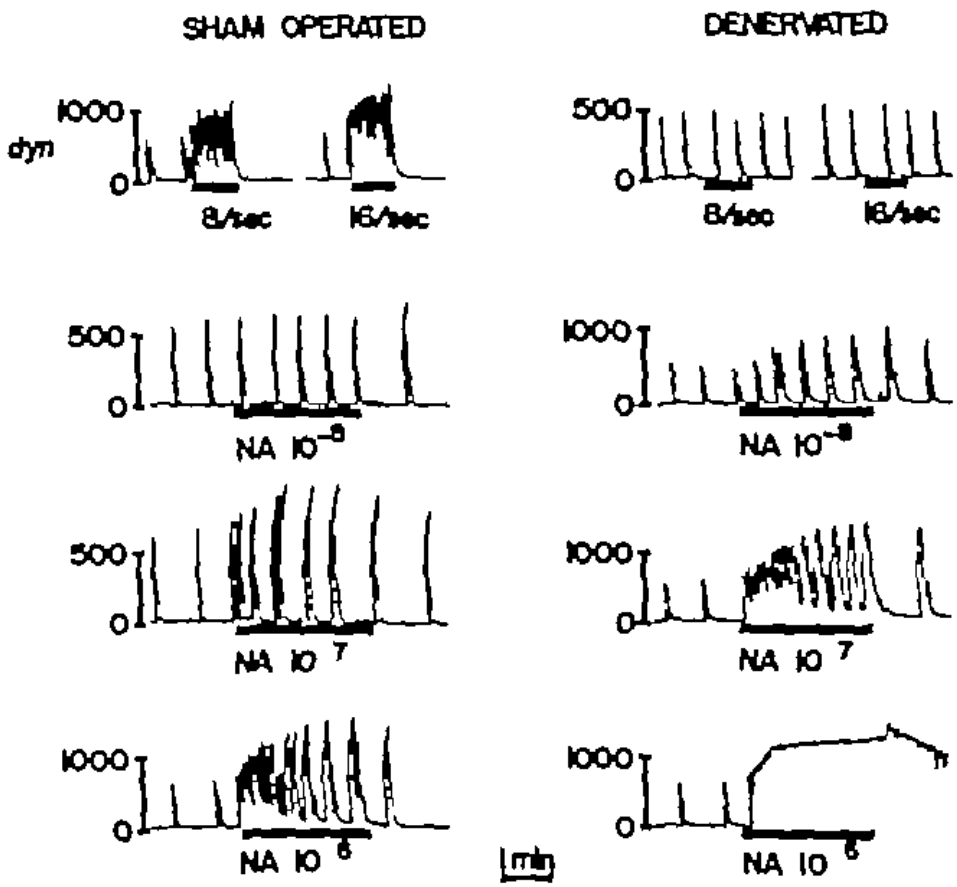


Fig. 9. Mechanical responses to electrical field stimulation (0.8 msec, 15 V) and to exogenous NA of the portal vein from one control (left) and one denervated preparation (right). Note absence of response to field stimulation and increased responses to NA after denervation.

Preparations from sham operated and non-operated animals did not differ in their responses to field stimulation or in histological appearance.

Fig 9 shows original recordings of mechanical responses to electrical stimulation and to three different NA concentrations in one denervated preparation and its sham operated control. The spontaneous myogenic activity typical of the portal vein is seen in both preparations during the control periods. Electrical field stimulation at 8 and 16 imp/sec elicited excitatory responses in the preparation from the sham operated but not in the one from the ganglionectomized animal. The denervated muscle showed a greater responsiveness to NA at all three levels illustrated.

The mean NA dose-response curves obtained in the present series of experiments are illustrated in Fig 10 The curve for six denervated vessels, examined 3 to 17 days after operation, is displaced to the left of that for the control group which consisted of 11 preparations from sham operated and four preparations from non operated rats (the two latter subgroups did not differ significantly from one another) In order to quantitate differences in NA responsiveness, values for ED_{50} were calculated by means of the computer program referred to under "Methods The

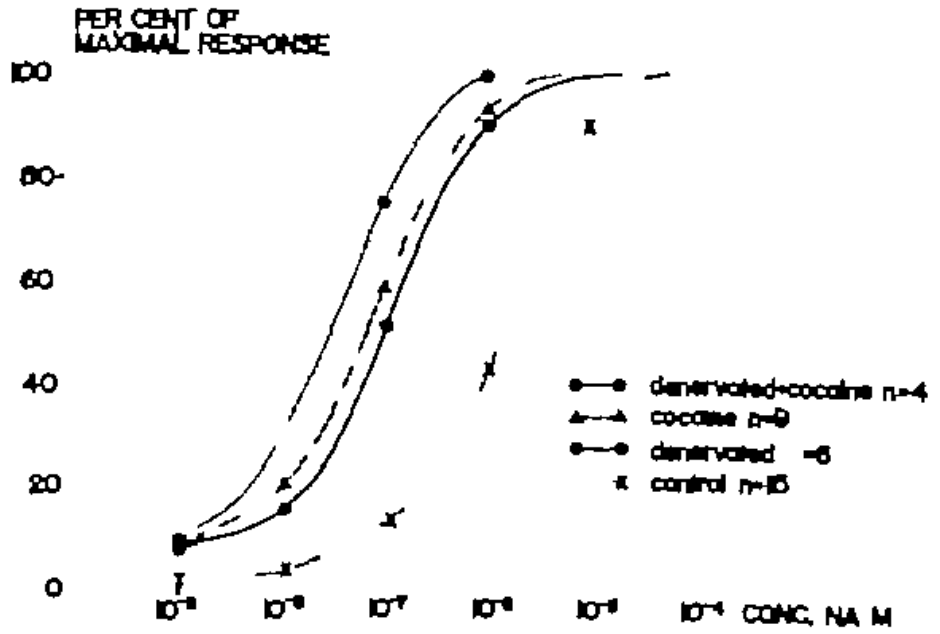


Fig. 10. Excitatory responses of the rat portal vein to NA under various experimental conditions, expressed in per cent of maximal response. Continuous β-receptor blockade. Least squares fit for a hyperbolic dose-response function calculated in each experiment (see "Methods"). The points in the figure represent mean values from number of experiments indicated.

ED_{50} for the control preparations was $(1.2 \pm 0.03) \times 10^{-}$ M (mean ± S.E.M.) and the corresponding figures for the denervated ones $(0.9 \pm 0.04) \times 10^{-}$ M. To give comparable responses normally innervated portal veins thus required 13 times higher NA concentrations in the bath than did the denervated muscles.

The individual values for ED_{50} in the denervated group indicated that the supersensitivity was already fully developed on the third postoperative day and no evidence was obtained of a further increase in responsiveness up to the 17th day. Some muscles from ganglionectomized animals were studied after longer intervals (20 to 35 days) but these preparations responded to field stimulation and showed fluorescent nerve terminals when examined microscopically, suggesting that reinnervation from remaining ganglion cells may have occurred.

The early onset of supersensitivity following denervation indicates that it is prejunctional in nature i.e. due to elimination of the neuronal NA uptake (see Trendelenburg 1966). To further elucidate this question, experiments were performed with cocaine which is considered to block the membrane uptake mechanism (for ref. see Iversen 1967). The mean NA dose-response curve of nine innervated portal veins treated with cocaine (10^{-6} M) is shown in Fig. 10. The ED_{50} of this group was $(0.4 \pm 0.02) \times 10^{-7}$ M. Fig. 10 also shows the mean dose-response curve of four denervated preparations treated with cocaine. ED_{50} for which was $(0.3 \pm 0.02) \times$ $\times 10$ M.

The difference between the mean ED_{50} of the control group and that of any of the three other groups is highly significant ($p < 0.001$). Cocaine treatment apparently results in a somewhat greater degree of supersensitivity than denervation as judged

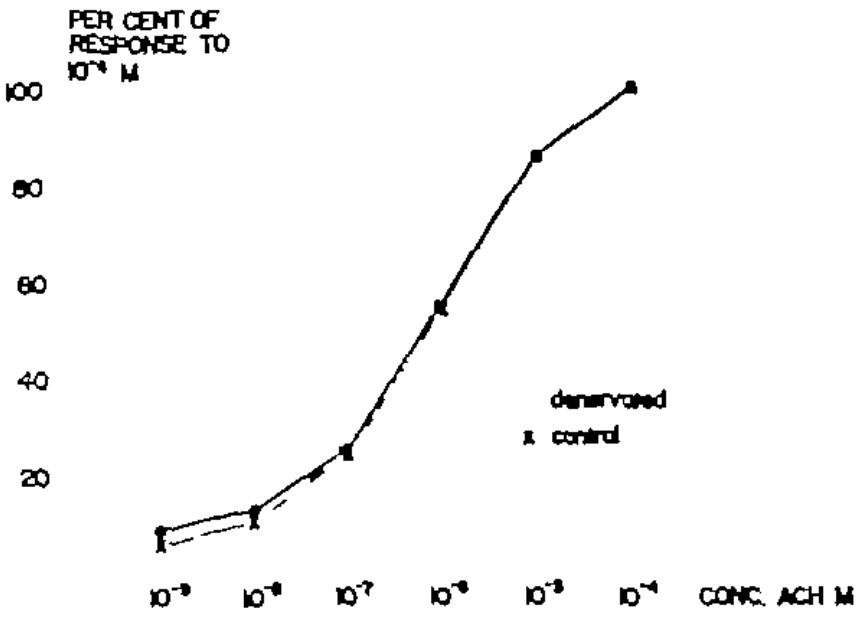


Fig 11 Excitatory responses to acetylcholine of the rat portal vein after denervation (●——●) as compared to control (× – – – ×). The points represent mean values from four experiments.

by the difference in ED_{50} ($0.05 > p > 0.01$) and the combination of the two procedures may accentuate the increased responsiveness in comparison with cocaine alone ($0.05 > p > 0.01$)

To investigate whether denervation of the portal vein also results in supersensitivity to non-adrenergic agents a series of experiments was performed with acetylcholine This substance produces excitatory responses of the portal vein which are blocked by atropine Acetylcholine dose-response curves for four preparations from sham operated and four preparations from ganglionectomized rats were obtained 8 to 20 days after operation. The results, illustrated in Fig 11 did not indicate any difference in responsiveness between the two groups.

Discussion

Chronic denervation of the rat portal vein caused a 13-fold increase in the sensitivity to exogenous NA in the present study Evidence that this supersensitivity was of the prejunctional type (i e due to elimination of the neuronal uptake mechanism) was given by the observation that it was fully developed already three days after the operation at which time the degeneration of nerve terminals is completed (Malmfors and Sachs 1965) and that cocaine treatment of normally innervated preparations caused a comparable or even greater supersensitivity The fact that cocaine caused a further shift of the NA dose-response curves to the left than did chronic denervation alone may suggest that cocaine also exerted some postjunctional

effect (Maxwell, Wastila and Eckhardt 1966 Bevan and Verity 1967 Kalsner and Nickerson 1969) No indication of development of unspecific postjunctional super sensitivity was obtained as no further increase in NA sensitivity was detected after the third postoperative day and as th responsiveness to acetylcholine was not altered after denervation. This is possibly due to the fact that the isolation procedure for *in vitro* studies may abolish the labile postjunctional supersensitivity (see Green and Fleming 1968)

Elimination of the neuronal uptake mechanism has been found to cause a more than 20-fold increase in sensitivity to NA in non vascular smooth muscle such as the nictitating membrane (Trendelenburg 1965) In most previous studies on blood vessels little or no supersensitivity has been obtained after denervation (see further below)

It is reasonable to seek the explanation for such differences in the extent of prejunctional supersensitivity in the organization of the sympathetic innervation The possible implications of the confinement of the adrenergic nerve terminals to a two-dimensional plexus in the arterial walls, as opposed to the dense three-dimensional distribution within the smooth muscle of the nictitating membrane have been discussed in a recent study by Haeusler and Haefely (1970). They concluded that these morphological differences in sympathetic nerve supply could explain their findings that neuronal uptake blockade produced a 15-fold increase in sensitivity of the nictitating membrane compared to only a 3-fold increase for an isolated mesenteric artery preparation. Further the distance between the nerve terminals and the nearest smooth muscle cells has been considered to be of importance for the degree of prejunctional supersensitivity This factor was discussed by Bevan and Verity (1967) as an explanation for the absence of denervation supersensitivity in isolated strips of elastic arteries where the neuro-muscular gaps exceed 4000 Å. This figure is smaller (about 1000 Å) in the peripheral vessels (see Lever Graham and Spriggs 1967) but it still exceeds values reported for densely innervated organs such as the vas deferens (Merrillees, Burnstock and Holman 1963)

The present data on supersensitivity in the portal vein indicate that th neuronal uptake mechanism in a control preparation so reduces the average NA concentration at the receptor sites, that the exogenous NA concentration in the medium has to be increased at least 13 times to compensate for the activity of th membrane pump.

The present histochemical analysis showed that the adrenergic nerve terminals are arranged in two-dimensional plexuses also in this vessel. Most of the cells in the longitudinal muscle layer which is 50 to 70μ thick, are thus widely separated from the nervous elements and it seems impossible that the NA concentration in their surrounding fluid should be directly influenced by the neuronal uptake It should be mentioned however that isolated perfused blood vessels have been found to be less sensitive to NA applied from the outside than to NA administered inside the lumen (Gillespie 1966, de la Lande Frewin and Waterson 1966, Crotty *et al.* 1969). De la Land Frewin and Waterson (1967) working with the central ear artery of

the rabbit, ascribed this difference to uptake of NA into the adrenergic plexus of nerve terminals at the adventitio-medial border On the other hand Crotty *et al.* (1969) found that this mechanism could not explain the differences in the sensitivity of the lateral saphenous vein of the dog to external and internal administration of NA. The existence of neural elements on both sides of the longitudinal muscle layer in the portal vein may seem to offer a possibility for a reduction of the NA concentration in the biophase below the bath concentration by a mechanism of the kind proposed by de la Lande and co-workers. Quantitative considerations of the rate of NA diffusion through vascular walls using the diffusion coefficient obtained by Török and Bevan (see Bevan Cheaher and Su 1969) and of the uptake capacity of adrenergic neurones (Trendelenburg, Draskóczy and Pluchino 1969) respectively show that the latter factor is *not* capable of reducing the mean tissue concentration in the portal vein sufficiently to explain the degree of supersensitivity This is also unlikely simply because of the scarcity of the outer nervous network (Fig. 2–4). Reduction in NA concentration corresponding to the degree of prejunctional supersensitivity can prevail only in the immediate vicinity of the adrenergic terminals *i.e.* at the very nearest layer of smooth muscle cells. Holman *et al* (1968) found that the terminal nerve structures were separated from the closest muscle cells by a distance of the order of 1500 Å in the rabbit portal vein.

The marked prejunctional supersensitivity obtained after denervation in the present study then seems to imply that the muscle cells which lie closest to the nerve terminals have a key function in eliciting the response also when the tissue is exposed to *exogenous* NA. These cells may function as pacemakers, they may have a more abundant supply of adrenergic a receptors or their receptors may have a greater affinity to NA. Once activity has been induced in these "key cells it can spread to the remainder of the media, so that forceful contractions ensue, due to the propagating ability of this vascular muscle (Johansson and Ljung 1967 1968, Ljung and Stage 1970) In view of the pattern of innervation such key cells controlling the longitudinal muscle layer may be located at its adventitial border in the innermost cell layers of this sheath or even in the circular muscle. In the latter case the longitudinal muscle would be activated by myogenic spread of excitation from the circular layer which in fact seems to receive the densest innervation.

In conclusion, the longitudinal smooth muscle of the portal vein develops a considerable prejunctional supersensitivity to NA despite the fact that nervous elements are found only at the borders of the muscle layer This pattern of innervation which characterizes most blood vessels has been held responsible for the low degrees of supersensitivity observed in arteries. Our results indicate that the *a*-adrenergic receptors which determine the response to exogenous NA are located *mainly* on the "innervated effector cells of the portal vein and from these cells the excitation spreads to the rest of the muscle by myogenic mechanisms. This situation may also prevail and give rise to high degrees of prejunctional supersensitivity in peripheral vessels with sparsely innervated but propagating smooth muscle

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Correlation between Noradrenaline Release and Effector Response to Nerve Stimulation in Rat Portal Vein in Vitro

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Abstract

HÄGGENDAL, J B JOHANSSON J JONASON and B LJUNG *Correlation between noradrenaline release and effector responses to nerve stimulation in the rat portal vein in vitro* Acta physiol scand. 1970 Suppl 340 17–3..

The purpose of the present study was to quantitate the release of adrenergic transmitter during sympathetic vasoconstrictor fibre stimulation as function of impulse frequency and to correlate this release to the magnitude of the contractile responses of the vascular smooth muscle. All experiments were performed *in vitro* using the neuroeffector system of the isolated rat portal vein. The preparations were first incubated in radioactive noradrenaline (³H NA) and during this 30 min uptake period the tracer equilibrated with an intraneuronal pool of NA corresponding to about 12 per cent of the total endogenous store, which was found to be approximately 2.2 μg NA/g tissue. In the subsequent phase of the experiments the tissue was superfused with Krebs solution, contractile activity was continuously recorded and the effluent from the muscle chamber was collected and analysed by liquid scintillation counting. In some experiments ³H NA and its main metabolites were separated and determined. The slow spontaneous release of radioactivity appearing in the absence of nerve stimulation consisted mainly of metabolites of ³H NA. The increased release obtained during electrical stimulation consisted almost exclusively of ³H NA itself The amount of ³H NA, released per impulse, was calculated as a fraction of the actual total content of radioactivity in the tissue At 4 imp/sec this fraction was 9.9×10 This could be estimated to correspond to total transmitter release per impulse of about 10 of the entire endogenous NA store. The fractional release of ³H NA per impulse seemed t be somewhat lower at 8 and 16 imp/sec and was significantly reduced at 32 imp/sec (3.9×10) The relat ve values obtained for total transmitt release at different frequencies and for the corresponding contractile re sponses could be summarized as release-response curv It indicated that the maximal con tractile response f the portal vein as determined in the first hand by postjunctional mechanisms and not by failure of the nerves to increase transmitter release at high frequencies.

For all parts of the vascular syst m the curves, relating th impulse rate to the effector response during vasomotor nerve activity are steep at low frequencies (1–8 imp/sec) Furthermore maximal constri tion is generally obtained at impulse

rates between 10 and 20 imp/sec (*e g* Folkow 1955[a] Celander 1954 Mellander 1960) As the mean impulse frequency in the vasomotor fibres under physiological conditions rarely seems to exceed 8–10 imp/sec (Folkow 1955[a]) the full range of nervous control of the blood vessels can be exerted by adjustments of the discharge rate within the steep part of the frequency response curve. A detailed understanding of the frequency response relationships in vascular neuroeffector systems requires knowledge of quantitative data for transmitter release from the vasomotor nerves at different impulse rates and the correlation between these amounts and the contraction of the vascular smooth muscle

Quantitative studies of vascular response and transmitter release under *in vivo* conditions are complicated by numerous factors. The vasoconstrictor response recorded as a change in pressure flow or volume can be affected by local metabolic influences and by geometrical factors involving differences in the wall to lumen ratio of the vessels. Furthermore, the amount of transmitter appearing in the venous blood varies with the flow rate which alters the conditions for diffusion (see *e.g* Rosell Kopin and Axelrod 1963 Carlsson Folkow and Häggendal 1964) *In vitro* preparations make it possible to study directly the contractile response of the vascular smooth muscle with well standardized conditions of diffusion However the amounts of endogenous noradrenaline (NA) released by nerve activity from a small strip of vessel are difficult to quantitate biochemically This problem of transmitter determination may be circumvented by studying the release of radioactive NA and this technique also permits analysis of ^{3}H metabolites of NA (for ref see *e.g* Jonason 1969 c) An important question is however to what extent the quantitative release of ^{3}H NA reflects the magnitude of the release of endogenous transmitter

The aim of the present study was to investigate the NA release in isolated preparations of the rat portal vein as a function of impulse frequency during electrical nerve stimulation and to correlate the amounts of released transmitter to the effector responses. The mechanical activity of the longitudinal muscle layer of the portal vein can directly be recorded (Axelsson *et al* 1967) and the intramural nerves of the preparation can be activated by electrical field stimulation (Johansson *et al* 1970) The rat portal vein is only about 100μ thick (Johansson *et al* 1970) and thus offers good possibilities for diffusion of liberated substances. In the present study the tissue was preincubated in ^{3}H NA before it was placed in a superfusion system which allowed recording of mechanical activity and continuous collection of released ^{3}H NA and its metabolites (*cf* Su and Bevan 1967 Hughes and Roth 1960)

Methods

I Procedures used for studying contractile responses and release of total radioactivity during field stimulation

Portal vein preparations from rats of the Sprague-Dawley strain, weighing 250–350 g and of either sex, were used in this part of the study The animals were killed by a blow on the neck.

The portal vein was isolated, cut open longitudinally and mounted in an organ bath for isometric recording of the mechanical activity as described previously (Arrhenius et al. 1967). A modified Krebs solution was used in all experiments. It had the following composition in mM: NaCl 123; KCl 4.73, $NaHCO_3$ 15.5; KH_2PO_4 1.19; $MgCl_2$ 1.19; $CaCl_2$ 2.49; glucose 11.5 and ascorbic acid 1.13 and was continuously bubbled with 4% CO_2 in O_2 at 38°C. After an accomodation period of about one hour dl-^3H-NA (specific activity 7.45 C/mmole; Radiochemical Centre, Amersham, England) was administered to the bath giving a NA concentration of 10^{-7} M. After 30 min incubation this organ bath was lowered, the muscle was rinsed for about 1 sec in non radioactive medium and a 1.5 ml bath was put in position. This small muscle chamber was continuously perfused at constant rate (approximately 1.3 ml/min) by pre-warmed and bubbled Krebs solution. Mixing of the fluid was obtained by bubbling the gas also through an inlet in the bottom of the chamber. The muscle chamber was supplied with a pair of platinum electrodes at a distance of 5 mm which allowed electrical field stimulation of the intramural adrenergic nerves as described elsewhere (Johansson et al. 1970).

The outflow from the perfusion system was continuously collected throughout the experiment according to the following time schedule. Sampling periods of 5 min duration were used in the initial 30 min of the wash-out. Following this, 1 min samples were taken for the first min before until the fifth min after each stimulation period which itself lasted for 2 min. The wash-out was then collected over two 5 min periods before the next cycle began (cf. Fig. 2 below).

The contractile activity of the portal vein was recorded continuously throughout the experiment. An integrating device was used to quantitate the force development during spontaneous activity and in response to nerve stimulation (see Fig. 1).

At the end of the experiment the muscle preparation was blotted between two layers of filter paper and weighed on a Cahn electrobalance. The tissue was homogenized in 1.5 ml of 0.4 N perchloric acid (PCA). Krebs and the extract was centrifuged for 20 min at 2000 rpm. For liquid scintillation counting 1.1 ml of the supernatant was taken. Further 0.1 ml of the ^3H-NA incubation medium and 1.0 ml of each wash-out sample, respectively, were diluted with PCA in Krebs to a final volume of 1.1 ml and a PCA concentration of 0.4 N.

The samples were counted in a liquid scintillator spectrometer after addition of 17 ml of scintillation fluid made up by 1 part methanol and 16 parts of a solution containing 120 g naphtaline; 7 g PPO (2,5-diphenoxyloxazole); 0.05 g POPOP (1,4-bis-2-(5-phenyloxazolyl)-benzene) in 1000 ml of dioxane. Quenching was corrected for by the use of internal or external standards.

II. Determination of radioactive NA and its metabolites

Some modifications of the wash-out procedure were introduced in experiments aiming at a differentiation between NA and its major metabolites. Three portal veins were mounted on the same muscle holder. One of them was connected to the transducer for recording of mechanical activity and the other two were merely stretched to their approximate in vivo length. During the 30 min uptake period the muscles were incubated in dl-^3H-NA as above or in l-^3H-NA (10^{-7} M with a specific activity of 2.34 C/mmole; Radiochemical Centre, Amersham, England). In the subsequent phase of the experiment, stimulation periods of 5 min duration were used and the perfusion fluid was collected over periods of 5 to 10 min in flasks containing 2 ml of 2 N HCl.

The samples were divided into two aliquots, one for determination of NA, deaminated metabolites and deaminated 3-O-methylated metabolites, and the other for the determination of normetanephrine (NM). Carrier substances were added (see Rutledge and Jonason 1967) and the samples were filtered and stored at −20°C.

The procedures used in this part of the study have been described in detail in previous publications (Rutledge and Jonason 1967, Jonason and Rutledge 1968) and will be presented here only in general terms. NA was adsorbed out and then eluted from Dowex-50 columns, by

acid and neutral metabolites passed through in the effluent The Dowex effluents were then passed over lumina columns and the effluents from these columns, containing the deaminated, 3-O-methylated metabolites were collected. Those products which were only deaminated were then eluted from the alumina columns by 0 2 N HCl. The metabolites from the acidified alumina effluents and eluates were extracted with ether the volumes were reduced under vacuum, scintillation fluid was added and radioactivity determined in a liquid scintillation counter

For determination of NM the neutralized samples were first passed over alumina where NA was adsorbed The alumina effluents, containing NM were then passed over a Dowex-50 column and eluted from the columns by N HCl. The amine eluates (NA and NM) were freeze-dried and the radioactivity was measured by liquid scintillation. Recoveries of the amines were determined in each sample by quantitating fluorimetrically the amount of carrier substance remaining in the eluate after the separation procedures. The values of the amines were calculated by correcting for efficiency recovery aliquot factors and the specific activity of the precursor The values of acid and neutral metabolites were calculated in the same manner except that no recovery values were assessed in these experiments.

III Determination of endogenous NA in the portal and mesenteric vein

Three experiments were performed in order to determine the total content of endogenous NA in the portal and superior mesenteric veins. Pooled preparations from 20 rats were used in each of these three experiments. The portal vein from the entrance of the gastro-splenic vein to the bifurcation at the hepatic hilus was dissected, cut open longitudinally rinsed in normal Krebs solution, blotted on filter paper and weighed on the electrobalance. A corresponding length of the adjacent superior mesenteric vein was treated similarly The tissues were homogenized in 10 ml ice-cooled 0.4 N PCA containing 20 mg ethylenediamine tetra acetate (EDTA) and 10 mg ascorbic acid. After the samples had passed columns of a strong cation exchange resin the NA was estimated fluorimetrically according to the modified trihydroxyindole reaction (Haggendal 1963). The recovery throughout the estimation procedures was determined by adding 100 pg NA to control samples. The recovery value, being about 70 per cent, was used for correction.

Results

I Endogenous NA levels

The endogenous NA levels in the portal vein were found to be 2.2 ± 0.24 μg/g wet weight (mean $\pm$ S.E.M.) and in the superior mesenteric vein 5.7 ± 0.13 μg/g

II Contractile responses and release of total radioactivity during field stimulation

The primary data obtained in this part of the study are exemplified in Fig. 1 The middle tracing illustrates the original recording of mechanical activity which demonstrates the typical spontaneous activity of the rat portal vein and the excitatory contractile responses to electrical field stimulation (0.8 msec; 15 V) at 4 and 16 imp/sec. The continuous integration of the mechanogram (upper recording in Fig 1) represents the accumulated area (dyn $\times$ min) under the curve of active force between each resetting As the integrator resets every 60 sec the tracing directly gives the mean force developed during each minute However as apparent in the stimulation periods, resetting also occurred when the integrator output reached full scale, which

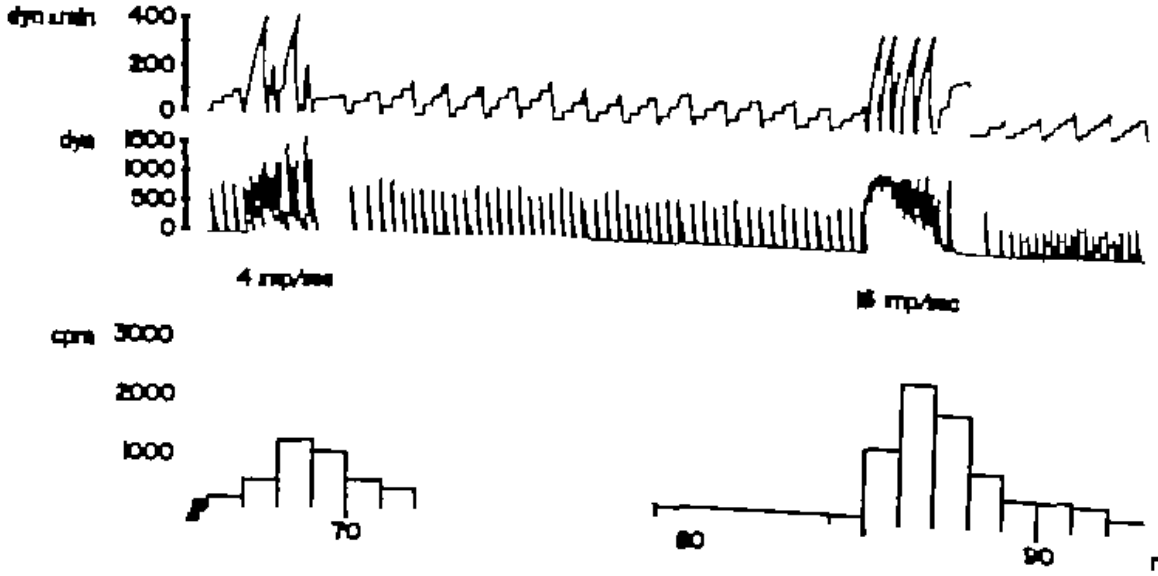


Fig 1. Mechanical activity of the portal vein, directly recorded (middle panel) and integrated (upper panel). Columns in lower panel indicate number of cpm and time for collection of each perfusate sample. Note increase in contractile activity and in output of radioactive tracer in response to electrical field stimulation (signal).

means that the average force then equals the sum of the deflections per min. The net mechanical response during nerve activity was calculated as the mean force developed during the stimulation period minus the mean force during the preceding two minutes of spontaneous activity. The absolute values of the neurogenic responses varied markedly between preparations. Therefore their magnitudes were in each experiment expressed in per cent of the initial response to 4 imp/sec, the frequency always used in the first stimulation period.

The lower part of Fig. 1 illustrates the total counts per minute (cpm) appearing in the perfusate collected over respective sampling periods. A low amount of radioactivity was present in the wash-out from the control state. During nerve activity this amount markedly rose to reach a maximum during the second minute and upon cessation of stimulation it returned to control values in 3–5 min.

The time course of the elimination of tracer from the tissue during the experiment could be reconstructed in detail by adding in the reverse order the total radioactivity of the different fluid samples to the number of cpm obtained in the ultimate muscle extract. Fig. 2 in this way illustrates the results of an experiment in which the effects of different impulse frequencies were studied.

A rapid fall in tissue radioactivity is found in the beginning of the wash-out period, most probably representing elimination of tracer from the extracellular fluid and perhaps, from other extraneuronal compartments. The subsequent slow phase has been interpreted to represent disappearance of tracer which, during the uptake period, has been incorporated into the intraneuronal "pool" of endogenous NA. A zero time value for this slow component was obtained by extrapolation, assuming an exponential decay (dotted line in Fig. 2). This figure, the weight of the tissue

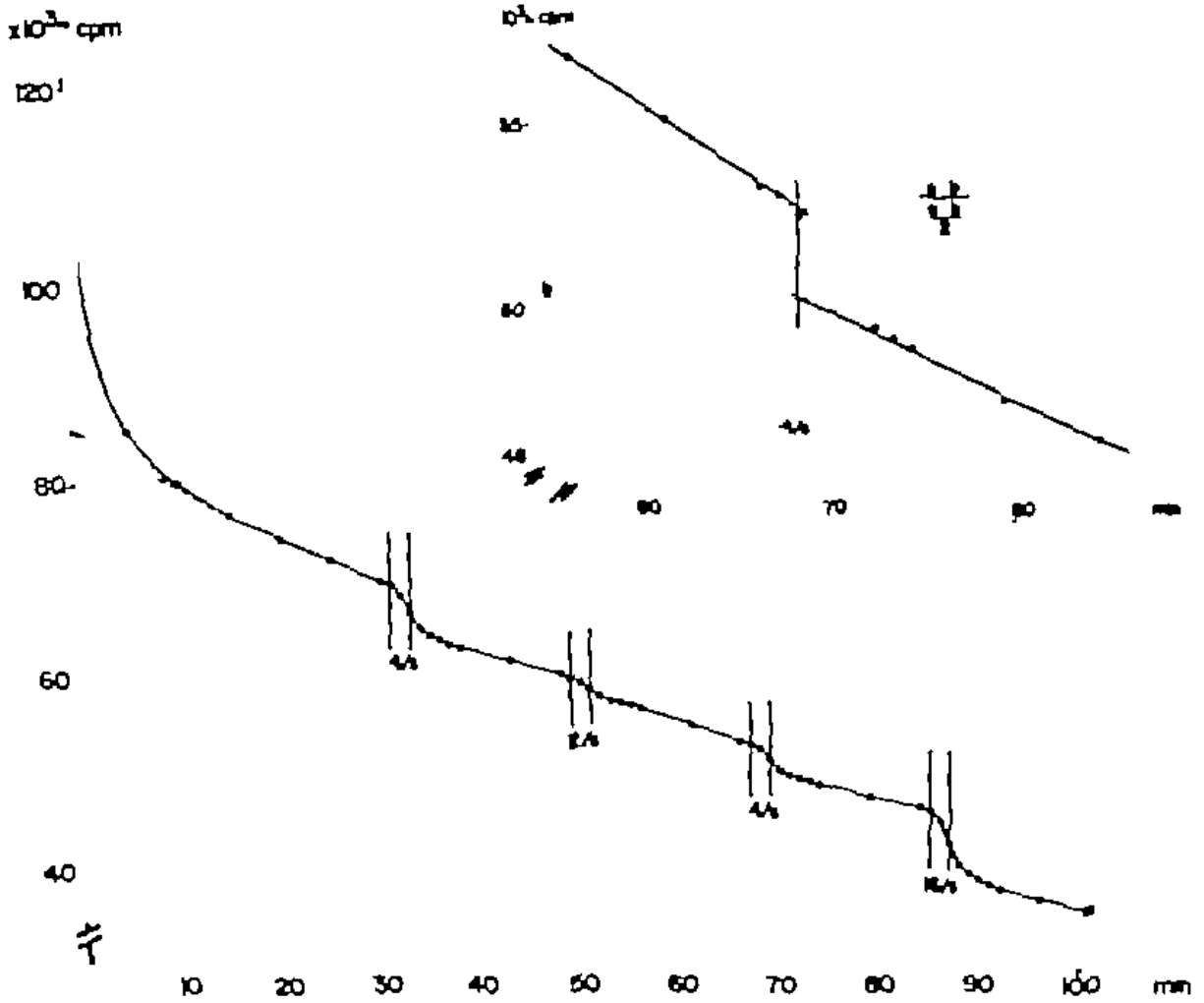


Fig. 2 Time course of the elimination of tracer from portal vein. The curve was constructed by adding, in reverse order the radioactivity of the perfusat samples t the cpm values for the ultimate muscle extract (open square symbol at the ight end) Release of tritiated transmitter in response t nerve stimulation was quantified as fraction (K) of the mean tissue content prevailing at the respective stimulation period. The magnified section of the curve illustrates how this calculation was done. For further details see text.

and the specific activity of the incubation medium made it possible to calculate the amount of labelled NA in relation to the endogenous NA store. In the experiment of Fig the zero time intercept was 85,200 cpm the muscle weight was 4.37 mg and the NA concentration of the medium 15.2 ng/ml giving 2,2*4 000 cpm/nl. This would correspond to a NA pool of 133 ng/g tissue. Since the determinations of total endogenous NA in portal veins indicated a content of 2.2 μg/g the specific activity of the NA n the tissue store in the particular case was only some 6 per cent of that of the incubation medium This figure is one of the lowest obtained the mean value for the whole group of 36 experiments was 1..3 per cent (±0.8 S.E.M.)

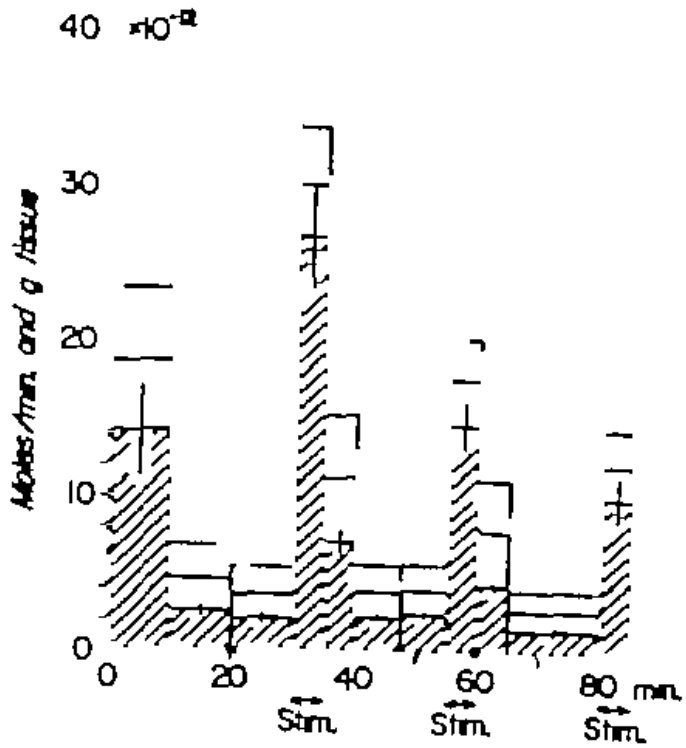


Fig. 3. Distribution pattern of radioactive *l*-NA and its metabolites in the perfusate. Filled bars represent labelled *l* NA; open bars represent the sum of *l* metabolites (calculated from *Fig. 4*) Experimental values of *l*-NA and NM corrected for recovery Within dotted lines, the calculated loss of acid and neutral metabolites through the separation procedures is given Mean values of two experiments (±S.E.M.) shown for noradrenaline. Stimulation at 4 imp/sec indicated.

Returning to the elimination curve of Fig * it can be seen that nerve stimulation was associated with an accelerated decrease of tissue radioactivity corresponding to the higher cpm values of the fluid samples obtained during and immediately after the stimulation period (*cf* Fig 1) This accelerated fall of tissue radioactivity is taken to represent transmitter release elicited by the nerve impulses, an interpretation which is supported by the results presented in section III We have chosen to quantitate this release in proportion to the actual total tracer content of the muscle (see further below) The procedure used in curve analysis and calculation is exemplified by the magnified section of the plot in Fig 2 The spontaneous wash-out rates preceding and following the nerve activity were extrapolated to the middle of the 2 min stimulation period (levels *a* and *b*) The number of cpm released by the train of impulses is then *a* minus *b* and the mean tissue content of radioactivity may be represented by the arithmetic mean of a and b The tracer release as a fraction of the total content is then given by *K* in Fig 2 Dividing by the total number of impulses during the stimulation period gives the fractional release per impulse

III Levels of labelled NA *and its metabolites in the perfusates*

The results presented in this section were obtained with labelled *l* NA. One control experiment with labelled *dl* NA showed the same general pattern The quantitative

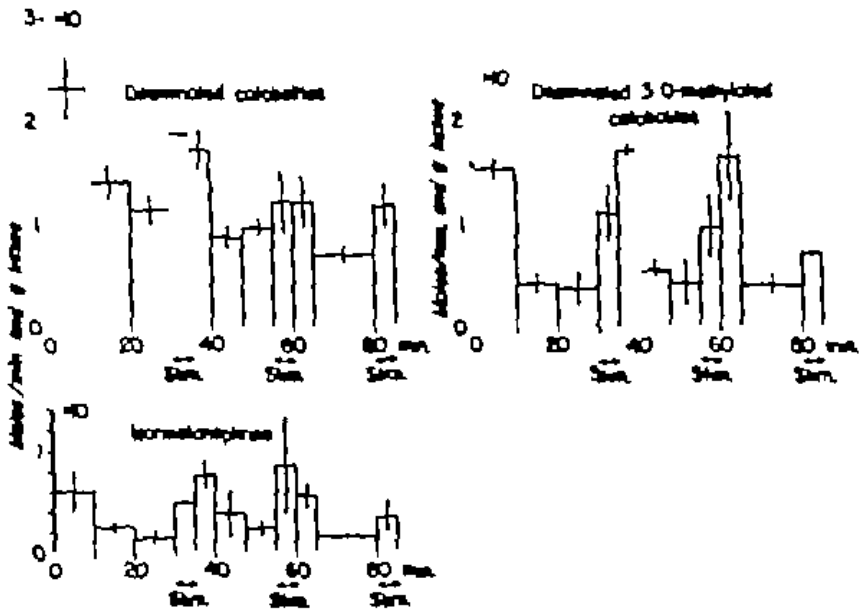


Fig. 4 Distribution pattern of the metabolites of radioactive *l* NA in the perfusate. Only NM corrected for recovery Mean values (±S.E.M.) of two experiments. Stimulation at 4 imp/sec indicated.

relationship between ^{3}H NA and its metabolites in perfusates from the stimulation and wash-out periods is demonstrated in Fig 3 The amount of ^{3}H NA (corrected for recovery) and the sum of the determined metabolites is presented. The dotted lines represent correction for loss of acid and neutral metabolites assuming a recovery of about 45 per cent on the basis of previous data (Jonason 1960 a, b) The major part of th radioactivity washed out during the first 10 min consisted of H NA During the following 20 min considerably smaller amounts of radioactivity were obtained the major part now being metabolites of labelled NA. During the three periods of field stimulation at 4 imp/sec there was a four to six fold increase in the release of total radioactivity and 70 to 80 per cent of this consisted of ^{3}H NA itself. In the samples taken immediately after the stimulation periods the total radioactivity was about 50 per cent of that obtained during stimulation and more than half of this activity emanated from metabolites. In the following wash-out fractions only small amounts of radioactivity were present, the major part being metabolites of NA

The pattern of th metabolites of labelled NA is presented in Fig 4 Only small amounts of NM were detected. The relative changes in the levels of NM during the different phases of the experiment were similar to the changes in NA The levels of the deaminated metabolites were higher than those of NM but the relative increase observed during stimulation was less pronounced (30 to 60 per cent) The levels of those products which were both deaminated and 3-O-methylated were in general higher than those of NM, but lower than those of the deaminated products.

However the increase of the amount of deaminated 3-0-methylated metabolites caused by electrical stimulation was relatively large (four to five times) and was maximal in the sample obtained immediately after stimulation.

In summary the results reported in this section have shown that the radioactivity of the perfusate in the absence of stimulation mainly consisted of metabolites of ^{3}H NA. The increased radioactivity associated with nerve stimulation is mainly in the form of ^{3}H NA proper If one assumes that an unchanged spontaneous leakage continues during stimulation (as has been done in the calculations of release exemplified in Fig 2) the present results imply that about 85 per cent of the increase during stimulation is constituted by ^{3}H NA. Furthermore, a large part of the remaining 15 per cent probably represents metabolites formed from ^{3}H NA after its release. In the following the increase in the output of total radioactivity caused by nerve stimulation will therefore be referred to as transmitter release.

IV Transmitter release and mechanical response at different impulse frequencies

Fig 4 showed that the H content in the tissue decreased rather rapidly with time and also that this decrease was influenced by the number of stimuli given. Furthermore, when the tissue contained less ^{3}H the absolute amount of radioactive transmitter released at stimulation also decreased (by about 70 per cent over 90 min) It might be more appropriate therefore to express transmitter release as a fraction of the prevailing tissue content of radioactivity as described in connection with Fig 4 A series of experiments was performed in order to elucidate whether this fractional release would vary in the course of the experiment as a function of time. Repeated stimulations at 4 imp/sec were given following the general schedule illustrated in Fig 2 The cumulated data indicated some tendency of the fractional release to decrease with time (about 20 per cent in 90 min) There were however relatively large variations between experiments so that no significant correlation between time and fractional release could be obtained. The mechanical responses showed some tendency to decline with time, on an average by about 25 per cent in 90 min. This fall was thus of about the same magnitude as the per cent decrease in fractional release of transmitter but considerably smaller than the decrease in absolute cpm values for release It is likely therefore that variations in the fractional release of H NA will reflect changes in the release of endogenous transmitter

The relatively large number of data ($n=45$) obtained with 4 imp/sec gives a value for fractional release per impulse of $(0.8\pm0.04)\times10^{-2}$ (mean$\pm$S.E.M.).

The dependence of the mechanical response on the amount of transmitter released was studied in experiments where different stimulation frequencies were used. The stimulation periods were arranged in such a way that the various impulse rates appeared in random order so as to minimize any possible effects of time and preceding impulses. For the same reason the results from the initial stimulation at 4 imp sec was only used as a reference and was not included in the comparison. Fig 3 illustrates

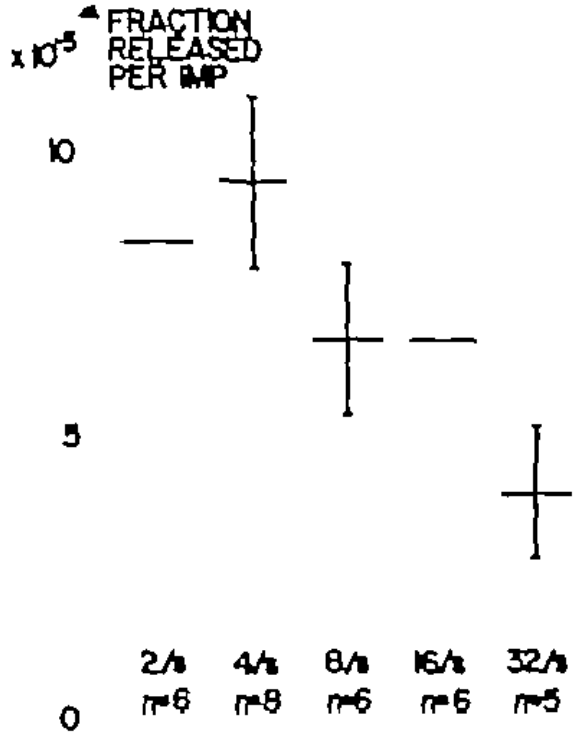


Fig. 5. Fraction of tissue radioactivity released per impulse at different frequencies of electrical field stimulation. Means ±S.E.M. indicated.

the fractional release per impulse at different frequencies of stimulation. The results suggest a moderate decrease in the transmitter release per impulse at 8 and 16 imp/sec compared to 4 imp/sec but the difference is not significant ($0.5>p>0.1$). A clear-cut decrease is found at 32 imp/sec when related to 4 imp/sec ($p<0.05$).

The quantitative data obtained at different impulse frequencies made it possible to analyse the contractile response as a function of the amount of labelled transmitter released. In the individual experiments relative values for the amount of liberated radioactivity and the mechanical response during each stimulation period have been expressed in per cent of the corresponding values obtained during the first period of stimulation as mentioned above this was always performed at a frequency of 4 imp/sec The results of this initial stimulation have thus been used as a reference for the subsequent stimulation periods in which different impulse rates were used in random order Thirty-one such randomized periods of stimulation have been obtained in 11 experiments and are represented in Fig 6 where the relative data for release and contractile response are plotted against each other

The total release of transmitter increased with frequency but not in proportion to the impulse rate obviously as a result of the variations in NA output per impulse indicated in Fig 5 The magnitudes of the mechanical responses in the frequency range of 2 to 8 imp/sec were closely related to the total amounts of NA released. At 16 and 3 imp/sec however the responses were only slightly enhanced despite the continued increase in transmitter liberation.

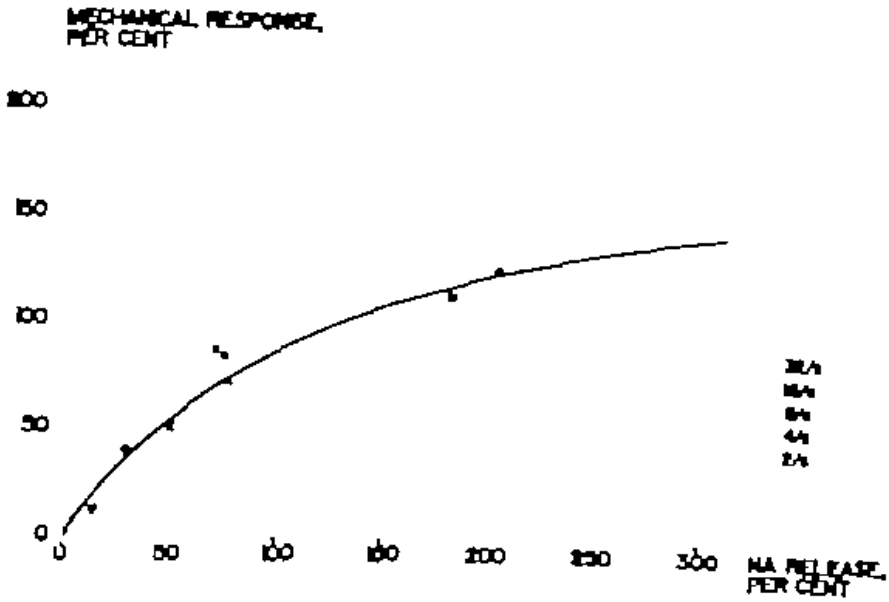


Fig 6. Relative data for ^{3}H NA release and mechanical activity in response to 2 min electrical field stimulation at different impulse frequencies. Same material as in Fig. 5. Line represents the best fit hyperbola. For further details see text.

Discussion

The NA content per unit tissue weight in the portal and mesenteric veins of the rat were rather high 2.2 and 5.7 μg/g respectively in comparison with values previously reported from several other but more thick walled blood vessels, which have ranged between 0.1 and 1.3 μg/g (von Euler and Lishajko 1958 Burn and Rand 1958 Mayer *et al.* 1968, Genest *et al* 1969) Since the innervation of most vessels, including the portal and mesenteric veins is arranged in an essentially two-dimensional way an expression of NA content per unit area may better correspond to the density of the adrenergic plexus. Considering the thickness of the portal and mesenteric veins which is about 100 and 30μ respectively and assuming a specific gravity of 1.0 both vessels contain approximately 20 ng/cm^2 vessel wall

It is now generally accepted that exogenous NA, both under *in vivo* and *in vitro* conditions, is taken up into the nerve terminals by the so-called membrane pump mechanism and stored there in the intraneuronal amine storage granules (*e.g* Hillarp and Malmfors 1964 Carlsson 1965, Malmfors 1965 Hamberger 1967) The characteristics of this uptake as studied by the use of ^{3}H NA have widely been analysed for different tissues (see *e.g* Iversen 1967 for review)

In the present experiments on the rat portal vein, uptake of H NA was obtained by 30 min incubation in 10 M labelled NA. Recent *in vitro* studies of the rat iri by Jonsson *et al* (1969) have shown that with such low concentrations of exogenous NA, tissue radioactivity reaches a relatively steady lev l within 30 min. The amount of ^{3}H NA remaining in the tissue after wash-out of the extracellular space did not change significantly when the uptake period was increased from 30 to 60 min in

their experiments. Evidence was presented that the labelled pool of NA was essentially intraneuronal but its size was considerably smaller than the total store of endogenous NA. The fact that a reasonably constant level of uptake was reached suggests, however that a subcompartment of the *total NA store had* been *equilibrated with* the extracellular tracer The calculations described in connection with Fig 2 above indicate that in the present experiments this subcompartment was about 12 per cent of the total endogenous NA content of the portal vein.

After ^{3}H NA has been taken up into the adrenergic terminals it can be released by direct stimulation of the nerves or by field stimulation as shown in a number of studies *in vivo* and *in vitro* respectively (*e g* Hertting and Axelrod 1961 Rosell, Kopin and Axelrod 1963 Gillespie and Kirpekar 1966 Baldessarini and Kopin 1967 Su and Bevan 1967) Electrical field stimulation of smooth muscle preparations has been shown to activate selectively the intramural nerve fibres (*Paton 1958* Paterson 1965) provided that impulse duration is kept low as in the present experiments.

Quantitative data on the release of labelled transmitter have been expressed above as a fraction of the actual tissue content of radioactivity and not as the absolute cpm values obtained in response to stimulation in the different stages of the experiment. The reason for doing this was that in control experiments with repeated stimulation at 4 imp/sec, the changes in the mechanical response seemed to correlate better with the changes in fractional release than with the changes in absolute ^{3}H release the latter decreasing markedly with time. If changes in the mechanical response at this physiological impulse rate signify variations in the total release of transmitter the latter variations should be reflected in the fractional release of ^{3}H NA. The fall in absolute release of ^{3}H NA in the course of the experiment is apparently compensated for by a corresponding increase in the release of endogenous transmitter probably as a result of new synthesis of NA (*e.g* Sedvall and Kopin 1967 Kopin *et al.* 1968) This may be associated with a redistribution of ^{3}H NA within the neuron However such redistribution appears to be of minor importance in the present experiments since the decrease in fractional release was small and comparable to the decrease in mechanical response

The fraction of the total tissue radioactivity which was released per impulse at a frequency of 4 imp/sec was found to be about 0.8×10^{-4} If this had originated from tracer which was evenly distributed in the entire store of endogenous NA the release of NA per impulse would also have been 0.8×10^{-4} of this total endogenous store. *In view of the above arguments that the* ^{3}H NA *is practically confined to a* smaller pool of NA, constituting some 1.. per cent of the total store there are reasons to believe that the total transmitter release per impulse is closer to 10^{-5} of the endogenous store This reasoning obviously rests on the prerequisite that released transmitter essentially comes from the pool of NA which was equilibrated with exogenous ^{3}H NA during the uptake period It is interesting to note that the figure for total release per impulse estimated in this way comes close to the value of 2×10^{-5}

for fractional endogenous release per impulse obtained by Folkow Häggendal and Lisander (1968) A figure of 5×10^{-4} for fractional endogenous release can be calculated from data obtained by Bevan, Chesher and Su (1969) in experiments on isolated pulmonary arteries treated with phenoxybenzamine which is known to increase overflow of transmitter

The overflow of radioactivity during stimulation measured in the present experiments may lead to an underestimation of the "true release because an unknown amount of released ^{3}H NA may have been reabsorbed into the nerve terminals by the "membrane pump Substances like desmethylimipramine and Lu 3-010 (1 phenyl 1 (3-methylaminopropyl) 3,3-dimethylphthalane) have been used experimentally to block this mechanism (*cf* Carlsson *et al.* 1969) Preliminary experiments with Lu 3-010 10^{-7} g/ml, using the present technique, did not result in any significant increase in the fractional release With a higher concentration (10^{-6} g/ml) there was even some decrease in the release per impulse, a phenomenon which is subjected to further analysis. Farnebo and Hamberger (personal communication) did not find any increase of the impulse-induced ^{3}H NA release from the isolated rat iris after treatment with desmethylimipramine at different concentrations. It appears that the so-called membrane pump blockers may influence also other mechanisms in the adrenergic neuroeffector system such as the suggested mechanism for variable transmitter release (Häggendal 1969 1970)

The present experiments concerning the pattern of H NA metabolism in the portal vein under the *in vitro* conditions showed that the major portion of the spontaneous release consisted of metabolites of NA, whereas ^{3}H NA itself was responsible for at least 85 per cent of the increased output of radioactivity during nerve stimulation Similar results were obtained by *e g* Rosell, Kopin and Axelrod (1963) studying the catecholamine release from th dog gracilis muscle *in vivo* Beside H NA the predominant compounds obtained in the perfusates from the isolated portal vein in the absence of nerve stimulation were deaminated metabolites. These probably reflect the action of th intraneuronal monoamine oxidase on ^{3}H NA which has leaked out into the axoplasm from the storage granules or been taken up by the membrane pump after primary spontaneous release into the neuromuscular gap (see *e.g* Jonason 1960 c) Metabolism by extraneuronal catechol-0-methyl transferase and extraneuronal monoamine oxidase is apparently of little importance during the spontaneous release, as suggested by the low lev ls of normetanephrine and deaminated, methoxylated products. Those latter compounds are, however responsible for the major part of the increase in metabolites obtained in connection with nerve stimulation It is likely that the NM originates from extraneuronal breakdown of released ^{3}H N A and that part of it has been further metabolized by tissue monoamine oxidase (*cf* Rutledge and Jonason 1968) It is worth noticing that deaminated 3-0-methylated products always reached their highest levels in the sample period immediately following nerve stimulation. This may suggest that they represent decomposition of H N A which has reacted with

the smooth muscle receptors before being 3-O-methylated and then deaminated (*cf* Eisenfeld *et al* 1967)

A major aim of the present investigation was to quantitate the transmitter release in the isolated portal vein as a function of stimulation frequency and to correlate these results to the simultaneously recorded effector responses. Such a correlation should help to explain the characteristics of the frequency response curves for adrenergically innervated effector systems

The amount of NA released per impulse did not change markedly when the adrenergic nerves were stimulated with frequencies in the range of 2 to 16 imp/sec. In conformity with previous studies (*e.g* Brown and Gillespie 1957 Kirpekar and Misu 1967 Davies and Withrington 1968) the output of NA fell at higher impulse rates. The mechanism of this decrease and the one also reported at frequencies of less than 2 imp/sec (Haefely Hürlimann and Thoenen 1965 Davies and Withrington 1968) does not seem to be known in detail Folkow (1952) demonstrated that normal vascular resistance is maintained at a discharge rate in the vasomotor fibres corresponding to 1–2 imp/sec and that during intense reflex vasoconstriction this discharge rate would rarely exceed 8 imp/sec. In this physiological range the total transmitter release thus seems to be proportional to the impulse rate.

As indicated in Fig 6 the contractile response of the portal vein is well correlated to the total amount of transmitter released by nerve stimulation at 2–8 imp/sec. Therefore, it is evident that the impulse frequency of vasomotor nerves very directly controls the state of contraction in the vascular smooth muscle within the physiological frequency range. At higher impulse rates, however the mechanical response does not increase much further in spite of continued increase in the total NA release. This implies that the limit for the maximal vasomotor response to nerve stimulation of moderate duration, as in the present experiments, is determined by some link of the effector system The levelling off of the frequency response curve above the physiological impulse rates therefore seems to be in the first hand a postsynaptic phenomenon.

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ACTA PHYSIOLOGICA SCANDINAVICA
SUPPLEMENTUM 349 33–68.

FROM THE DEPARTMENT OF PHYSIOLOGY UNIVERSITY OF GÖTEBORG, SWEDEN

Nervous and Myogenic Mechanisms in the Control of a Vascular Neuroeffector System

An integrated survey of a series of studies on the portal vein

BY
BENGT LJUNG

GÖTEBORG 1970

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Introduction

The regional blood flow resistance as well as other vascular functions in the peripheral circulation are determined by the state of constriction, or tone of smooth muscle in the various types of blood vessels, and this tone is regulated by vasomotor nerves by circulating vasoactive hormones and by local factors (for ref see the recent review by Mellander and Johansson 1968) It is known that acute elimination of the nervous influence results in decreased resistance to blood flow in most vascular beds (for ref. see *e.g.* Folkow 1955) However after acute denervation some vascular tone persists in arteriolar resistance vessels, in precapillary sphincters and, to some extent in small veins. On the other hand, large arteries and veins and arterio-venous anastomoses may become fully dilated after the vasomotor nerves have been cut. The vascular tone that does prevail after acute denervation can only to a limited extent be ascribed to humoral vasoconstrictor agents but appears to reflect inherent myogenic activity in the smooth muscle (*e.g.* Folkow 1962 1964 a)

Direct microscopic observations on the vascular bed of suitable structures, such as the rat mesoappendix and the bat wing, have shown that the diameter of small arteries and veins and their branches rhythmically increases and decreases (for ref see *e.g.* Wiedeman 1963) Chambers and Zweifach (1944) coined the term vasomotion to describe this activity Vasomotion has been shown to persist in the distal arterial and venous vessels after acute denervation (*e.g.* Wiedeman 1963) whereas the major vessels then markedly increase in diameter Considerable evidence has thus accumulated from studies with haemodynamic approaches and vital microscopy that the smooth muscle in several sections of the peripheral circulatory system maintains basal vascular tone by spontaneous synchronized contractions. Such rhythmic behaviour requires that both initiation of activity and cell to cell propagation occur within the muscle tissue. This tissue then would belong to the single unit type of smooth muscle according to Bozler's classification (Bozler 1948) Myogenic tone in some vascular sections may be based on similar fundamental characteristics of the smooth muscle without giving rise to easily observable rhythmic contractions simply because the activity is asynchronous or of a tetanic type.

Much earlier work on isolated preparations of vascular smooth muscle has been performed on strips or segments of large arteries and veins and the lack of myogenic automaticity in such vessels lead to the conclusion that vascular muscle in general would belong to the multi unit type (Bozler 1948) a view which seemed deeply rooted as late as in the beginning of the sixties.

This summary is based on results reported in the following papers.

I. Sympathetic control of rhytmically active vascular smooth muscle as studied by a nerve-muscle preparation of portal vein. Johansson, B and B Ljung Acta physiol scand. 1967 *70* 299–311

II Spread of excitation in the smooth muscle of the rat portal vein Johansson B and B Ljung Acta physiol. scand. 1967 *70* 312–322.

III Role of myogenic propagation in vascular smooth muscle response to vasomotor nerve stimulation Johansson B and B Ljung Acta physiol scand 1966 *73* 501–510

IV Local transmitter concentrations in vascular smooth muscle during vasoconstrictor nerve activity Ljung, B Acta physiol scand. 1969 *77* 212–223

V Adrenergic excitatory influences on initiation and conduction of electrical activity in the rat portal vein. Ljung B and L. Stage. Acta physiol.scand. 1970 In press.

VI. Prejunctional supersensitivity in the rat portal vein as related to its pattern of innervation Johansson, B B Ljung, T Malmfors and L. Olson. Acta physiol scand 1970 Suppl 340 5–16

VII Correlation between noradrenaline release and effector response to nerve stimulation in rat portal vein in vitro Häggendal, J B Johansson, J Jonason and B Ljung Acta physiol scand 1970 Suppl. 340 17–32.

The papers are referred to in the text by their Roman numerals.

transmitter may spread by cell to cell propagation to deeper portions of the media

The aim of this article is to summarize the results and conclusions presented in papers I to VII and to discuss the relative importance of these two mechanisms during vasomotor nerve activity in the portal vein. Possible implications of the present results for the control of vascular muscle in other parts of the circulatory system will also be discussed briefly

The separate studies have aimed at a stepwise analysis of the properties of this neuroeffector system. In paper I methods for direct recording of the mechanical activity of nerve-muscle preparations of the portal vein *in vivo* and *in vitro* were described and the adrenergic nerve supply was identified by pharmacological analysis. The importance of myogenic mechanisms for the synchronisation of the spontaneous activity (II) and of the excitatory neural responses (III) were then studied qualitatively Further aspects on pacemaker function and synchronization during adrenergic excitatory responses were elucidated in V A study on the development of prejunctional supersensitivity to noradrenaline (NA) after chronic denervation (VI) yielded information regarding the distribution of the adrenergic α receptors that are of importance in producing the response to exogenous NA. In the same study a description of the morphological characteristics of the rat portal vein and its innervation was included Quantitative studies on the release and stimulating effects of the adrenergic transmitter substance, NA, were performed in IV and VII.

Methodological considerations

Studies of intact vascular beds with methods for recording of pressure flow volume capillary exchange etc have greatly deepened our knowledge of the dimensions and the control of the circulatory system. In view of this wealth of information it may seem that experiments on isolated vascular preparations would involve a step in the direction of a more unphysiological approach with little prospect of bringing useful contributions to cardiovascular physiology The virtue of the *in vitro* system is, however that it permits analysis of cellular mechanisms behind observed phenomena to an extent which is not always possible *in vivo* and also that it permits quantitative studies under strictly standardized conditions. It is obvious that data from experiments on isolated vessels are more valuable if some evidence can be mustered to show that the principles revealed are applicable also to the intact circulation. An attempt was made to base the present studies of the neuro-muscular function of the isolated portal vein on the *in vivo* situation by comparing the responses of the isolated preparation to those of the portal vein *in situ* (I) The subsequent investigations dealing with propagation, electrophysiology morphology of innervation, transmitter release etc in the portal vein, have not aimed at a detailed analysis of each of these different aspects but have intended to clarify their respective roles in the primary function of the effector ... in the contractile response

The various experimental procedures employed in the individual studies have been described in detail in the respective papers. Here only some general methodological aspects will be discussed.

Animals As to the choice of species cats and rabbits were used in I for studies on the portal vein *in situ* and for the development of the technique for isolation of the nerve-muscle preparation. In papers II to VII *in vitro* studies of portal veins from rats have been performed. No qualitative dissimilarities between the mechanisms controlling the activity of this vessel in the different animals have appeared However species variations in the quantitative effects of nerve stimulation and of exogenous NA may exist. All quantitative data discussed in relation to release and effects of NA (IV VI VII) refer to the conditions in the rat.

Recording of contractile activity The mechanical activity has been recorded isometrically in the direction of the long axis of the vessel as the majority of the muscle cells are longitudinally arranged. As demonstrated in paper VI however an inner thin layer of the media consists of circular muscle The contractions of this layer have not been considered in those experiments.

The phasic contraction pattern constituting the spontaneous activity and remaining also at low and moderate degrees of induced excitation seemed to create some problems with regard to the quantitation of excitatory responses. However integration of the mean force development per unit time was of great help in this respect and was accomplished either by a gravimetric method (I) or by the use of an electronic integrating device (IV VI VII) When responses to nerve stimulation and to exogenous excitatory agents were measured, the mean spontaneous force developed in the preceding control period, was always subtracted, thus allowing estimations of net excitatory effects. Variations in the spontaneous activity may obviously lead to inaccuracies in the measurements of weak responses. However in the present studies quantitative comparisons between excitatory responses under varying experimental conditions (IV VI) have not been based on differences in threshold values but on relative changes of moderate and strong responses (in IV) or on shifts in dose-response curves (ED_{50} calculated in VI) Under these circumstances it is improbable that variations in spontaneous activity could have influenced the results and conclusions.

Recording of electrical activity Extracellular recordings of electrical activity have been performed simultaneously with registration of mechanical activity in II and V

In the former study the sucrose-gap method was applied in order to analyse qualitatively the effects of a hyperosmotic solution on the vascular muscle The sucrose gap method first utilized for recordings from nerve by Stämpfli (1954) was modified for studies on smooth muscle by Burnstock and Straub in 1958 More recently the technique has widely been used for investigations on effects of ions and drugs on membrane phenomena in vascular smooth muscle The experimental

procedure as applied to the portal vein has been described in detail by Axelsson *et al* (1967)

In V the main purpose of recording electrical activity was to provide a means of detecting the sequence of excitation in different parts of the preparation Recordings were made between a common electrode and each of three glass pipettes filled with Krebs solution and placed in contact with the portal vein preparation By correlating the temporal relationship between the activity recorded via the three electrodes, initiation and spread of activity in the smooth muscle could be analysed Similar techniques have been used in studies on electrical activity of gastro-intestinal muscle (see Prosser and Bortoff 1968) The recorded electrical activity represents volume-conducted potentials which are known to reflect the time course of membrane current flow under the electrode when this is close to the tissue (*e.g* Medrano *et al* 1967) The configuration of the potential changes in the electrograms were not analysed in detail in V since the purpose of the study was merely to elucidate the pathways of propagation of the electrical activity and this could be done by observing the sequential appearance of activity in the three leads.

Electrical stimulation of vasomotor nerves in vitro Two different methods were applied for electrical stimulation of the sympathetic postganglionic nerve supply of the isolated portal vein. In I, III and IV a nerve-muscle preparation was studied which consisted of the portal vein and the bundle of nerve fibres running towards it from the right coeliac ganglion. The nerves were directly stimulated in these experiments via a fluid electrode (see I) based on the principles described by Garry and Wishart (1951) Such a nerve-muscle preparation allows a safe analysis of the selective influence of nerve activity upon the effector organ However the delicate nerve fibres are inevitably subjected to some trauma during the dissection and in the course of an experiment. Therefore, this method would be less suitable for quantitative studies regarding the effects of nerve stimulation on transmitter release and effector responses which have been performed in VII. Instead the method of transmural electrical field stimulation was employed in VII and as a control of nerve degeneration, in VI (*cf* Paton 1955 Paterson 1965 Hughes and Vane 1967). Control experiments demonstrated that stimulation with pulses of 0.8 msec duration and 15 V in amplitude were supramaximal for nerve activation and that responses to such stimuli could be blocked by guanethidine, phenoxybenzamine or chronic denervation (see Fig. 9 in VI) The smooth muscle was comparatively insensitive to direct stimulation, less than 10 per cent of the neurogenic response was obtained when the preparation was stimulated after adrenergic blockade with pulses of 10 times longer duration (8 msec) It is thus safe to conclude that the intramural nerves were selectively activated by electrical field stimulation at the low impulse duration used in VI and VII.

Estimation of transmitter release. An indirect pharmacological analysis of the effect of transmitter concentration during nerve activity was performed in IV

Some aspects of methodological interest will be considered below in the discussion of the functional implications of the results of this study

Experiments aimed at direct quantitation of NA release from the adrenergic nerve terminals were done in VII. As shown in that study the total endogenous NA content in one rat portal vein (weighing 3 mg) is only about 6 ng. In view of the limited amounts thus available for release, labelled NA was studied as an indicator of the endogenous amine Quantitative conclusions regarding the release of endogenous NA based on data from such experiments are, of course, only justified if a relationship can be established between tracer release and release of endogenous NA. This matter will be considered below in connection with a discussion of the results and conclusions attained in VII

Results and conclusions

The main results and conclusions presented in I to VII will be summarized and discussed here in four subsections. The first part (A) deals with the functional characteristics of the smooth muscle of the portal vein. In section B the organization of the vasomotor nerves in the vascular wall will be described and their primary function during activity *i.e.* the release of transmitter will be discussed quantitatively In the third part (C) the distribution of the transmitter within the effector and the kinetics of transmitter receptor interaction are considered. In the final section (D) the results presented in the foregoing subsections are correlated quantitatively to the effector response so as to give an integrated picture of the function of the neuroeffector system in the portal vein.

A. Properties of the smooth muscle in the portal vein

Morphology Some histological data were presented in VI *The media of the vessel* was shown to consist of two muscle layers. The inner one which is about 15μ thick is arranged in an approximately circular fashion and appears to be a continuation of the media of the more thin walled mesenteric veins The outer media layer of the portal vein approximately 60μ thick is composed of bundles of muscle fibres arranged longitudinally It thus forms a comparatively heavy coat which extends from the liver hilus to the proximal part of the superior mesenteric vein (Fig 1-4 in VI) Assuming an average cell diameter of 4μ (*cf* Rhodin 1959 1967) and considering the large extracellular space (45 per cent of the wet tissue weight, determined with ^{14}C-sucrose by Arvill, Johansson and Jonsson 1969) it can be estimated that the longitudinal muscle sheath will contain 10 to 15 layers of cells

Electron microscopic studies of rat portal vein have shown regions of fusion between adjacent muscle cell membranes, nexuses" (Barr personal communication, Funaki 1967 See also Dewey and Barr 1968)

The rabbit portal vein has been shown to have morphological characteristics similar to those found in the rat (Holman *et al* 1968)

Spontaneous activity The portal vein contracts spontaneously both *in vivo* (I) and *in vitro* (I to VII) In the isolated preparation from the rat the automaticity is characterized by 3 to 8 phasic contractions per min and these have been shown electrophysiologically to be associated with bursts of action potentials (Funaki and Bohr 1964, Axelsson *et al.* 1967 see also II and V) Between these excitation periods there is electrical and mechanical inactivity The peak tension of the phasic spontaneous contractions often corresponded to more than half of the maximal tension obtainable in response to exogenous vasoactive agents (see *e.g* Fig 9 in VI) This indicates that the majority of the muscle cells become activated during each spontaneous contraction.

The presence of this spontaneous phasic activity indicates very strongly that excitation. I is rhythmically induced in some part of the muscle and 2. becomes propagated within the tissue.

Initiation of activity The frequency of the spontaneous contractions was not markedly affected by adrenergic neuron blockade (I) adrenergic α and β-receptor blockade (I, IV) or by chronic denervation (VI). This demonstrates that the vascular smooth muscle has an inherent ability to discharge rhythmically The membrane potential of cells in the portal vein has been shown to undergo gradual depolarization which seems to lead to rhythmic firing of bursts of action potentials (Funaki and Bohr 1964 Axelsson *et al* 1967) In view of the conducting properties of the muscle (see below), areas where the cells discharge simultaneously may serve as "pacemakers" for the entire preparation. From the results reported in V it appeared that the functioning pacemaker could be located in any part of the preparation and that shifts of pacemaker area frequently occurred during the spontaneous activity The pronounced synchronization of the spontaneous activity implies that the area which at a given moment has the most rapidly "sliding" membrane potential will elicit the excitation in the muscle.

Results from a set of unpublished experiments also illustrate this principle. Fig. 1 shows the mechanical activity of the hepatic (h) and mesenteric (m) ends of the rat portal vein when recorded separately with the middle part of the vessel immobilized (*cf* II and III). The polygraph channel recording activity in (m) had been reversed The two ends of the strip resulted in different fluid compartments utilizing the experimental arrangement described in III. In the upper panel of Fig. 1 the two ends of the preparation contracted synchronously in normal fashion. By local inspection at high paper speed (right part) the hepatic end could be seen to begin to contract slightly before the mesenteric one indicating that the actual pacemaker was located in (h). When the local calcium concentration was increased on the hepatic side the pacemaker with lower frequency located in the mesenteric end, gradually took over control of activity while the mesenteric half of the preparation was also exposed to the same increase in calcium concentration (panel C), the hepatic end regained control but

elements and that the concentration then rapidly declines so that it does not exceed 10^{-8} M at a distance corresponding to the diameter of one muscle cell.

Interaction between transmitter and adrenergic α receptors The concentration of transmitter which interacts with the adrenergic α receptors to produce the excitatory response to nerve stimulation was analysed using an indirect approach in IV. In principle this estimation was based on the finding that the degree of reduction of responses to exogenous NA produced by partial α receptor blockade with low concentrations of phenoxybenzamine was inversely related to the level of NA in the bath. Responses to nerve stimulation at 4 and 16 imp/sec were less reduced than were responses to exogenous NA at concentrations of 10^{-6} M or lower. However the neural responses were relatively more affected than the responses to 10^{-6} M exogenous NA. The per cent response remaining after partial phenoxybenzamine blockade was graphically represented as a function of the concentrations of exogenous NA in each experiment. By interpolation to these curves it was possible to deduce the NA concentrations which would be blocked to the same extent as were the responses to nerve stimulation. The values obtained 8×10^{-8} M at 4 imp/sec and 2×10^{-8} M at 16 imp/sec, were interpreted to represent the transmitter concentrations which had been reacting with receptors initiating the neural effector responses. It appears that the only factor which could make this value erroneously *high* would be the neuronal uptake mechanism which was not blocked in these experiments. It was later found in VI that chronic denervation of the portal vein caused a supersensitivity of the prejunctional type corresponding to a 13-fold decrease in ED_{50}. This implies that when the adrenergic nerves are intact the uptake mechanism reduces the NA concentration at the α adrenergic receptors to such an extent that the NA concentration in the bath has to be increased 13 times to give a response comparable to that of a denervated vessel. Therefore the local concentrations of exogenous NA at the receptors were below those in the bath when the effective transmitter concentrations were determined in IV. However at high exogenous NA levels the limit of the capacity of the uptake mechanism is reached and therefore the concentration in the entire biophase approaches that in the bath (see Fig. 10 in VI and also Langer and Trendelenburg 1969).

It thus seems safe to conclude that the actual transmitter concentration which activates the receptors during nerve stimulation at 4 imp/sec is, at least 10^{-8} M. Returning to the calculated concentration values presented in Fig. 2 it can be seen that NA levels of this order of magnitude only prevail for short periods at distances from the site of release which seem to include only one single layer of muscle cells. Although this reasoning is based on a number of assumptions which still remain to be experimentally verified in the portal vein it is evident that during nerve activity a very limited portion of the muscle cells of the media in this vessel can be directly activated by the transmitter.

It is striking that the effective transmitter concentration as determined in IV

corresponds rather closely to the peak values of the transmitter concentration (see Fig 2) despite the fact that the concentration peaks are of very short duration This indicates that the stimulating action of the transmitter is prolonged at the postjunctional level, so that its effectiveness in producing the contractile response to nerve stimulation is increased The time constants characterizing many of the links in the series of events leading to the contractile response may contribute to this enhancement. However the process of interaction of NA with the α receptors is of particular interest and will be discussed in detail in a later publication (Johansson, et al., to be published) The characteristics of the hyperbolic function relating the concentration of exogenous NA to the contractile responses of the denervated portal vein presented in VI imply that the process of formation of NA—α receptor complexes is about 10^7 times faster than the reverse reaction leading to the breaking of the complexes.[1]

Receptor kinetic reasoning (modified from Waud 1968) [ ] symbolizes concentration; k association rate constant; k_d dissociation rate constant.

$$\underset{[D]}{NA} + \underset{[R]}{\alpha\text{-receptor}} \underset{k_d}{\overset{k}{\rightleftharpoons}} \underset{[DR]}{NA-\text{receptor complex}} \tag{1}$$

$$[DR]+[R]=[R]_t = \text{total receptor population} \tag{2}$$

According to the law of mass action, (1) and (2) give at equilibrium.

$$\frac{[DR]}{[R]_t} = \frac{1}{1 + \frac{k_d/k}{[D]}} \tag{3}$$

Assuming that the mechanical response is proportional to the fractional receptor occupancy the ED_{50} for the denervated portal vein, approximately 10^{-6} M, gives 50 per cent receptor occupancy

$$\frac{50}{100} = \frac{1}{1 + \frac{k_d/k}{10^{-6}}} \qquad k_d/k_1 = 10^{-6} \tag{4}$$

The equation characterizing the drug receptor interaction can be given in the following general form:

$$\frac{d[DR]}{dt} = k_1[D][R] - k_d[DR] \tag{5}$$

replacing k with k_1 10^{-6} (4) gives

$$\frac{d[DR]}{dt} = k_1([D][R] - [DR]\ 10^{-6})$$

During rising and high concentrations of NA the rate of [DR] formation thus is high and roughly proportional to the NA concentration whereas the rate of [DR] dissociation at any moment is about 10^6 times slower and proportional to the concentration of the drug receptor complex.

In view of the rapid rate of binding of NA to the receptors, a high degree of receptor occupancy can develop despite the short duration of the peak concentrations of transmitter in the neuro-muscular gaps following the nerve impulses. Dissociation of the receptor-agonist complexes in the intervals between the peaks will lead to a gradual decrease in occupancy but the rate of this decay will be much slower than the fall of transmitter concentration calculated by the diffusion equations. It is evident that the use of partial α-adrenergic blockade to estimate the concentration of transmitter as done in IV will give a mean effective concentration which is more closely related to receptor occupancy than to mean concentrations of free transmitter" in the extracellular fluid phase. The fact that this effective transmitter concentration comes close to the peak values obtained in the left panel of Fig 2 can be understood from the above considerations of the kinetics of the receptor-agonist reactions. During repetitive stimulation receptor occupancy seems to be determined mainly by the peak concentration of the transmitter at the nearest muscle cells and by the time interval between pulses.

D Transmitter release and effector response at different impulse rates

The frequency-response curve The consideration of the release of the transmitter and its reaction with the α receptors, in the foregoing section, was mainly based on data pertaining to nerve stimulation at 4 imp/sec. The relationship between impulse rate and mechanical response has been presented in I for the cat portal vein *in situ* (Fig 5 in I) and for the rabbit portal vein *in vitro* (Fig. 7 in I) and in IV for the isolated nerve-muscle preparation of the rat portal vein.

In a series of unpublished experiments the responses of the isolated portal vein to stimulation of the intramural nerves were recorded. Electrical field stimulation with pulses of 15 V amplitude and 0.3 msec duration was used to selectively activate the intramural nerves (see VI for details in method) Each preparation was stimulated periods of _ min at different impulse rates ranging from 0.5 to 32 imp/sec applied in random order The resulting mean increases in force, expressed as a per cent of the response to 10 M exogenous NA, are illustrated in Fig 3.

The pattern of the effector response to nerve stimulation (*cf* section A) was similar in the various preparations and under the different experimental conditions The shapes of the frequency response curves likewise were in good agreement although the relative magnitude of the maximal response to nerve stimulation (that obtained at 16 imp/sec in most experiments in each series) varied with the technique used In all different *in vitro* preparations the delicate postganglionic nerve fibres have been subjected to some trauma but the functional consequences of this should be greatest in those experiments where the nerves were actually dissected and pulled into the fluid electrode This is the most likely reason for the smaller maximal response to nerve stimulation (in per cent of the maximal NA response) in these preparations. Electrical field stimulation should be more effective for activation of

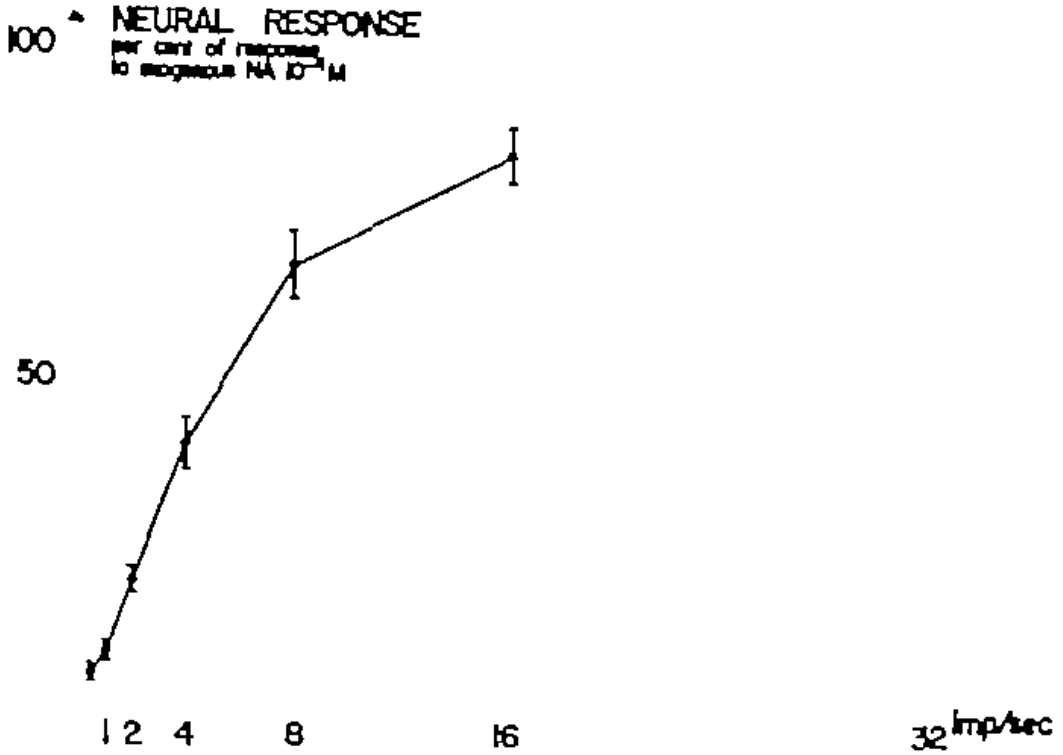


Fig. 3. Excitatory responses of rat portal vein to electrical field stimulation (0.8 msec 15 V) for 2 min at graded impulse rates. Frequencies applied in random orders in different experiments. Responses expressed as a per cent of responses to exogenous NA 10⁻⁴ M. Mean ± S.E.M. from 20 experiments.

all th nerve fibres supplying the vessel and the results obtained with this technique (Fig 3) will therefore be considered representative of the optimal neural control of the portal vein

The maximum response to nerve stimulation in the portal vein is obtained at 16 imp/sec and consequently the frequency response curve is steep at low frequencies. As a rule, no detectable contraction was produced by one single impulse of supra maximal intensity but repetitive stimulation at 0.3–0.5 imp/sec was required to obtain a measurable increase in contractile activity (unpublished results) Thus the full range of nervous control of the effector system can be exerted by varying the impulse rate by a factor between 30 to 50 times. For comparison, the rat o between the threshold concentrati n of exogenous N \ and the concentration necessary for maximal response is about *10 000 This principal difference between the frequency*-response and the dose-response relationships is often found in sympathetically innervated *effector* organs. In both cases the final common path in the production of the effector response starts with the NA–α receptor reaction. Th hyperbolic dose-response function obtained when N \ is exogenously administered to the portal vein (VI, IV) can be accounted for on the basis of current theories of drug receptor int raction (see review by Waud 1968, and footnote above) It seems reasonable also to seek the explanation for the shape of the frequency response curve in the kinetics of the reactions between the α receptors and the local concentration of transmitter during nerve activity at different impulse rates.

As to the amounts of transmitter released per impulse in the portal vein the experiments with ^{3}H NA in VII did not show any statistically significant variations when the impulse rate was altered between 2 and 16 imp/sec. In some previous studies on the perfused spleen of the cat a clear-cut decrease in the NA output per impulse has been observed at the lowest impulse rates, 1–3 imp/sec and at supra physiological frequencies (Brown and Gillespie 1957 Gillespie and Kirpekar 1966 Haefely Hürlimann and Thoenen 1965 Kirpekar and Misu 1967 Davies and Withrington 1968) Considering the obvious difficulties involved in quantitative collection and determination of the minute amounts of NA released at the lowest impulse rates, it appears that the transmitter release per impulse might be constant at all frequencies within the physiological range over short periods of stimulation.

It is a common feature of all the frequency response functions obtained for the portal vein (I, IV VII, Fig 3 above) that an approximately linear relationship exists in the frequency range of 1—8 imp/sec. Consequently the mean receptor occupancy during stimulation with these impulse rates *should* increase in proportion to the change in frequency Further the responses to 1 and 8 imp/sec correspond to about 10 and 70 per cent, respectively of the maximal response to exogenous NA (Fig 3) which shows that the mean level of receptor occupancy indeed rises very steeply over this frequency range This in turn can be understood in the light of the discussion in the last paragraph of section C When the interval between impulses is gradually reduced below say 1 sec the foot of the curve representing receptor occupancy *due to a subsequent impulse will climb higher and higher up on the* decay of the curve originating from the preceding impulse. The top values for the concentration of NA receptor complexes will not increase markedly since they are determined by the amplitude of the brief peaks of transmitter concentration (Fig 9 A) which seems to remain reasonably constant during repetitive stimulation. Furthermore the characteristics of the agonist-receptor association at these high NA levels imply that moderate changes in the absolute value of the peak NA concentration would only very slightly affect the resulting degree of receptor occupancy

At impulse rates above 8/sec the frequency response curves level off in spite of the continued increase in total transmitter release demonstrated in VII It may be tempting to ascribe this phenomenon to a decrease in the amount of NA released per impulse which appears to occur at high frequencies in the portal vein (VII) as well as in other effector organs (Brown and Gillespie 1957 Gillespie and Kirpekar 1966 Kirpekar and Misu 1967 Davies and Withrington 1968) However the high effective transmitter concentration 2×10^{-5} M obtained for 16 imp/sec in IV suggests that postsynaptic mechanisms may be responsible for the levelling off and the subsequent fall of the response at high frequencies As judged from the dose-response relationships published in VI, an effective transmitter concentration of 2×10^{-5} M appears to be sufficiently high to produce maximal excitation of those *effector* cells which become directly activated by the transmitter An optimal excitatory influence upon the innervated muscle cells may thus be reached at

the summit of the frequency response curve *i.e.* around 16 imp/sec. The tendency of the neurogenic responses to decline at supra-optimal impulse rates (*see IV*) also has its correspondence in the responses to exogenous NA, which decrease if the NA concentration is markedly increased (VI).

Localization of action of transmitter and exogenous NA. It can be seen from Fig. 3 above that during vasomotor nerve stimulation at 16 imp/sec the response constituted on an average 85 per cent of that to 10^{-4} M exogenous NA which could be considered maximum under the actual experimental conditions. The method of field stimulation appears to effectively activate the intramural nerve fibres but it is inevitable that some of these may have become injured during the isolation procedure and in the course of the experiments. It seems likely therefore that under ideal conditions responses of similar magnitude could be obtained by nerve stimulation and by administration of exogenous NA. This supports the above conclusion that during the maximal response to nerve activity an optimal NA concentration prevails at those effector cells which become directly activated by the transmitter. It also indicates that a comparable mass of muscle is excited by nerve activity and by exogenous NA.

It was demonstrated in a previous section that the transmitter can only reach one or two out of the 10 to 15 layers (see above) of muscle cells in concentrations which are compatible with the magnitude of the effective transmitter concentrations observed in IV. Furthermore, it was concluded from the results of VI that those receptors which initiate the response to exogenous NA must be close to the nerve terminals, suggesting that the primary action on the effector system is located at the same sites whether NA is released from nerve terminals or administered exogenously.

This conclusion was based on an analysis of the development of supersensitivity to NA after denervation of the rat portal vein. Chronic denervation by bilateral removal of the coeliac and mesenteric ganglia caused a 13-fold increase in sensitivity to NA as revealed by a parallel shift of the dose-response curve. The supersensitivity was found to be of the prejunctional type *i.e.* due to elimination of the neuronal uptake mechanism which thus normally reduces, by a factor of 13, the average concentration of exogenous amine in the extracellular fluid surrounding those effector cells which become primarily affected by NA. Considering the spatial relation between the two-dimensional adrenergic plexuses and the muscle cells of the media, also studied in VI and regarding the capacity of the NA uptake process in adrenergic nerves, it was concluded that a reduction in NA concentration corresponding to the degree of prejunctional *supersensitivity could prevail only in the* immediate neighbourhood of the nerve structures (for details and references see VI).

From this reasoning it follows that the same small portion of the muscle cell becomes primarily excited both when endogenous NA is released during nerve-

activity and when exogenous NA is administered In the former case this restriction in NA influence is apparent from the dynamics of transmitter distribution during nerve activity whereas in the latter it is a consequence of the properties of the effector As mentioned in VI the particular properties of the innervated cells may involve quantitative or qualitative aspects of receptor supply or functional membrane characteristics manifesting themselves as differences in excitability Since the high effective concentrations can occur only at very short distances from the sites of release (Fig _) and since the cells directly responding to NA are close to the nerve plexuses it seems justified to speak of the neuro-muscular junction as a functional entity in this type of vascular smooth muscle. A similar specialization of the innervated" cells in smooth muscle from other organs has been suggested by Burnstock (1968)

Myogenic spread of adrenergic excitation In spite of the limited portion of the effector cells which primarily become activated by nerve impulses or exposure to exogenous NA the magnitude of the tension response indicates that the entire effector system is engaged, a conclusion which is supported by comparison with responses to other means of excitation (K^+ hypotonicity etc.) Consequently the *majority* of the muscle cells of the longitudinal layer appears to become secondarily excited by myogenic spread of electrical activity This is possible by virtue of the conducting properties of this smooth muscle as described previously Myogenic spread of adrenergic excitation was shown to occur over considerable distances in the experiments reported on in III and in Fig 6 of V In the former series of experiments excitatory responses were induced by nerve stimulation or by administration of exogenous NA to one half of a portal vein preparation, the two ends of which resided in different chambers of the organ bath. After treating the other half of the vessel with phenoxybenzamine so as to exclude any direct stimulation of its receptors, it was demonstrated that part of the adrenergic response induced on the non treated side was propagated to the other side, so that forceful, synchronous contractions of increased frequency occurred in both parts of the preparation. Application of hypertonic sucrose to the central section of the vessel was used to support the myogenic nature of this propagation (Fig 3 in III) The conduction distances involved in these experiments are obviously many times greater than those which are relevant in the transmural spread of excitation through the media.

In conclusion it seems likely that the majority of the smooth muscle cells in the portal vein become secondarily affected during responses both to nerve stimulation and to exogenous NA. Those cells where the membrane potential primarily becomes reduced under the NA influence seem to be rather densely distributed along the vessel and therefore these graded potential changes have to spread over only short distances to be of importance for the recruitment of the remainder of the muscle during vasomotor nerve activity (*cf* section A above)

General discussion

The aim of the preceding section was to unify the results obtained in the present series of studies into an overall view of the neural and myogenic mechanisms which operate in the portal vein. In this section the emerging picture of the functional properties of this neuroeffector system will be summarized and its general implications on the control of the system will then be briefly discussed.

The vascular smooth muscle of the portal vein displays spontaneous phasic contractions both *in vivo* and *in vitro*. The mechanical activity is closely associated with electrical spike discharge. Initiation of this activity is due to rhythmic excitation of muscle cells in pacemaker areas, the discharge rate of which can be considerably increased by NA either after its release from the adrenergic vasomotor nerves or when exogenously administered. The muscle cells fire bursts of spike potentials which can spread over the entire length of the preparation by virtue of low resistance connections between the muscle cells, the so called nexuses. The latter allow conduction of individual action potentials over the bundles of smooth muscle. Nerve stimulation and the influence of exogenous NA improve the conditions for propagation.

During nerve activity NA is released from the varicosities of the terminal adrenergic axons which are arranged in two-dimensional plexuses and do not penetrate into the bundles of smooth muscle cells which constitute the effector. As a consequence of this geometrical arrangement and of the amounts of NA released per impulse during nerve stimulation the released transmitter primarily activates only the very nearest muscle cells. This however is accomplished by exposure of these cells to short lasting high concentration peaks which give rise to rapid formation of transmitter receptor complexes causing an immediate high degree of receptor occupancy. In contrast to the swift dilution of NA in the fluid surrounding the innervated effector cells, the breaking of the drug receptor complexes is a slow process implying a prolongation of the stimulating effect of the quantity of transmitter released per impulse. For this reason the mean receptor stimulation obtained during repetitive nerve activity becomes comparatively high and roughly proportional to the impulse rate in the physiological frequency range (1–8 imp/sec).

The sensitivity of the effector to exogenous NA seems to be essentially confined to those muscle cells which are reached by the transmitter in effective concentrations during nerve activity. This differentiation of the muscle cells indicates a specialized postjunctional link in the neuro-muscular connections of this vessel. Because of electrotonic coupling between the muscle cells the direct neural influence on the limited portion of the effector cells becomes conducted so as to affect the entire media.

This organization of the neuroeffector system resembles in many respects that described in other organs supplied with smooth muscle of the single unit type such as the gastro-intestinal tract although the excitatory nerves in the

course are cholinergic in nature (*cf* Gillespie 1962 a, b see also reviews by Holman 1968 Bennett and Burnstock 1968)

As to the nervous control of other blood vessels Bevan and co-workers have performed a thorough analysis of the neuro-muscular mechanisms operating in an isolated preparation of a large elastic vessel, the rabbit pulmonary artery (see Bevan 1962 Su Bevan and Urquilo 1964 Bevan and Verity 1966 Verity and Bevan 1968 and Bevan *et al* 1969) In brief, that particular vascular effector was shown to respond very slowly to stimulation of the adrenergic vasomotor nerves and after a relatively long latency The induced contractile activity seemed to be independent of electrical changes in the muscle membrane and consequently myogenic conduction should not occur The average neuromuscular distance was found to be very large (19000 Å) and the transmitter seems to activate the muscle cells gradually by diffusion into the media from the terminal adrenergic plexus located at the adventitio-medial border The muscle layers throughout the media of elastic arteries have been shown to be supplied with α receptors (Bevan and Verity 1967) which would make it possible for diffusion of transmitter to recruite the deeper layers of the media in the neural response

Experiments under *in vivo* conditions have also led to the conclusion that diffusion *of transmitter is of importance for activation of the media in large conduit arteries* (Gerová *et al* 1967 Gerová and Gero 1969)

The fundamental discrepancies between the present findings regarding the nervous control of the smooth muscle in the portal vein and those mentioned here for elastic arteries probably reflect true differences in the functional organization of the neuro-effector systems in respective sections of the vascular system. It is conceivable that the portal vein with its pronounced single-unit behaviour and discrete neurogenic influence enforced by the myogenic conduction of activity on one hand and the pulmonary artery with multi unit type of smooth muscle and more diffuse spread and action of the transmitter on the other represent the extremes in a spectrum of vascular neuroeffector organization. Between these ultimate types, most vessels may occupy intermediary positions with regard to rhythmicity propagating ability receptor distribution and intimacy of neuro-muscular connecti (*cf* Bohr 1965 Furchgott 1964 Rhodin 1967 see also reviews by Holman Somlyo and Somlyo 1968)

The extent of nervous influence in the parallel-coupled vascular circuits different tissues shows variation which seem purposeful in meeting the demands of the respective organs as well as the cardiovascular homeos whole organism (see review by Mellander and Johansson 1968) Su differences in the control of circulatory functions apparently reflect rather than qualitative dissimilarities in the neuro-muscular organi the individual vascular circuits Each of the latter however is form of series-coupled sections of widely different morphological and f teristics. As pointed out in the Introduction" above the present ex

the portal vein was prompted by the expectation that this neuroeffector might serve as a model of the nervous control of haemodynamically more important sections of the peripheral circulation where the vessels are less accessible to direct studies. It therefore seems appropriate to conclude this summary by considering the applicability to different sections of a vascular bed of the principles presented with respect to the portal vein.

There are several reports on the functional properties of large and medium sized arteries i.e. those with "Windkessel" and distributing functions. The results are to some extent contradictory which may reflect variations between different vessels within this group. The large arteries from most species generally show no spontaneous myogenic activity and action potentials have but rarely been recorded in response to exogenous NA or nerve stimulation (for ref. see Holman 1969). Evidence has been presented that transmitter diffusion within the media is responsible for spread of activity during vasomotor nerve stimulation (Bevan *et al.* 1969, Gerová *et al.* 1967, for further ref. see above). However, it cannot be excluded that myogenic spread of excitation could play a role also in large arteries since conduction of electrical activity has been demonstrated in strips of rat aorta (Biamino and Kruckenberg 1969) and since morphologically regions of membrane fusion between the muscle cells have been shown to exist in this vessel (Cliff 1967). Further, it is known that vasodilatation in the peripheral resistance vessels of skeletal muscle is associated with an ascending dilation of the cognate conduit arteries (see Hilton 196..) and inhibition of myogenic propagation might be a likely explanation (*cf.* Folkow 1964 b). It is probable that the smooth muscle in vessels within the Windkessel and conduit sections shows regional individualities in function but in general they lack inherent activity and are predominantly controlled by extrinsic nervous influences.

In contrast, the precapillary resistance vessels, *i.e.* small arteries and arterioles and to some extent also the postcapillary resistance vessels, *i.e.* venules and small veins, are known to possess considerable myogenic activity which haemodynamically manifests itself as a basal vascular tone which persists after acute denervation but can be inhibited by vasodilator agents (see Folkow 196.. 1964 a and b). Vital microscopic studies have revealed rhythmic calibre changes both on the arterial and venous side in these sections although the contraction waves seem to fuse into a less phasic pattern of activity as the muscle mass increases in the more proximal resistance vessels (Wiederman 1968, for further ref. see Wiederman 1963). Johansson and Bohr (1966) demonstrated phasic contractions in isolated strips from arteries with an outer diameter of 200 to 500 μ. It is thus established that the smooth muscle in the resistance sections possesses ability to initiate and conduct activity. Electrophysiologically spontaneous action potentials have been recorded from small arteries and arterioles (Funaki 1961, Steedman 1966). Further, as the density of nexuses has been demonstrated to increase with decreasing vessel diameter (Rhe... 1967) it is reasonable that the smooth muscle in the peripheral resistance v... functional characteristics similar to those of the portal vein. Folkow has

functional importance of myogenically active vascular smooth muscle in the resistance vessels, in view of its sensitivity to stretch in explaining the cellular mechanisms contributing to blood flow autoregulation (Folkow 1962 1964 a, b) In the present series of studies another important functional aspect of the electrotonic coupling between the effector cells is indicated, that of myogenic spread within the media of neurogenically induced activity The adrenergic nerve terminals are confined to the adventitio-medial border of the resistance vessels which again, implies that only a limited portion of the effector cells are in close connection with the muscle cells (Ehinger *et al* 1967 Rhodin 1967) The minimal neuro-muscular distances in these peripheral vessels appear to be less then those considered above for the portal vein (Appenzeller 1964 Matthews and Gardner 1966 Lever Graham and Spriggs 1967) In fact, the neuro-muscular relationship seems to become increasingly intimate with decreasing vascular size at least on the precapillary side (Rhodin 1967) Further some studies indicate that prejunctional supersensitivity after denervation can develop in the resistance vessels in contrast to the large elastic arteries (*cf* VI and Reynolds and Imig 1963 Haeusler and Haefely 1966 Palmer and Norberg 1969) This also supports the idea that the general neuroeffector organization in the resistance vessels may closely correspond to that described in the portal vein. It thus seems that also in resistance vessels myogenic conduction in the smooth muscle contributes to the effectiveness of the neural influence. In this connection it deserves mention that the myogenic enforcement of the nervous response does not require that electrotonic conduction occurs over great distances in order to synchronize the electrical activity in large sections of the vasculature It may also be operating in vessels which do not show phasic contractions as the only prerequisite is electrotonic coupling between the muscle cells.

The vessels in the precapillary sphincter section regulate the number of patent capillaries. Their smooth muscle usually is circularly oriented and consists of only a few interconnected muscle cells (Rhodin 1967) which show inherent rhythmicity and vasomotion before and after acute denervation (for ref see Wiedeman 1963) Due to the limited muscle mass it appears that myogenic conduction may be of little importance for the magnitude of the neural influence. As in the arteriolar resistance vessels the activity in the precapillary sphincters seems to be profoundly affected by local vasodilator factors formed in the tissue. In the precapillary sphincter section this influence also seems particularly strong in counteracting the neurogenic constriction This illustrates the role played by myogenic automaticity in providing the background activity on which excitatory and inhibitory influences act in a competitive way (see Mellander and Johansson 1968)

In the cutaneous arterio-venous shunts involved in thermoregulation, and also in the large veins with predominant capacitance function, the smooth muscle does not show spontaneous activity with the exception of the large splanchnic veins. There appears to be little experimental evidence available which would allow direct evaluation of the importance of myogenic conduction in arterio-venous shunts and

capacitance vessels. It seems safe to conclude however that extrinsic nervous influences are very dominant in the regulation of the contractile activity in these vessels. They respond to nerve stimulation at very low impulse rates and in fact one single impulse in the vasomotor nerves causes a clear-cut reduction in cutaneous blood flow due to constriction of the arterio-venous shunts (Sivertsson 1970) The frequency response curves for both arterio-venous shunts (Folkow and Sivertsson 1964) and capacitance vessels (Mellander 1960) are very steep in the low frequency range.

The low impulse rates required for threshold responses in these vessels and the steep frequency response curves in the low frequency range resemble in many respects the frequency response relationships described for the sympathetically innervated, non vascular smooth muscle of the multi unit type for instance in the nictitating membrane and the dilator muscle of the iris (Celander 1954) In these organs the adrenergic innervation is very dense and appears to directly reach most of the individual muscle cells (Malmfors 1965 1968) and this can account for the characteristics of their frequency-response relationships. The data available at present concerning the pattern of innervation of arterio-venous shunts and capacitance vessels do not seem to justify the conclusion that the vasomotor nerves directly influence all effector cells of the media. The frequency response curves of these vascular sections may also be explained in the face of an adrenergic nerve supply restricted as in most blood vessels, to the outer layers of the media.

The fact that these blood vessels respond to nerve stimulation at very low impulse rates could be a consequence of a multi-unit arrangement with limited electrotonic interconnection between the effector cells In such muscle the depolarization induced by the transmitter can only affect the individual unit. Due to the absence of a large current sink in non-conducting muscle the local depolarization caused by a certain degree of receptor occupancy for a limited period of time, would be greater than in single-unit smooth muscle If such a mechanism contributes to the low threshold frequency in the cutaneous arterio-venous shunts and the capacitance vessels, another consequence of such multi unit behaviour would be that only muscle cells activated by the transmitter would participate in the contractile response. It is well known that the range of variation in regional flow resistance obtained by nervous activation of the cutaneous arterio-venous anastomoses is extremely great, but this could be compatible with activat on of muscle cells only in the outer media as the wall t lumen ratio in these vessels is high (Folkow and Sivertsson 1964).

The steepness at low stimulation rates of the frequency response curves for the comparatively thin walled capacitance vessels has to be explained by other mechanisms. Several factors may contribute. In some large veins adrenergic nerves are found within the media indicating that also its deeper layers of muscle cells in fact receive some innervation (Ehinger et al 1967) Further when expressed as a per cent of maximum th apacitance responses, recorded as changes in blood volume will appear exaggerated in comparison with resistance

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FACTORS INFLUENCING THE AUTONOMIC COMPONENT OF THE DEFENCE REACTION

BY
BJÖRN LISANDER

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This summary is based on studies reported in the following papers:

I Folkow B., B. Lisander R. S. Tuttle and S. C. Wang. Changes in cardiac output upon stimulation of the hypothalamic defence area and the medullary depressor area in the cat. *Acta physiol scand* 1968 *72* 220—233

II Djojosugito, A. M., B Folkow B. Lisander and H. Sparks. Mechanism of escape of skeletal muscle resistance vessels from the influence of sympathetic cholinergic vasodilator fibre activity *Acta physiol scand* 1968 *72* 148—156.

III Djojosugito, A. M., B. Folkow P H. Kylstra, B. Lisander and R. S. Tuttle. Differentiated interaction between the hypothalamic defence reaction and baroreceptor reflexes. I. Effects on heart rate and regional flow resistance. *Acta physiol scand* 1970. *78* 376—385

IV Kylstra, P H. and B. Lisander Differentiated interaction between the hypothalamic defence area and baroreceptor reflexes. II Effects on aortic blood flow as related to work load on the left ventricle. *Acta physiol scand* 1970. *78* 386—392.

V Lisander B. and J Martner Cerebellar suppression of the autonomic components of the defence reaction. *Acta physiol scand.* 1970 In press.

The papers are referred to by their Roman numerals in the text.

Introduction

During the last decades there has gradually emerged an increased knowledge concerning the "anticipatory" visceromotor adjustments that are elicited *e g* in situations of challenge or manifest threat. With respect to the cardiovascular system these adjustments, often initiated by telereceptors and calling for corticohypothalamic integration, include an augmented heart activity and an increased muscle blood supply as combined with blood flow restrictions in most other tissues. It was, no doubt, evident already from the classical studies of Hess, Bard, Ranson and their groups (see *e g* Bard 1960) that the response patterns characterizing *e g* rage or fear are associated with widespread and intense sympathetic discharge. However most of our present knowledge concerning the complex cardiovascular adjustments, associated with the so called *defence reaction*, has gradually developed from studies concerning the sympathetic cholinergic vasodilator fibres and their functional significance, since they appeared to be involved only in the defence reaction. It was therefore initially often taken for granted that these fibres represented the most dominant and essential link of this reaction but, interestingly enough dilator fibres are now known to be present only in some species (see below) but nevertheless, powerful and efficient defence reactions seem to exist in virtually all mammals.

The existence of a sympathetic vasodilator fibre system suggested already in 1880 by Dastre and Morat but clearly demonstrated first by Bülbring and Burn (1935) has aroused much interest and debate during the last few decades. Further studies by Folkow and Uvnäs (1948 1950) and Folkow Haeger and Uvnäs (1948) suggested that such fibres were distributed to the skeletal muscles only that their transmission was uniformly cholinergic and that they were not involved in the steadily operating reflex homeostasis of the circulatory system. Earlier speculations that sympathetic dilator fibres might be supplied to the coronary vessels as well (see *e g* Uvnäs 1960 a) have recently been challenged (Feigl 1967). However the vagal nerves seem to convey dilator fibres to the coronaries of the dog but their functional significance is still unknown (Feigl 1969)

It now appears clear from many studies that the sympathetic cholinergic vasodilator fibres impinge upon precapillary resistance vessels only and

have no direct effects on the precapillary "sphincter" sections or on the capacitance vessels. Thus, the clearance of ^{131}I and the uptake of oxygen did not increase in muscles in association with activation of their vasodilator fibres (Hyman *et al* 1959 Rosell and Uvnäs 1962) and studies of the filtration process and of the pre- and postcapillary responses in connection with activation of the dilator fibres indicated a distribution confined to the precapillary resistance vessels (Folkow Mellander and Öberg 1961) The finding that the clearance of radioactive tracers from the muscle tissue did not increase in connection with the cholinergic dilatation led first to a discussion whether the dilator fibres might open shunt vessels (Uvnäs 1960 b) However other studies, including those mentioned above and experiments where the transit of microspheres was determined (Piper and Rosell 1961) indicated that no true shunts were involved but that the restriction of the dilator fibre impact to larger precapillary resistance vessels only could, in fact, cause "functional shunting" by creating uneven capillary perfusion. Moreover acetylcholinesterase-rich terminals, in all probability belonging to the sympathetic vasodilator fibres, were recently demonstrated in the precapillary vessels of skeletal muscles in cats and dogs (Bolme and Fuxe 1970). These axon terminals were indeed restricted to arterial vessels 30—100 μ in diameter in contrast to the adrenergic nerve terminals that are known to be spread over a

ly wider spectrum of vessels on the arterial as well as the venous

1951 it was shown by Eliasson *et al* that the sympathetic dilat
could be activated from a restricted "centre" in the anterior h
thalamus of the cat, as part of a characteristic and powerful re
pattern involving intestinal and cutaneous vasoconstriction heart
ation and usually a blood pressure rise. Later vasodilator ne
ways have been traced from the amygdala (Hilton and
and from the motor cortex, to descend via the hypothalar
centre and a mesencephalic synaptic relay station to the
neurons in the spinal lateral horns (Eliasson, Lindgren
Lindgren 1955 Lindgren *et al* 1956, Abrahams, Hilton
1960) The cholinergic vasodilator fibres may also be
epithalamus (Reinert 1964)

Thus the cholinergic vasodilatation, as evoked from
"defence area" forms part of a characteristic
that involves adrenergic excitation of most vascular
as combined with an activation of the adrenal medull
1951 Rosén 1961 Feigl Johansson and Löfving 1964

1964). Further there seems to be a concomitant activation of the adrenal cortex (Folkow *et al* 1967) Also the sympathetic supply to the gastrointestinal tract proper is excited inducing a prompt suppression of this organ system, mainly by means of an adrenergic inhibition of the intramural cholinergic ganglion cells (*e g* Folkow and Rubinstein 1965 Jansson, Lisander and Martinson 1968). Piloerection, pupillary dilatation, *etc.* are other signs of this widespread but well-coordinated sympathetic discharge pattern.

As to the functional significance of the sympathetic cholinergic dilator fibres, it was suggested already in the thirties by Bülbring and Burn (1935) that they might be activated in connection to muscular activity They are not engaged in the steadily operating homeostatic reflexes emanating from the baro- and chemoreceptors (see Uvnas 1960 a) although it has recently been reported that they may be activated from intrathoracic receptors (Bergel and Makin 1967 Bevan 1968) When the hypothalamic representation of the sympathetic vasodilator fibres was shown, their role in "emergency situations" and perhaps also in the initiation of exercise, was suggested (Eliasson *et al.* 1951). It was further shown that hypothalamic stimulation in awake cats evoked the mentioned visceromotor changes which were then found to be regularly linked to an arousal reaction that escalated into flight or attack if the stimulation was intensified (Abrahams, Hilton and Zbrozyna 1960) Based on these as well as on subsequent observations (Abrahams, Hilton and Malcolm 1962, Abrahams, Hilton and Zbrozyna 1964) Hilton and associates proposed that the autonomic changes form a preparatory cardiovascular reflex, activated already in the alerting stage of the defence reaction. Further Bolme and Novotny (1969) showed that the cardiovascular components of the defence reaction can be elicited as an orienting reflex or as a conditional reflex to either painful cutaneous stimulation or to exercise on a treadmill. These results are, in general terms, in agreement with the results and ideas of Hilton and his group, as outlined above. Bolme (1969) suggested that "the sympathetic vasodilator nerves play a role in the cardiovascular homeostatic mechanisms to regulate the arterial pressure prior to exercise" Recently however the concept that vasodilator fibres are activated as part of the alerting response and manifest "emergency situations" was challenged by Adams *et al* (1969) who found no evidence for a vasodilator fibre activation during fighting in cats. It should perhaps in this context be stressed that several autonomic response patterns of perhaps somewhat different organization might be elicited from diencephalic levels, each coupled to particular and partly

different types of emotionally charged" situations. If so, there might be no true controversy between these apparently different results.

Marked species differences appear to exist with regard to the supply of sympathetic vasodilator fibres. These fibres have been shown in cats, dogs, sheep, goats and foxes but not, for instance, in rats nor in different kinds of monkeys (see Bolme *et al* 1970) As far as man is concerned, the question is not definitely settled. However cholinergic nerve terminals could not be found around the vessels in human skeletal muscle (Bolme and Fuxe 1969) On the other hand, during mental arithmetics an increased blood pressure, heart rate and cardiac output, associated with an increased muscle blood flow and a reduction of renal and cutaneous blood flows, have been observed (Fencl *et al* 1959) Moreover Blair *et al* (1959) observed a prompt and marked increase of muscle blood flow in acutely alarmed human subjects, a response that seemed to be considerably reduced after atropine. In fact, in 1945 Barcroft and Edholm suggested that sympathetic dilator fibres might be involved in man in situations of vaso-vagal syncope produced by bleeding. In any case, in species lacking or having a poor supply of cholinergic sympathetic vasodilator fibres, such as the rat, a closely similar cardiovascular pattern nevertheless appears to be evoked in stressful situations (Folkow and Rubinstein 1966) The increase in muscle blood flow seems here to be caused by a selective and regional inhibition of vasoconstrictor fibre tone, perhaps reinforced by the vasodilator action of bloodborne adrenaline.

The aim of the present series of studies has been to elucidate *how the cardiovascular component of the defence reaction, as induced by hypothalamic stimulation is altered by some influences of local reflex and central nervous origin* In paper I the interest was focussed upon the changes in cardiac output and its preferential distribution to the skeletal muscles. It was observed that, in spite of continuous hypothalamic stimulation, the cholinergic vasodilatation tended to fade away rapidly ("escape ') and, along with this the increase of cardiac output as well.

This escape was subjected to further analysis in paper II. It appeared to be due to a purely *local* "autoregulatory response" of the precapillary vascular smooth muscles that efficiently curtailed the neurogenic blood flow increase within the skeletal muscles.

Further during a defence reaction the heart rate as well as mean arterial and pulse pressures almost invariably increase and the question arose as to what extent the consequent baroreceptor activation is able to reflexly counteract the defence reaction. This question was investigated in paper III. Evidence was here obtained suggesting a differentiated interaction between

the defence reaction and the baroreceptor reflexes which could have interesting consequences for cardiac output.

For such reasons, the effects of this interaction with particular regard to the cardiac output was studied in detail in paper IV Because of this differentiated interaction, the reflex inhibitory response from the baroreceptors appeared, in fact, to act in "haemodynamic synergism" with the excitatory defence reaction, if anything bolstering the cardiac output increase for a given neurogenic drive and load on the heart.

Finally in search for neurogenic influences that might have a purely suppressing effect on the defence reaction and on the vasodilator fibre activity it was found that stimulation of parts of the cerebellar cortex exerted such an overall inhibitory influence (paper V) markedly contrasting to that elicited by the inhibitory baroreceptor reflex.

Methods

General considerations In the present series of studies anesthetized animals, often under the additional influences of muscle-paralyzing drugs and hence artificially ventilated, were used to examine how some factors of local, reflex and central nervous origin influence the hypothalamically induced defence reaction.

It might appear that studies of such a type would be better performed in awake animals with implanted devices for recording and stimulation. It is, for example, obvious that neuronal excitability and synaptic transmission, especially of cortical and diencephalic structures, are markedly affected even by superficial anesthesia, implying that brain stem mechanisms may be secondarily depressed, or conversely released by the elimination of tonic excitatory and inhibitory influences from higher centres. Too deep an anesthesia will also markedly suppress the activity of such lower brain stem structures that directly control vascular tone and cardiac activity Further anesthesia may in other more indirect, ways affect the activity levels and responses of the different effector organs. For example, what might look like a differentiated autonomic discharge response to stimulation as judged from the effector adjustments, may in fact be a uniform one (or *vice versa*) depending upon the initial level of autonomic activity and the current state of the effectors. Thus, if autonomic nerve activity is initially very high, a further excitation, even if uniform may cause quite different degrees of effector responses than is normally the case. These different hemodynamic effects will be obtained, simply because the hyperbolic frequency response curves for resistance vessels, capacitance vessels and for the heart have different shapes. Lastly whenever artificial respiration is administered, care must be taken to adjust tidal volume and frequency to prevent hypo- or hyperventilation which, because of the consequent shifts in P_{O_2}, P_{CO_2} and pH, influences not only the cardiovascular effectors directly but also their nervous control.

Several reasons have, however contributed to the choice of the present experimental approach. In awake animals, autonomic nervous activity and cardiovascular homeostasis are subject to so many unpredictable influences that the mechanisms under study may be more or less masked in an uncontrollable way Further anesthesia tends to exclude such secondary

influences that can appear in awake animals in the form of conditioned reflexes. It should also be realized that topical stimulations of autonomic brain stem structures in awake animals are likely to excite *ascending* pathways in the reticular formation as well. These may *via* cortical or diencephalic structures, elicit secondary autonomic responses, masking those that were intended to be elicited by direct excitation of *descending* pathways or their relay stations. Besides, the present type of experiments permits an isolation of the autonomic nervous adjustments from somatomotor influences on the cardiovascular system by the use of curarization. Apart from the obvious ethical objections, the use of awake and curarized animals for studies of this type would only invite non-specific and unpredictable defence reactions that may well create more disturbances than the use of a well controlled light anesthesia. Attempts have also been made to minimize the adverse effects of anesthesia by using different anesthetic agents preferentially however chloralose was used since this drug appears to affect autonomic nervous control less than other types of anesthesia. As far as the recordings of the autonomic responses are concerned, a purely electrophysiological approach seems less feasible for the present problems, for reasons discussed in detail by Löfving (1961).

Material and anesthesia. The various experimental procedures used have been described in detail in the respective papers. In the present context only some general methodological aspects will be outlined. The investigations were carried out in a total of about 175 cats and 7 dogs, the latter reported in papers I and II. In one cat an electromagnetic flow probe had in an earlier operation under pentobarbital anesthesia been placed around the aortic root to permit a comparison of aortic flow in the awake, intact animal with that during anesthesia and following the various experimental procedures (see IV). All other experiments were performed on anesthetized animals, where in most cases chloralose, 30—60 mg/kg b.w. was used. In some animals chloralose-urethane (25—50 and 100—200 mg/kg b.w. respectively) or pentobarbital sodium 30—40 mg/kg b.w. was given instead. The anesthetic agents were usually administered intravenously following induction with ether but also the intraperitoneal route was used, or the drug was given to the awake animal *via* an injection into the cephalic vein.

Recordings of the cardiovascular parameters. Heparin was used as an anticoagulant. *Mean arterial blood pressure* was recorded continuously by a catheter in one of the brachial or femoral arteries by means of a Statham (P23AC) strain gauge pressure transducer or with a mercury manometer. *Central venous pressure* was followed by means of a catheter connected to a low pressure Statham (P23BC) transducer.

When recorded, *cardiac output* was in most cases determined with the thermodilution method (see Korner 1965) This has the advantage of leaving the central circulation undisturbed and at the same time permitting fairly frequent determinations. The tip of the thermistor was placed in the ascending aorta. Small slugs of saline at room temperature were injected *via* a cannula with its tip placed close to the right atrium The thermodilution curve obtained was plotted semilogarithmically and corrected for the slight recirculation after which the area under the curve was determined and cardiac output calculated. The thermodilution method has, however the disadvantage that cardiac output cannot be recorded continuously Therefore, an electromagnetic flowmeter Medicon 2000 was used in other experiments although the thorax had to be opened in a fairly traumatic operation. Each probe was calibrated by perfusion with saline. By this technique cardiac output minus the coronary blood flow was recorded.

Muscle blood flow was measured as the outflow from the deep femoral vein, mainly draining the calf muscles, by means of a drop recorder unit operating either an ordinate writer or a step recorder Circulation below the ankle was always occluded by a tight ligature and sometimes the calf was skinned, the bone marrow opened and plugged and all collateral veins at the knee level were ligated. *Skin blood flow* was recorded, by the same technique, as the venous outflow from the hind paw by means of a cannula inserted in the great saphenous vein, all other major collateral veins being ligated. *Intestinal blood flow* was measured as the venous outflow from a 20—30 g jejunal segment following laparotomy and exteriorization of other parts of the intestines. All drop recorders were initially primed with dextran-Tyrode solution, which was also administered if bleeding occurred.

In several experiments vascular flow resistance was directly recorded by means of an electronic device (for details see paper III) In a few control experiments these regional blood flows were also followed by an electromagnetic flow meter placed on the arterial side.

For calculation of the capillary filtration coefficient (CFC) in skeletal muscles, a method was used that enables simultaneous recording of arterial inflow pressure, venous outflow pressure, blood flow and changes of tissue volume in the calf (see Kjellmer 1964) The calf was placed in a saline-filled plethysmograph and CFC was determined by raising the venous outflow pressure abruptly by a known amount and noting the rate of outward filtration resulting from the increased capillary hydrostatic pressure. This value was expressed as the flow of filtrate across the

capillary membrane in 100 g of tissue per unit pressure change; ml/(min×100 g×mm Hg)

Stimulation techniques For topical stimulations in the central nervous system the animal's head was fixed in a modified Horsley-Clarke apparatus. The *defence area* was reached stereotaxically and stimulated by a thin monopolar electrode, isolated except for its tip. The *medullary depressor area* was sometimes reached stereotaxically through the cerebellum but more often the latter structure was gently lifted or completely or partly removed by suction. A similar type of electrode was used. The *anterior cerebellar lobe* was generally stimulated by unidirectional pulses between a pair of electrodes 2 mm apart, or sometimes by a concentric bipolar electrode.

All central nervous stimulations were performed by a Grass S5 stimulator or by a stimulator specially designed for these purposes (constructed by Erving Lars Stage), allowing for either constant current stimulation or constant voltage in the form of unidirectional rectangular pulses.

Following repeated defence area stimulations the responses sometimes tended to decay gradually. Signs of deterioration appeared first by the gradual elimination of the vagally mediated post stimulatory bradycardia and, later by a decline of the cholinergic muscle vasodilatation. As the cholinergic muscle vasodilatation was found to be resistant to the baroreceptor reflex influences and could only be depressed by cerebellar cortical stimulation, in contrast to most other components of the defence reaction, it was used as the criterion that the defence area was still largely intact after the various experimental procedures. As soon as the cholinergic vasodilatation showed signs of decaying rapidly upon repeated stimulations, the experiment was no longer used for "matching" the defence reaction against other influences.

During stimulation thermal and/or electrical injuries often occur and this, together with polarization phenomena, may lead to changes in resistance and capacitance in the electrode circuits. Such changes were, however often followed in the present experiments by the use of an oscilloscope and an unchanged cholinergic vasodilatation proved to be a sensitive criterion that the defence area was largely intact as changes in muscle vasodilatation as a rule paralleled changes in electrode circuit resistance. For such reasons defence area stimulations with constant voltage or with constant current yielded essentially similar results in the present experiments.

For baroreceptor stimulation a Moussejeff (1926) preparation, connected to a pressure reservoir containing oxygenated dextran-Tyrode solution was utilized. The artery transformed into a blind sac, could then be closed

towards the heart by a clamp on the common carotid artery so that the baroreceptor region was either exposed to any desired level of steady pressure by means of the pressure system or when the carotid clamp was removed, to the prevailing pulsatile arterial pressure. Other baroreceptor stations were in most experiments excluded. In some experiments the central end of the cut vagal nerve was stimulated in the proximal direction by a bipolar silver electrode using stimulation parameters that induced a well-graded and reproducible depressor response caused by generalized sympathetic inhibition and, in cases where the other vagus nerve had been left intact, vagal bradycardia.

Results and Comments

Changes in the central circulation caused by activation of the defence area as compared with depressor area stimulation.

In paper I the hypothalamic defence area, as well as the bulbar depressor area and related hypothalamic depressor (sympathoinhibitory) structures, were stimulated. Changes in cardiac output, heart rate, stroke volume, central venous and arterial pressures were studied. Muscle blood flow was recorded as well, partly to indicate an appropriate activation of the defence area. These two centrally elicited response patterns were chosen as they contrast in several respects (see *e g* Folkow Heymans and Neil 1963) the important defence reaction involving a widespread but differentiated sympathetic *excitation* while a generalized sympathetic *inhibition* is elicited from the mentioned depressor regions.

Defence area stimulation caused as much as a five-fold increase in skeletal muscle blood flow in the anesthetized, usually curarized animals. This flow increase often occurred in two steps with a fairly slight initial increase due to inhibition of vasoconstrictor fibre tone and a secondary usually far larger flow increase that was abolished by atropine (see also papers *III* and *IV*) As long as the adrenal medullae and their innervation were left intact the adrenaline release could somewhat prolong and occasionally even enhance the neurogenic muscle vasodilatation, as evidenced by its usually slight modification when the β-receptors of the muscle blood vessels had been blocked by administration of a β-adrenergic blocking drug In the majority of the experiments, however in which the adrenals had been denervated or eliminated to start with, the cardiovascular changes, including the muscle vasodilatation, were principally the same as when the adrenals were intact. This observation confirms earlier findings that the influence of circulating catecholamines is of fairly subordinate importance with respect to cardiovascular effector organs as compared with that of the direct nervous control, at least in the cat (Celander 1954 Mellander 1960) It was, on the other hand, noted that the cholinergic muscle vasodilation, though usually very intense to start with, was fairly transient even if prolonged stimulation of the defence area was performed. The

background of this "fading" effect of the cholinergic vasodilator fibres was separately studied in paper II (see below)

With respect to the central circulation, there was often a brief initial *decrease* in aortic flow at the onset of the defence area stimulation largely coinciding in time with a very prompt resistance increase in circuits like those of the skin, the kidneys and the intestines. This was made clear by some experiments where an electromagnetic flow meter was mounted on the arterial side of the mentioned vascular beds. An immediate vasoconstriction occurred in these circuits simultaneously with the initial, transient fall in aortic outflow (Lisander unpublished) and this vasoconstriction which could imply as much as a threefold increase in resistive load for the left ventricle, preceded the full onset of muscle vasodilatation by about 5—8 seconds. This sudden but transient resistive load, which seemed to be established even before the increased venous return and the neurogenic effect on the heart had become fully developed, probably explains the brief initial fall in left ventricular output. This fall might, however partly be due to relatively poor myocardial function, as it was seldom observed to any considerable extent in later experiments when more preparative skill was attained.

After this transient reduction and in phase with the onset of the muscle blood flow increase, cardiac output rose well beyond control and could at its peak increase twofold, coinciding with the maximal increase of muscle blood flow which could be fivefold. In this situation both stroke volume and heart rate were substantially increased and mean blood pressure was raised about 50 per cent. This means that total peripheral resistance fell by some 25—30 per cent despite the often extensive vasoconstriction in the skin, gastrointestinal tract and the kidneys. It also means that the left ventricle within a few seconds could be faced by roughly a threefold increase in work load, due to the twofold increase in output combined with the 50 per cent pressure increase. Central venous pressure could either rise or fall slightly but stroke volume increased independently compatible with a considerable inotropic effect on the heart of neurogenic origin. Apart from the somewhat larger increase in heart rate due to the decreased vagal influence on the heart, when the vagal nerves were intact, the response pattern was in principle the same after vagal section as long as one or both of the carotid baroreceptor areas were left intact.

However after administration of a β-adrenergic blocking drug (alprenolol Aptin® Hässle) the response pattern was changed in some functionally important respects. In animals with intact vagi there was still a slight

or moderate tachycardia but this was now entirely dependent upon an inhibition of prevailing vagal tone as it was absent after vagotomy. Cardiac output nevertheless rose to some extent as the result of an increase in stroke volume which was regularly related to a rise in central venous pressure. Evidently the stroke volume increase was in this situation partly or mainly a consequence of the Frank Starling relationship. Again, the substantial rise in central venous pressure, which was now seen, may be due to several factors. It is clear from other studies (*e.g.* Folkow, Mellander and Öberg 1961) that a considerable venoconstriction forms part of the defence reaction as does contraction of the spleen, both factors that *will enhance venous return. Moreover it is possible that the marked* cholinergic vasodilatation in the skeletal muscles might *per se* raise central venous pressure to some extent, in a similar manner as is seen upon opening of an artificial arteriovenous shunt. Whichever the case, in the intact animal such effects appear to be well balanced by the prompt onset of an increased sympathetic influence on the heart so that only slight shifts in central venous pressure occur in this situation.

It is of interest to note that for comparable excitations of the defence area the increase in the load on the left ventricle was reduced by approximately 50 per cent as a result of the β-adrenergic blockade on the heart. The increase of cardiac output *per se* was reduced to less than half of that present before the β-adrenergic blockade. This illustrates the extent that the central nervous drive on the heart directly contributes to the cardiac changes.

These effects of defence area activation should be compared with those produced by stimulation of the medullary depressor area and related hypothalamic structures, from which inhibition of sympathetic activity and activation of the vagal heart fibres is induced. Such stimulations were carried out on animals that were "curarized" and artificially ventilated, vagotomy was performed in the course of the experiments. As a rule the common carotid arteries were occluded during some of a series of central stimulations to create a high initial level of sympathetic activity.

When the depressor area stimulation was started, arterial blood pressure fell rather abruptly to begin with. Simultaneously muscle blood flow and cardiac output exhibited transient and usually slight *increases* that were both unaffected by atropine. Also heart rate, central venous pressure and total peripheral resistance fell during this phase. All these events must be ascribed to a generalized inhibition of sympathetic activity, the result of which becomes particularly striking with respect to muscle blood flow resistance since sympathetic constrictor fibre tone is usually most intense

in this vascular bed in the actual experimental situation (see e g Löfving 1961) For these reasons a sudden generalized inhibition of sympathetic tone can lead to such a drastic fall in flow resistance within the skeletal muscles that their blood flow *increases* transiently despite the concomitant fall in perfusion pressure. Simultaneously with this phasic muscle blood flow increase, cardiac output could exhibit a slight but transient increase, which, however was effectively curtailed when central venous pressure fell and the sympathetic inotropic effect was withdrawn Soon therefore, a gradual fall in output below control ensued which first after a minute or two of stimulation reached a stable and often very low level. Sometimes this fall in output could be further enhanced by tilting the animal with the hind legs down. An even more powerful influence in this respect, sometimes causing acute cardiovascular collapse, could be obtained by so adjusting the respiration pump as to increase intrathoracic pressure moderately Also bleeding, so small that it hardly influenced "resting blood pressure, was found to intensify the decrease in cardiac output caused by depressor area stimulation. All these maneuvers tend to reduce venous return and are normally compensated for by a reflexly increased sympathetic activity Due to the centrally induced sympathetic inhibition the animals were, however largely unable to perform such reflex compensation *for a peripheral pooling of blood.* — *The hypothalamic depressor (sympathoinhibitory)* area was stimulated instead of the bulbar one in a few experiments. The changes were essentially the same but usually less pronounced in extent.

In general, therefore, the influences on the central circulation caused by depressor area stimulation were in direction "mirror images" of those induced by defence area stimulations, while both exhibited a muscle vasodilatation which, however had a different background in the two situations.

The "escape" from the vasodilator fibre influence

In paper I was observed that increases in muscle blood flow and in cardiac output showed a close syncronicity during defence area stimulation, both tending to fade away despite continued hypothalamic stimulation. In paper II this "escape" of the muscle resistance vessels from the dilator fibre influence was subjected to further study

Irrespective of whether the cholinergic vasodilatation is elicited centrally or peripherally there is usually a rapid return of vascular resistance during continuous stimulation (Lindgren 1955 Folkow Mellander and Öberg 1961 Renkin and Rosell 1962 Dorr and Brody 1965 Bolme 1969) Some

of the possible mechanisms that might explain this escape seem to be *a priori* unlikely. Thus it cannot be due to compensatory reflexes in the isolated vascular bed studied as it occurs also when the cut sympathetic trunks are stimulated directly. Furthermore, it appears to take place irrespective of the method used for elimination of the vasoconstrictor fibre influence and can therefore hardly be ascribed e.g. to insufficient α-receptor blockade. Neither is it likely to be due to smooth muscle tachyphylaxis to acetylcholine since prolonged infusion of this agent results in a fairly sustained vasodilatation (see *e.g.* Mellander and Johansson 1968). Moreover, the escape can hardly be ascribed to transmitter depletion at the nerve endings because acetylcholine can be detected in considerable amounts in eserinized perfusates even after several minutes of sympathetic stimulation (Folkow, Haeger and Uvnäs 1948). Lastly, local damage to the stimulated sympathetic nerves seems to be ruled out, first by the fact that well sustained vasoconstrictions are obtained by the same type of stimulation when no α-adrenergic blocking drugs are given and, second, by the fact that an escape takes place also when a cholinergic dilatation is elicited by hypothalamic stimulation.

In the present experiments, direct stimulation of the lumbar sympathetic chain was utilized in order to minimize the number of neuronal links involved. Further, the ability of the denervated vascular bed to autoregulate flow in the face of altered perfusion pressure was assessed by changing the arterial inflow pressure in a stepwise fashion. In this way the "reactivity" of the smooth muscle in the resistance vessels could be repeatedly tested. Thus, when perfusion pressure was suddenly returned to control after a brief and moderate lowering there was normally a brisk but transient reactive hyperemia, revealing the extent of the compensatory vascular relaxation that had taken place during the pressure reduction. However, it was observed that whenever normal blood flow autoregulation was impaired, the escape from sympathetic vasodilator fibre influence was less pronounced too. These two phenomena were so closely correlated as to strongly suggest that the escape phenomenon is simply due to a local myogenic response.

Measurements of the capillary filtration coefficient (CFC), reflecting the responses of the precapillary sphincter sections, were therefore performed to clarify where within the muscle vascular circuit such a local counterregulation occurred. To allow for CFC measurements at the peak of the neurogenic vasodilatation, it was as a rule necessary to prolong the dilator response somewhat, which could usually be achieved by large administrations of phentolamine. This drug, when given, in doses supra

maximal for constrictor fibre blockade, directly to the vascular bed of the calf muscles, often proved to decrease markedly both flow autoregulation to pressure changes and the escape from the dilator fibre influence, without causing too drastic reductions in the tone of the resistance vessels and precapillary sphincters in the "resting" state. It remains unclear by what mechanism phentolamine acts in this respect. It was in any case not related to its blocking effect on the adrenergic constrictor fibres, as this was already achieved by the combined use of guanethidine and substantially smaller doses phentolamine. The completeness of the blockade of the α-receptors could be checked by supramaximal sympathetic stimulation after atropine administration. The large doses of phentolamine sometimes appeared to reduce smooth muscle reactivity in the resistance vessels to such an extent that autoregulation of flow was completely absent in some experiments, in which cases well sustained neurogenic vasodilatations could be induced during stimulation periods lasting 10 minutes or more.

CFC was found to *decrease* somewhat during the neurogenic vasodilatation. This is in contrast to the results obtained when acetylcholine was infused intraarterially when CFC increased along with the flow increase. However as soon as an escape from the dilator fibre influence occurred, CFC again returned towards control, in phase with the escape. Had a compensatory contraction of the precapillary sphincter sections been responsible for the escape, CFC would have become *reduced* in phase with, and in proportion to the escape. It further seems excluded that the escape occurs in postcapillary vessels since this would have led to a *rise* in capillary pressure during the escape, which certainly was not the case. Moreover veins in general exhibit only little myogenic automaticity (see e g Ljung 1970) and do not display any significant autoregulation to e.g. pressure changes (Folkow and Öberg 1961) Hence, by exclusion, the myogenic counterregulation responsible for the escape phenomenon must occur in essentially the same parts of the precapillary vessels where the *dilator fibres exert their action (see Introduction)*.

Based on these findings, the hypothesis was put forward that the sympathetic vasodilator fibres act by inhibiting the activity of only a limited number of precapillary smooth muscle cells along the precapillary resistance vessels that act either as "pacemakers" for the establishment of precapillary myogenic tone or/and are of importance for the upstream myogenic propagation of the pacemaker activity to the largest precapillary resistance vessels. Such a type of restricted dilator fibre action would be somewhat analogous to the vagal influence on the heart, which is obviously very efficient despite a vagal fibre distribution that is mainly

limited to a few key points in the myocardium. A profound and widespread dilatation of the precapillary resistance vessels would thus be possible by depriving those resistance vessels that are placed upstream to the pacemaker regions of the myogenically propagated excitation. Just as the heart may escape from a vagal inhibitory influence, an escape would also easily occur in the resistance vessels when their reactivity is pronounced, then induced from normally silent "ectopic" pacemakers located in such vascular sections that are not directly affected by the dilator fibres.

Interaction between the defence reaction and baroreceptor reflexes.

A defence area stimulation practically always elicits a prompt increase both in mean arterial pressure and in pulse pressure. The question then arises if and to what extent, the baroreceptor reflexes counteract the hypothalamically induced changes. Hilton (1963 1965) measuring blood pressure and heart rate in cats, found that a carotid sinus distension which normally caused marked bradycardia and blood pressure fall had no such effects when the defence area was stimulated simultaneously He concluded that the baroreceptor reflexes suffer a temporary abrogation during the defence reaction. On the other hand, in paper I it was observed that the blood pressure rise caused by a standardized defence area stimulation was almost the same after β-adrenergic blockade. Since the cardiac output increase was now considerably smaller total flow resistance must, in return, have contributed relatively more to the pressure rise, suggesting a reflex modulation. Moreover some earlier studies also indicate that the systemic vessels also during defence reactions can be affected by the baroreceptor reflexes (e.g. Feigl Johansson and Löving 1964 Folkow Öberg and Rubinstein 1964). These results together suggest the possibility of a modified operation of the baroreceptor reflexes during the defence reaction and this problem was further elucidated in III and IV

Experiments with standardized defence area stimulations were carried out where the animals had one carotid sinus prepared according to Moussejeff (1926) while other baroreceptor stations were excluded Blood flow was recorded in muscle, skin or intestine, together with heart rate and blood pressure. The conclusions drawn were as follows: If a baroreceptor reflex was elicited alone there was, as expected a fall in blood pressure and heart rate combined with systemic vasodilatation. If however a defence area stimulation was performed simultaneously the reflex inhibitory effects on the heart were almost completely suppressed, while those on the peripheral vascular beds were about the same as before. As

far as the muscle vessels were concerned, the conditions were here particularly complex. As earlier found by Folkow, Öberg and Rubinstein (1964) vasoconstrictor fibre tone had to be more or less eliminated before the cholinergic vasodilatation could be fully developed. This was achieved by means of a baroreceptor reflex inhibition of prevailing vasoconstrictor fibre activity initiated by the rise in mean and pulse pressures as a result of the defence reaction. Such a mechanism is quite effective because the vasoconstrictor fibres to the muscle resistance vessels do not appear to be significantly activated from the defence area directly.

In other experiments standardized defence area stimulations and baroreceptor fibre stimulations were matched against each other. In still other experiments the defence area stimulations were performed with and without the baroreceptors being activated in the normal way by the ensuing pressure rise. Broadly speaking, these results too suggested that the baroreceptor reflex effect on the heart was almost completely suppressed by the defence reaction, even when the vagal heart fibres had been left intact, in agreement with Hilton's results (1963, 1965), whereas the reflex modulation of the peripheral vascular beds remained largely unhampered. In other words, there appeared to be a *differentiated* interaction between the excitatory defence reaction and the secondarily involved inhibitory baroreceptor reflexes, where only the reflex inhibitory influence on the heart was suppressed centrally by the defence reaction. The consequences of this differentiated interaction on the cardiac output and the work load of the left ventricle were further explored in a preparation where aortic blood flow was also measured by means of an electromagnetic flowmeter applied around the aortic root (paper V). From these experiments it became evident that the efficiency of the heart as an "output pump" during a defence reaction was clearly *improved* by the action of the baroreceptor reflexes. Depending mainly on the intensity of the defence area stimulation a standardized activation of these reflexes could either decrease the load on the heart for a given cardiac output, or increase the output for a given load on the heart. It was further confirmed that the reflex inhibition of constrictor fibre tone to the muscle resistance vessels was a prerequisite for an efficient vasodilator fibre influence, at least in the cat. Therefore, the secondary involvement of the baroreceptor reflexes had also the important consequence that it helped to direct the major part of the output increase to the skeletal muscles.

In summary, the net hemodynamic result of the differentiated interaction between the defence reaction and the baroreceptor reflex was an undiminished central drive on the heart, while the vascular bed was still

reflexly modified. For such reasons a larger cardiac output could be achieved from a given drive and load on the heart and in such a way that the blood supply of the skeletal muscles was preferentially favoured.

Cerebellar modification of the defence reaction.

As it was found in IV and V that the differentiated interaction between the excitatory defence reaction and the inhibitory baroreceptor reflexes actually led to improved haemodynamic effects of the defence reaction, it was explored whether some other inhibitory influence might exert an overall suppressing effect on the defence reaction. It is known that cerebellar cortical stimulation can reduce the reflex responses induced by modulations of the baroreceptor activity (Moruzzi 1940), while cerebellectomy seems to exert opposite effects (Reis and Cuénod 1965) neither procedure influencing "resting" blood pressure significantly Furthermore sham rage, which can be induced by even trivial stimuli in the decorticate cat, can be inhibited both by cerebellar stimulation (Moruzzi 1947) and by baroreceptor activation (Bartorelli *et al* 1960)

For such reasons a series of experiments were performed in order to explore if and then in what way the cardiovascular components of the defence reaction induced by hypothalamic stimulation, were influenced by stimulation in the cortex of the anterior cerebellar lobe. The preparation was largely the same as in IV except for arrangements for topical cerebellar stimulation. Besides, in some experiments cross circulation of the skeletal muscle vascular bed was performed where adrenergic blockers could be given intra arterially to the cross-circulated calf muscle vascular bed.

Cerebellar stimulation, which alone had only insignificant cardiovascular effects, proved to reduce the increase in heart rate and in blood pressure as well as the muscle blood flow augmentation as elicited by defence area stimulation The interest was focused mainly upon the cerebellar suppression of the muscle vasodilatation. This phenomenon could *a priori* be due to a decreased activation of the cholinergic vasodilator fibres or to an impeded reflex inhibition of constrictor fibre tone in this vascular bed, which, as mentioned, seems to be a prerequisite for the full development of the cholinergic dilatation.

There was no evidence of any muscle vasoconstrictor fibre activation that could be ascribed to the cerebellar stimulation and which could have masked the vasodilatator fibre impact. On the other hand, the concomitantly decreased augmentation of mean arterial pressure and pulse pressure must imply a reduced stimulus for the baroreceptors. It therefore seemed

possible that the less pronounced pressure changes, induced by combined defence area and cerebellar stimulation, could cause a reduced reflex inhibition of vasoconstrictor fibre tone and for that reason lead to a decreased cholinergic dilatation. From experiments where baroreceptor activity could be "stabilized" it was, however evident that such a mechanism played no significant role for the decreased muscle vasodilatation.

The possibility remained, however that the reflex effect of a given baroreceptor activation might be diminished by the cerebellar stimulation. If so, the incomplete inhibition of vasoconstrictor fibre tone might have interfered with the cholinergic vasodilatation. In order to definitely exclude any involvement of adrenergically mediated phenomena in the cerebellar suppression of the muscle vasodilatation, the adrenergic system was *pharmacologically blocked, the vagi cut and the adrenals* eliminated in a series of experiments. Even under these conditions, however the atropine-sensitive muscle vasodilatation induced by defence area stimulation was markedly reduced by a concomitant cerebellar stimulation. Hence, the cerebellar suppression of the muscle vasodilatation involves a considerable suppression of the cholinergic vasodilator fibre activity *per se* Baroreceptor activation, on the other hand, had no such effects. Thus, when a cross-circulated calf region had been adrenergically blocked, even strong activations of the baroreceptors did not interfere with the vasodilator fibre influence evoked by hypothalamic stimulation.

To summarize, while the inhibitory baroreceptor reflex and the defence reaction by their differentiated interaction work in "haemodynamic synergism" (see III and IV) the cortex of the anterior cerebellar lobe appears to suppress in a generalized way the defence reaction by inhibiting essentially all its cardiovascular components, including the activation of the cholinergic vasodilator fibres.

General Discussion

Smooth muscle tone of the skeletal muscle vascular bed is determined predominantly by local myogenic mechanisms, as modified mainly by vasoconstrictor nerve activity The balance between these two factors varies, however considerably in the different series-coupled vascular sections. Further while the adrenergic vasoconstrictor fibres reach more or less all vascular sections where smooth muscle is present, the cholinergic vasodilator nerve endings seem to be confined only to the larger precapil lary resistance vessels of skeletal muscle (for ref see Mellander and Johansson 1968) The vasoconstrictor nerves, which in contrast to the vasodilator ones are tonically active, appear to affect the venous capacitance vessels and the larger precapillary resistance vessels to a relatively more dominating extent than is the case with the precapillary sphincter sections, even though adrenergic fibres can be traced in these sections too

In agreement with such findings in the muscle vascular circuit, microscopic observations concerning other vascular beds indicate that, following sympathectomy vascular tone is best retained in the immediate precapil lary sections (for ref see Wiedeman 1963) A recent study where intravital microscopy was combined with direct recordings of blood flow in skeletal muscle of the cat agrees with these findings (Eriksson and Lisander 1969) indicating also that the vasoconstrictor fibre impact is especially marked in somewhat larger precapillarly vessels with diameters that are about 50 microns in the denervated "resting" state.

Thus, sympathetic denervation leads to a considerable reduction in tone particularly in that variety of vascular smooth muscle that, according to Bozler's (1948) terminology may be called multi unit" type (mainly veins and larger arteries) whereas a considerable tone remains in the "single-unit" type of smooth muscle (mainly in arterioles, precapillary sphincters, in some venular sections and in the portal vein) In the latter type of muscle a "basal tone" seems to be generated by myogenically active "pacemaker" cells from which the activity is propagated in a cell to-cell fashion to myogenically less active regions largely in a similar way as impulse generation and propagation occurs in the heart. Thus, pacemaker activity seems to be especially well developed in vascular sections adjacent to the proximal end of the capillaries, from which it may be myogenically propagated

upstream with chances for fusion of activities where vascular branches meet. If so such a functional arrangement could explain the more steady tone in larger precapillary vessels (Folkow 1964) and also the occurrence of ascending dilatation in such vessels when e.g. vasodilator metabolites have suppressed pacemaker activity in more peripheral branches.

As to the detailed arrangement of the nervous supply to the muscle vessels, the adrenergic nerve terminals seem to be arranged in a two-dimensional plexus that does not penetrate the media but makes direct contact only with its adventitial layer (*e.g.* Bolme and Fuxe 1970). Recent *in vitro* studies of a "model" of single unit type of vascular smooth muscle (portal vein) indicate that the relatively sparse supply of adrenergic terminals in such vessels nevertheless allow for a very efficient nervous control. The reason is that the direct nervous influence, though exerted on only a fraction of the smooth muscle cells, by means of myogenic cell-to-cell propagation to non-innervated muscle cells "recruits" virtually the entire media to participate in the neurogenically induced activation (see Ljung 1970). It seems likely that a similar organization exists in the haemodynamically important precapillary resistance vessels.

As to the sympathetic cholinergic vasodilator fibres, haemodynamic experiments, briefly outlined in the introduction, indicate that they exert their action by dilating mainly or only the larger precapillary resistance vessels with no direct influence on *e.g.* the precapillary sphincter sections or on the postcapillary vessels. The experimental results presented in II lend further support to this view. Thus, the fall in blood flow resistance induced by activation of these fibres was accompanied by a constriction of the precapillary sphincter sections, as judged by a decrease in CFC. This latter response should probably be ascribed to a myogenic counter-regulation triggered by the neurogenically induced flow increase and the consequent rise in local transmural pressure. The evidently secondary nature of this oppositely directed response in the precapillary sphincter sections may explain some earlier observations where the diffusion events have been continuously followed during activation of the cholinergic vasodilator fibres. In the very earliest phase of the neurogenic dilatation there is evidence of an improved capillary exchange (Barlow and Walder 1965, Bolme and Edwall 1970), but it becomes reduced below the resting value during more prolonged dilator fibre activations (Rosell and Uvnäs 1962). Further evidence against any direct effect on the size of the perfused capillary surface area by the cholinergic dilator fibres is given by the fact that the ^{86}Rb clearance remains largely unchanged during dilator fibre activation if blood flow is kept constant throughout (Renkin and Rosell

1962) As a contrast to this evidently restricted effect of the cholinergic dilator fibres, infused acetylcholine increased both the blood flow and the number of perfused capillaries (II) in agreement with several other studies (see Mellander and Johansson 1968)

Recent morphological studies have further supported this view concerning a restricted dilator fibre impact as indicated by the mentioned physiological studies. Thus, in cat skeletal muscle the cholinergic sympathetic nerve endings, which like the adrenergic ones appear to be confined to the border between the media and the adventitia, are largely restricted to arteriolar vessels with diameters of 30—100 μ in contrast to the widespread supply of adrenergic fibres (Bolme and Fuxe 1969) Further the density of their terminals appeared to be considerably lower than that of the adrenergic nerves, also in the mentioned vascular sections.

Despite their evidently sparse and restricted distribution, the dilator fibres are nevertheless able to produce an almost maximal reduction of flow resistance in skeletal muscle at least in certain favourable situations (see *e g* Folkow Mellander and Uberg 1961) suggesting an impressive and widespread effect on virtually all the precapillary resistance vessels. This may be accomplished in two principally different ways Either the diffusion of transmitter is so extensive that almost all smooth muscle cells along the vascular arborization are reached, or the restricted supply of dilator fibres is concentrated to such smooth muscle sections that are particularly important for the establishment of myogenic vascular tone, *i.e* on pacemaker cells or/and on key points for the myogenic propagation of pacemaker activity

The first alternative seems highly unlikely both because the cholinergic transmitter is generally most efficiently destroyed at the site of release and, should it have a chance to diffuse away it would like the adrenergic transmitter (see Ljung 1970) become so rapidly diluted that it would probably be able to influence only closely adjacent muscle cells. In this particular case the transmitter would by diffusion have to also reach also larger precapillary resistance vessels situated several millimeters upstream from the site of release.

As to the other possibility *i.e* that the dilator fibres primarily interfere only with pacemaker cells or/and with some restricted key points for the myogenic spread of excitation from the pacemakers, such an explanation seems to best fit both the present and most earlier findings. Such a neurogenic suppression of the precapillary myogenic activity at, or near its normal source of origin would deprive more proximal vascular sections of their excitation. However in case usually silent "ectopic" pacemakers

upstream with chances for fusion of activities where vascular branches meet. If so such a functional arrangement could explain the more steady tone in larger precapillary vessels (Folkow 1964) and also the occurrence of ascending dilatation in such vessels when e.g vasodilator metabolites have suppressed pacemaker activity in more peripheral branches.

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fibre stimulation, it is not surprising that the cholinergic vasodilator fibres have never been shown to exert any *tonic* influence upon the cardiovascular system even in situations of *e g* sustained alarm etc. (see Uvnas 1960) Because of the escape phenomenon, an intermittent dilator fibre activity or a sudden increase of such activity would be needed to reveal any clearcut vascular effect. Conversely a prevailing tonic activity in the dilator fibres would hardly be revealed by such procedures as nerve section, atropinization or administration of eserine, again, due to the normally efficient escape phenomenon.

Thus, in several respects the sympathetic vasodilator fibres pose many interesting questions and their study has led to a greatly improved knowledge not only concerning the important cardiovascular changes taking place in alarm situations but has also helped to clarify intricate problems concerning precapillary vascular control It is then somewhat ironic that many species, perhaps including man, have a poor supply of these interesting fibres or even lack them entirely It has, however been suggested that such species, which nevertheless seem to display a characteristic and efficient defence reaction with increased muscle blood flow may do so by inhibiting the vasoconstrictor fibre tone in this vascular bed often reinforced by blood borne adrenaline and possibly facilitated by the presence of a relatively low myogenic tone in the muscle resistance vessels. Actually in case the adrenergically controlled vascular smooth muscle cells are in part responsible also for the generation in myogenic tone in such cases, pacemaker activity might be brought to a temporary quiescence by the sudden withdrawal of excitatory nervous impulses. Such a hypothetical mechanism would, in fact, haemodynamically function in a similar way as the vasodilator fibres do in species where the myogenic precapillary tone is pronounced in the skeletal muscles, as in the cat and the dog. However little is known about these problems but quantitative studies of muscle blood supply during alarm situations in species lacking sympathetic dilator fibres may be of considerable interest, considering the biological importance of the defence reaction.

According to Löfving (1961) depressor responses elicited from the cingulate gyrus and the hypothalamic sympathico-inhibitory area might be involved in emotional fainting in man and in the "playing dead" reactions of some animal species. Those structures, in turn, project upon the medullary depressor area from which a general inhibition of sympathetic tone is elicited (Löfving 1961 Porszasz 1964 Porszasz-Gibitzer 1965) In general agreement with these findings, inhibition of sympathetic tone appeared to be solely responsible for the

increase in muscle blood flow that took place initially when the medullary depressor area was stimulated (I) This flow increase could transiently even surpass that seen after sympathectomy which is seemingly suggestive of an active dilatation but it was completely unaffected by atropine. Further in the cross circulation experiments described in V in which the defence area was to be stimulated, the nearby hypothalamic sympathoinhibitory area was occasionally activated separately on insertion of the needle electrodes. This led to a depressor response in the recipient, but no vasodilatation was seen in its cross circulated calf muscles after that adrenergic blockers had been given to this vascular bed (Lisander and Martner unpublished)

Initial increases in muscle blood flow have been observed in man during fainting by Barcroft and Edholm (1945) and it was by them suggested that it might be due to an activation of cholinergic sympathetic vasodilator fibres in connection with the fainting response. Furthermore, it has been proposed that muscle vasodilator responses conveyed by the medullary depressor area have an active component, mediated by histaminergic vasodilator fibres in the sympathetic trunks (Beck 1961) In general, transient outflows of muscle flow beyond that present after acute sympathectomy have often been considered as strong evidence of vasodilator fibre activation. It is beyond the scope of the present survey to discuss at length the evidence for or against these views. It should, however be stressed that the muscle vasodilatations, seen upon depressor area stimulation or baroreceptor activation, were completely unaffected by atropine but were abolished in cross-circulated calf muscles after regional administration of adrenergic blockers. It is, moreover frequently seen that a sudden interruption of constrictor fibre activity is followed by a transient "reactive hyperaemic response, a type of reactive hyperaemia, simply as a result of the previous neurogenic flow restriction The fact that such transient dilatations can be abolished or reduced by antihistaminics (Beck 1961 1965) might after all be due to unspecific actions of the drugs used in analogy of the phentolamine effects described in II Doses of the latter drug, which exceeded those required for complete α adrenergic blockade, were sometimes found to abolish reactive hyperaemia by interfering with smooth muscle "reactivity Such an unspecific interference with phenomena like automaticity and myogenic propagation in blood vessels may well be exerted by several drugs it is, for instance, seen after administration of quinidine and urethane (Lisander unpublished)

Except for the initial increase in muscle blood flow the defence reaction and the depressor response are opposite to each other in virtually all re-

spects (see Introduction) The defence reaction comprises an increased discharge in virtually all adrenergic fibres, with exception of those to the muscle resistance vessels, where instead the cholinergic vasodilator fibres are activated, as combined with a central suppression of the vagal activity to the heart. The depressor response, on the other hand, consists of a generalized inhibition of sympathetic adrenergic fibre activity as combined with an increase in vagal tone to the heart.

Also the changes in cardiac output caused by these two centrally induced response patterns are each other s contrasts, to the extent that they are al most mirror images as described in I. Upon defence area stimulation a shortlasting fall in cardiac output preceded the often pronounced rise that characterizes this excitatory response pattern. Depressor area stimulation, on the other hand, led to an initial brief increase in output which was followed by an often profound reduction. The shortlasting initial fall in cardiac output observed upon defence area stimulation was synchroneous with the prompt vasoconstriction in several important vascular beds preceding the slightly delayed muscle vasodilatation. The heart is able to expel the same, or nearly the same, stroke volume against a wide range of resistances, due to the heterometric and homeometric autoregulation mechanisms (the Frank Starling effect and the Anrep effect, respectively see Sarnoff and Mitchell 1962) Both mechanisms may be expected to be involved after a sudden rise in peripheral resistance, the homeometric autoregulation, however first after a somewhat longer interval. During defence area stimulation there is, in addition, the increased sympathetic inotropic and chronotropic influence on the heart which is powerful and rapidly established (Rosén 1961) Under conditions of high inotropy in the myocardium a sudden rise in aortic resistance may cause a transient fall in output lasting a few beats, followed by a rise beyond the initial value, at an increased diastolic left ventricular volume (Bugge-Asperheim and Kiil 1969).

At the peak of the muscle vasodilatation, total systemic resistance returned to or below the resting value, with a maintained neurogenic drive on the heart. In addition to this direct excitatory influence on the myocardium, an increased venous return adds to the situation and is indeed a prerequisite for maintaining the output at a high level. Here the constriction of the spleen and of the systemic (Folkow Mellander and Öberg 1961) as well as of pulmonary capacitance vessels (Anderson and Brown 1967) are of importance, the former illustrated in the present experiments by the considerable rise in right atrial pressure seen when the defence area was stimulated following β-adrenergic blockade.

Observations on the control of cardiac output as exerted by cortico-hypothalamic structures are fairly scarce in the literature especially when compared to the amount of studies concerned with the central control of other facets of the circulation. Rushmer and Smith (1959) found that cardiac and peripheral circulatory changes mimicking those during exercise could be induced by stimulation in the fields of Forel in the diencephalon of the dog structures which seem to correspond to the defence area and/or its projections in the cat. Further the present finding (I) that cardiac output rises during defence area stimulation in the cat has been independently made by Anderson and Brown (1967)

The results obtained in III and IV suggest a differentiated interaction between the excitatory hypothalamic defence reaction and the inhibitory baroreceptor reflexes, with a selective suppression of the inhibitory reflex influence on the heart while the reflex modulation of vasoconstrictor fibre activity was little, if at all, affected. Contributing to this differentiated interaction, the different shapes of the hyperbolic frequency response curves, characterizing the neuro-effectors of the various cardiovascular compartments, may in some situations help to reinforce the differentiated influence by a peripheral action. Thus, the curves characterizing the adrenergic control of the heart and the capacitance vessels are decidedly more steeply hyperbolic than those of most resistance vessels (Folkow Löfving and Mellander 1956 Mellander 1960) If for example, a very intense vasoconstrictor fibre discharge is induced from the defence area and this is moderately inhibited to a uniform extent by the baroreceptor reflex, this reflex modulation will substantially damp the centrally induced constriction of the resistance vessels but hardly that of the capacitance vessels. The reason is that the reflex discharge modulation will in this latter case still be operating largely on the horizontal "plateau" level of the markedly hyperbolic frequency response curve characterizing the capacitance vessels. Such differences as to the frequency response curves must, incidently be of great importance for the efficient mobilization of the capacitance vessels for priming the heart and for the neurogenic activation of the heart as well. The particularly steep frequency response curves, which characterize the adrenergic control of both these cardiovascular compartments, will help to keep the heart well filled and also to keep the myocardium at an almost maximal degree of inotropy even in situations where the centrally induced sympathetic discharge is as moderate as 4—5 impulses/sec.

It is, however clear that the differentiated interaction between the defence reaction and the baroreceptor reflexes does not depend only on

peripheral factors such as the mentioned differences in the neuroeffector characteristics. It also involves a true nervous "occlusion" of the reflex cardiac control at some central level. It would otherwise be difficult to explain e g the virtual absence of any baroreceptor reflex activation of vagal heart fibres during the defence area stimulations. These results confirm those of Hilton (1963 1965) and are also in agreement with the recent findings of Gebber and Snyder (1970) Both these groups found that vagal bradycardia induced by baroreceptor activation was completely inhibited by hypothalamic stimulation Gebber and Snyder noted, in addition, that the baroreceptor modulation of the sympathetic outflow was still efficient during hypothalamic stimulation, in agreement with the present results concerning the blood vessels. They also recorded action potentials in the external branch of the superior cervical ganglion, the inferior cardiac branch of the stellate ganglion and in the splanchnic nerve. Hypothalamic stimulation caused a rise in activity in these nerves which was partially reduced by superimposed baroreceptor activation.

Hilton (1963 1965) recording only blood pressure and heart rate, found that carotid sinus distensions that under resting conditions caused a marked blood pressure fall and bradycardia had no detectable effects when performed during defence area stimulation, not even on the blood pressure. In this latter respect there is an apparent discrepancy between Hilton's data and those presented in III and IV It may however be explained by the fact that the baroreceptor activation described in IV did sometimes not reduce the pressure rise but could instead further enhance aortic blood flow particularly if the defence area stimulation was very pronounced. If only blood pressure and heart rate had been recorded in such experiments the mentioned baroreceptor reflex influence on the vascular bed would have passed unnoticed.

Since the baroreceptor reflexes do not interfere with the discharge of the cholinergic dilator fibres, but may instead greatly favour their impact on the muscle resistance vessels by inhibiting prevailing constrictor fibre activity these reflexes have also the important consequence that they particularly favour the blood supply to the skeletal muscles with respect to the *distribution* of the increased cardiac output. Haemodynamically therefore, the defence reaction and the baroreceptor reflexes may be said to act in synergism with respect to creating an appropriate enhancement of the nutritional supply during emergency situations at reasonable metabolic costs for the heart.

The inhibitory influence that can be elicited from the cerebellar cortex, on the other hand, was shown to interact with the defence reaction in a

principally different way by exerting an *overall* inhibitory influence, thus including the discharge of the cholinergic vasodilator fibres as well. For example, after complete pharmacological blockade of all adrenergic effects on the cardiovascular system defence area stimulation caused a marked, atropine-sensitive muscle vasodilatation and this dilatation could be considerably reduced by a concomitant cerebellar stimulation. No other type of neurogenic influence that specifically inhibits sympathetic vasodilator fibre activity appears to be known at present.

The only corticofugal neurons of the cerebellum emanate from the Purkinje cells and they convey only inhibitory impulses. Nevertheless, cerebellar cortical stimulation may cause not only inhibitory but also excitatory responses in the cerebellar nuclei and the nucleus of Deiters. These excitatory effects seem to be due to antidromic activation of afferent neurons which by means of collaterals reach both the cerebellar cortex and the mentioned nuclei (see Eccles, Ito and Szentágothai 1967) Hence, the studied cerebellar inhibition of the defence reaction may have been caused either by stimulation of Purkinje fibres or by antidromic activation of the mentioned afferents. It is, on the basis of the present experiments alone, impossible to exclude any of these alternatives as a possible mechanism for the inhibition of the defence reaction. One appealing possibility is, however that this effect is due to excitation of true Purkinje fibres exerting an inhibitory influence on cells in the fastigial nucleus, which, in turn, exert a tonic excitatory influence directly or indirectly on the hypothalamic defence area. It is, for example, known that sham rage can be facilitated by stimulation of the fastigial nucleus (Zanchetti and Zoccolini 1954) and other findings suggest that some of its neurons may have a tonic activity This cerebellar nucleus is further known to project upon the brain stem reticular formation (for ref see Eccles, Ito and Szentágothai 1967) According to Lindgren (1955) the descending axons from the neurons comprising the defence area, at least those controlling the vasodilator fibres, have synaptic relays in the mesencephalon and in the spinal cord. It is not unlikely that the cerebellar inhibitory action on the defence reaction is exerted on more than one of these three neuronal stations.

It has been reported that distension of the carotid sinus may inhibit sham rage in decorticate cats (Bartorelli *et al* 1960) It was therefore investigated whether baroreceptor reflexes exerted any direct inhibitory influence on the sympathetic vasodilator fibre connections when these are activated by topical stimulation of the hypothalamic defence area. However no such action could be found which emphasizes the suggestion

that the baroreceptor reflexes act in haemodynamic synergism with the cardiovascular defence reaction when they are secondarily excited in the natural way by the blood pressure rise (see III and IV) Thus, although both baroreceptor reflexes and cerebellar cortical stimulation may inhibit sham rage, only cerebellar stimulation interferes with the dilator fibre discharge, as elicited by topical stimulation of the hypothalamic defence area. The reason may be that the inhibitory influence of baroreceptor activation upon the sham rage response has its impact e.g on ascending pathways necessary for the activation of the hypothalamic defence area, which in the present experiments was directly activated.

Summary

1 Myogenic, reflex and central nervous modifications of the hypothalamically induced defence reaction have been analyzed in cats and dogs.
2 The effects of the defence reaction on the central circulation were explored, in comparison to the effects of an opposite type of centrally elicited response, the depressor reaction

Defence area stimulation induced up to twofold increases in cardiac output and about threefold increases in calculated work load for the left ventricle. β-adrenergic blockade could reduce this increase in left ventricular work load by almost 50 per cent. In skeletal muscle, active cholinergic vasodilatation increased flow up to fivefold whereas gastrointestinal renal and cutaneous blood flows were promptly reduced. Since these neurogenic flow reductions slightly preceded the muscle vasodilatation, the cardiac output increase could be preceded by a very brief phase of decrease.

Depressor area stimulation produced an often profound reduction in cardiac output, sometimes preceded by a brief increase. Flow resistance fell, especially in skeletal muscles, where an initial flow increase coincided with the transient increase in cardiac output- further there was a pooling of blood in the capacitance section. All these vascular effects appeared to be due to inhibition of vasoconstrictor fibre tone only Blood loss or raised venous transmural pressure greatly potentiated these effects of depressor area stimulation to the extent that acute cardiovascular collapse could ensue.

3 The cholinergic muscle vasodilatation, whether elicited centrally or peripherally tended to fade away rapidly despite continued dilator fibre stimulation. However this "escape" took place only when vascular smooth muscle reactivity" was pronounced, otherwise, sustained neurogenic vasodilatation could be obtained. Precapillary sphincter activity was secondarily increased as a result of the neurogenically reduced flow resistance, but returned to control when the escape developed, suggesting that this phenomenon takes place upstream to the sphincter sections of the precapillary vessels. A hypothesis was put forward that the cholinergic vasodilator fibres have a very restricted site of action, suppressing only "pacemaker" smooth muscle cells and/or key sites for the upstream myo-

genic propagation from such pacemakers. A neurogenic inhibitory influence at such sites would lead to widespread dilatation upstreams, but escape would ensue whenever latent pacemakers in these nonninnervated proximal sections of the resistance vessels become active, thus reestablishing myogenic tone.

4 The interaction between the hypothalamic defence reaction and the homeostatic baroreceptor reflexes was explored with respect to cardiac as well as to vascular effects. The results suggest that defence area stimulation suppresses the baroreceptor reflex inhibition of the heart while that influencing vasoconstrictor fibre discharge remains largely the same. As one of the consequences, skeletal muscle blood supply could be considerably enhanced since the reflex inhibition of regional constrictor fibre tone greatly facilitated the impact of the cholinergic vasodilator fibres. As to cardiac performance, the differentiated interaction between the defence area and the baroreceptor reflexes enabled the heart to deliver a given output at a lower load, or conversely a higher output for a given load on the left ventricle. Thus, the baroreceptor reflexes, rather than damping the defence reaction may be said to act in "haemodynamic synergism" with this central excitatory response, thanks to the mentioned differentiated interaction.

5 Cerebellar cortical stimulation, on the other hand, interacted with the hypothalamically induced defence reaction in quite a different manner. While cerebellar stimulation alone had only slight cardiovascular effects, it considerably reduced the increases in heart rate and blood pressure as well as the muscle blood flow increase as elicited by standardized defence area stimulations. The cerebellar suppression of the muscle vasodilatation was mainly due to an interference with the cholinergic vasodilator fibre activation as elicited from the defence area. Thus, the mentioned cerebellar structures appear to antagonize essentially *all* the elements comprising the reaction in contrast to the effects induced from the baroreceptors and with a markedly different net cardiovascular response as a result.

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Postnatal Development of Spinal Anterior Horn Neurones in Normal and Undernourished Rats

A Quantitative Cytochemical Study

BY

MATTI HALTIA

STOCKHOLM 1970

ACTA PHYSIOLOGICA SCANDINAVICA
SUPPLEMENTUM 352

FROM THE NEUROPATHOLOGICAL LABORATORY DEPARTMENT OF PATHOLOGY I, AND
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SWEDEN

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CONTENTS

I Introduction 5

II Material and methods 7

A. Experimental animals 7

B Production of undernutrition and rehabilitation 8
1 Choice of method 8
2. Production of undernutrition 9
3. Rehabilitation 11

C. Cytochemical methods 11
1 Choice of methods 11
2. Sampling 13
3. Preparation of material for cytochemical analysis 13
4. Determination of neuronal dry mass 14
5 Determination of neuronal RNA content and composition 16

D Statistical methods 18

III Dry mass and RNA content of rat spinal anterior horn neurones during normal postnatal development 19

A. Review of the literature 19

B. Results of the present investigations 21
1 Neuronal dry mass 21
2. Neuronal RNA content and composition 26
3. Interrelations of neuronal dry mass and RNA content during postnatal development 32

C. Discussion 34

IV Neuronal dry mass and RNA content in rats subjected to postnatal undernutrition 41

A Review of the literature 41

B. Results of the present investigations 42
1 Neuronal dry mass 42
2. Neuronal RNA content 45

C. Discussion 40

V Neuronal dry mass and RNA content in rehabilitated rats 48
A. Review of the literature 48
B Results of the present investigations 48
C. Discussion 50

VI Summary and conclusions 51

Acknowledgements 53

References 55

Tables 61

I. INTRODUCTION

Cell differentiation and growth are closely related to the transcription of the genetic code, that is, to RNA synthesis, and to the process of translation or protein synthesis (cf Mandel and Jacob 1970) Information concerning the cellular RNA and protein metabolism during development would thus appear essential for the understanding of the processes of growth and differentiation under both normal and pathological conditions. Obviously this also applies to the nerve cells.

The unique biological properties of the neurone, especially as regards the dynamics of that specialized cell (Weiss 1960), suggest that concepts based on observations of the development of other cell types need not be valid for nerve cells. The early ultraviolet spectrographic and volumetric studies of Hydén (1943) led to the conclusion that embryonal mammalian nerve cells, unlike most other somatic cells, pass through a second period of growth, characterized by continuous accumulation of nucleolar and cytoplasmic nucleotides and proteins. Similar observations were later made by Sourander (1953) and Hughes (1955) on the mass and nucleic acid content of the anterior horn neurones of chick embryos. Studies on rabbit retinal ganglion cells (Brattgård 1952) demonstrated that nucleoprotein and protein concentrations differed in young and adult or stimulated and unstimulated animals but because of discontinuous sampling, only very limited conclusions could be drawn as to the rate of change at various ages. The same applies to the study of Ringborg (1966) on the RNA content and base composition of the pyramidal cells of rat hippocampus.

The available information is not sufficient to create an integrated picture of the postnatal changes in the nucleic acid and protein content of the mammalian neurone For such a picture to emerge systematic quantitative cytochemical *information covering the whole period of postnatal development of the nerve* cell from birth to maturity is needed Such information concerning the basic structural and functional unit of the nervous system would be desirable both for a better understanding of the normal growth and maturation of the nervous system, and as a prerequisite for studies of neuronal development under experimentally *varied conditions.*

In view of recent systematic morphological and functional studies on the postnatal development of the mammalian motor neurones (cf Skoglund 1969) it would be of particular interest to study the chemical differentiation of these cells.

Since the function of these neurones is intimately connected with skeletal muscle (Eccles 1963, Guttman 1963) a major determinant of body mass (Donaldson 1924, Miller 1969), an understanding of the development of spinal anterior horn neurones in relation to somatic growth is also of interest.

Except for Brattgård's (1952) study little is known about the influence of environment on the postnatal chemical maturation of the neurone. During recent years increasing interest has been shown in the effects of early postnatal nutritional deprivation on the developing nervous system (e.g. Scrimshaw & Gordon 1968, McCance & Widdowson 1968). This interest has been largely stimulated by the realization that the incidence of protein-calorie deficiency (W.H.O 1962) in human infants is widespread and on the increase (Béhar 1968) and that it is apparently associated with impaired intellectual function (Cravioto *et al.* 1966, Stoch & Smythe 1963, Mönckeberg 1968) Experimental studies, on the other hand, have led to the formulation of a hypothesis of vulnerable periods in the development of the central nervous system during which unfavorable environmental conditions, e.g undernutrition, might be particularly harmful (Dobbing 1968). It has been shown in animal experiments that undernutrition during early postnatal period results in impaired behavioral characteristics. A number of physical, chemical and histological changes, such as reduced brain weight, reduced DNA-content of the brain and retarded myelination have also been observed in the central nervous system of human infants or animals subjected to undernutrition during early postnatal development. These changes were partly irreversible by rehabilitation (cf. Winick 1969). It is evident that the entire process of structural growth and chemical differentiation of the nerve cells as of any other cells must in some way depend on the nutrients available (Coursin 1968). Virtually no information is available, however on the effects of nutritional deprivation on the development of the neurone

The aim of the present study was to draw a coherent picture of the changes of the RNA and protein content of the nerve cell as a function of age and in relation to somatic growth during postnatal development. An attempt was also made to investigate the effects of caloric restriction imposed during the period of most intensive developmental change, and the effect of subsequent rehabilitation.

For this purpose, spinal anterior horn neurones were isolated from normal, undernourished and rehabilitated rats at frequent age intervals, and used for determinations of the neuronal dry mass after lipid extraction, reflecting the cellular protein content, and for determinations of the neuronal RNA content and composition. In these determinations, use was made of techniques allowing direct analysis of the absolute amounts of RNA and dry mass per cell.

II. MATERIAL AND METHODS

A. *Experimental animals*

The rat was chosen as the experimental animal because it offers several advantages from the point of view of the present study The rat is a spostnatal brain developers (Davison and Dobbing 1968) and can thus be easily subjected to varying experimental procedures during the most intensive period of development of its nervous system. Furthermore, since rats have been extensively used in studies of protein-calorie deficiency several methods for nutritional manipulation of even young rats have been devised (e.g. Sugita 1918, Stewart 1918, Kennedy 1957 Chow & Lee 1961, Miller and Dyrnza 1963). Finally early concepts concerning the nucleic acid and protein metabolism of developing nerve cells were largely based on observations of spinal neuroblasts of rat embryos (Hydén 1943).

Male albino rats of the Sprague-Dawley strain bred at the Department of Histology University of Göteborg, were used throughout the study Their parents were delivered by AB Anticimex, Stockholm, Sweden.

The animals were kept in metal cages on sawdust and soft shavings at a temperature of about 20° C. The pregnant rats were placed in separate cages four to five days before term. Fine wood wool was provided as nesting material. The animals were controlled twice daily to insure determination of the time of birth to within ± 12 hours. The gestation period for rats varies usually between 21 and 23 days (Porter 1967). On the day following birth the young rats were weighed and sexed (see Porter 1967) and the litter size was adjusted to eight pups. All abnormal pups and redundant females were discarded. The animals were weaned at 21 days of age. The rat mothers and young rats were given water and a standard diet *ad lib* The development of the rats was closely followed by inspection and weekly weighings, and the least suspicion of illness meant that the animal was immediately discarded.

Miller (1969) has discussed the significance of the discontinuous nature of many developmental processes in experimental design. Frequent sampling is mandatory during periods of rapid change. Thus, during the present study animals were sacrificed at eleven different ages for the cytochemical analysis of neuronal development in normal rats. The distribution of the animals in the various age groups is presented in detail in Table I The 6 rats in each age group were usually derived

from two different litters. Altogether 68 animals from 24 litters were thus used for studies of neuronal development in normal animals.

For cytochemical studies of the effect of undernutrition and subsequent rehabilitation on neuronal development an additional 32 rats were used, sacrificed at 4 different ages (see Table II and X for details of the experimental design)

B. Production of undernutrition and rehabilitation

1 Choice of method

As noted above, several techniques for producing undernutrition in infant rats have been described. Sugita (1918) and Stewart (1918) limited the daily suckling time of young rats. Based on the previous observations of Parkes (1929) on mice, Kennedy (1957) developed his »litter adjustment method« to produce undernourished rats, later extensively used by e.g. Widdowson & McCance (1960), Dobbing (1964) and Winick and collaborators (Winick and Noble 1966). More recently Chow and Lee (1964) described a method in which the rat mother was restricted to 50 per cent of her total calorie requirement. Finally Miller & Dyrnsza (1963) reported a more rigidly controlled technique for modifying the diet of the postnatal suckling rat. They fed the neonatal animals intragastrically using a specially designed needle. Their method also involved the use of nonlactating foster mothers. With this method a variety of diets can be fed to the neonate but the selection of diets is restricted, for example by the necessity of adjusting the osmolarity of the diet (Miller & Czajka 1967).

The advantages and drawbacks of the methods of Kennedy and Chow and Lee were discussed by Miller (1960). There are considerable difficulties in obtaining reproducible results with these methods as the extent of the undernutrition will vary a great deal depending e.g. upon such factors as the size and the aggressiveness of the individual pup. The method of Miller and Dyrnsza allows exact control of the quality and quantity of the diet ingested, but this technique is very laborious and is out of the question if a relatively large number of undernourished animals is to be produced.

In the present study it was important that the degree of undernutrition as reflected by body weight, could be exactly controlled, and that undernourished pups from several litters could be combined to make a uniform experimental group. The principle used by Sugita and Stewart appeared to allow direct and relatively easy control of the increase in body weight of each pup by regulating the daily suckling time. Furthermore control pups from the same litter can be fostered by the same mother. The rat mothers are not subjected to such untoward conditions as excessively large litters or dietary restrictions which might affect their ability to take care of the young. This principle was, therefore, adopted for the preliminary experiments.

After several trials the following procedure was finally accepted.

Before parturition the rat mothers were accustomed to daily handling. The time of birth was observed but the mother and pups were left undisturbed during the first day. At the age of one day the litter size was adjusted to 9. The pups were weighed and sexed and divided into an experimental and a control group consisting of 6 and 3 pups, respectively. Both groups were weighed daily.

The experimental animals were daily isolated from the mother and kept in conditions simulating those in a rat's nest (Gustafsson 1948). They were placed in a small plastic box with soft shavings as bedding material. This box was kept in an incubator with a relative humidity of approximately 60 to 70 per cent and a temperature of from 35° C to 22° C, depending on the age of the animals (Gustafsson 1948). The daily suckling time was adjusted so as to maintain a steady albeit very slow increase in body weight. In practice, this time varied from 6 to 9 hours daily. It was found that the body weight had to be allowed to increase at a rate of not less than 20 per cent of the control rate to assure acceptable survival at weaning. This meant that the undernourished rats weighed about 11 to 15 gms. at weaning. Some rat mothers showed inability or unwillingness to take care of the undernourished young and were discarded. »Good» mothers succeeded well with most of their litters, the survival rate being about 80 to 100 per cent. Only male rats from such litters were included in the actual experimental series.

After weaning at 21 days of age the undernourished rats were kept in metal cages on sawdust and soft shavings at about 20° C. They were given a defined, balanced diet in restricted amounts twice daily and water *ad lib*. The diet (for composition of diet see Table III) contained 20 per cent protein and all necessary minerals and vitamins in amounts exceeding the established minimum requirements calculated on a body weight basis (McCoy 1963). The amount of food was adjusted so as to allow a very slow increase in body weight. The undernourished rats used for the cytochemical analyses were allowed to reach the following body weights: about 10 g at 15 days of age, about 20 g at 30 days of age and about 30 g at 90 days of age. The total mean amount of diet consumed was 2.7 g *per* rat *per* day as compared with 11.2 g per rat and day for rats fed the same ration *ad lib*. The caloric intake of the undernourished rats was thus *restricted to about 24 per cent of that of control animals*.

Except for their small size and rather thin hair the undernourished animals did not show any obvious signs of disease, in particular no signs of any known vitamin deficiency. They were very lively with an apparently intact motor performance as also documented cinematographically (Fig. 1).

The control siblings (see table V) remained with the mother until weaning at 21 days of age. Thereafter they were given the experimental diet and water *ad lib*.

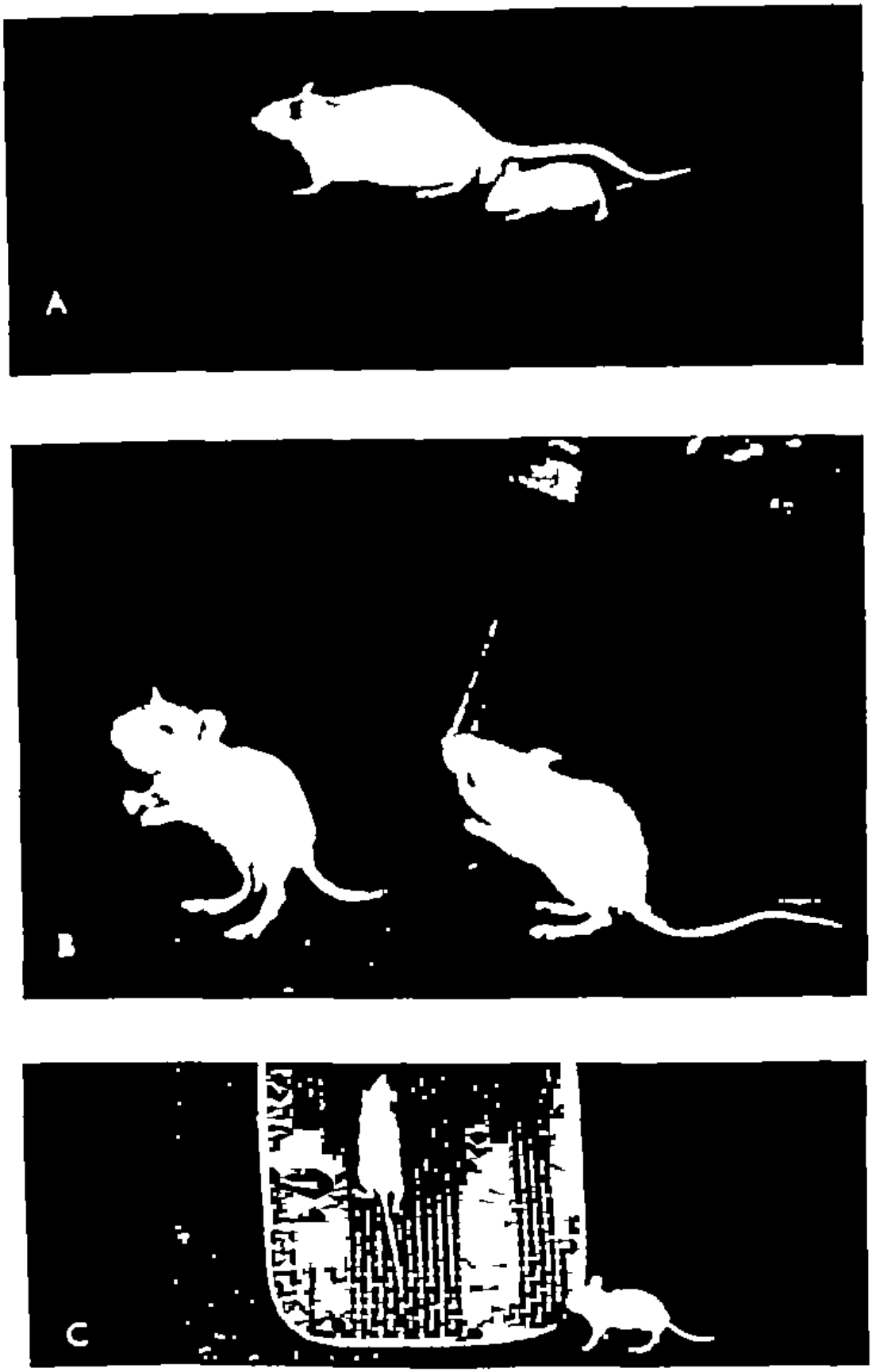


Fig. 1 a. A 3-month-old undernourished rat (body w ight 30 g; t the right) and control littermate (body w ight 380 g; t the l ft) b Two undernourished rats balancing on hind legs and drinking. An undernourished rat limbing on ertical rface.

5. Rehabilitation

For rehabilitation the previously undernourished rats were given the experimental diet and water *ad lib*. After a delay of a few days this resulted in a rapid increase in body weight, which however even after maximal rehabilitation, did not reach normal values for their age. In other respects the rehabilitated animals did not present any obvious abnormalities in appearance, motor performance, or behaviour at simple clinical inspection.

C. Cytochemical methods

1. Choice of methods

The purpose of the present study was to investigate variations in the RNA and protein content of nerve cells at various ages. An apparently simple and attractive way would have been to rely on various types of histochemical estimations in tissue sections (cf. Adams 1965) and calculate the relative changes in cellular RNA or protein content on the basis of measurements of cell volume. In a developmental series such an approach might, however, give rise to considerable difficulties.

As recently pointed out by Mellström and Skoglund (1969), the same histological treatment may cause markedly differing degrees of shrinkage in the nervous tissue from animals of different ages, thus distorting the results of the volume measurements. Furthermore, for calculation of volume of a large number of neurones, various mathematical idealizations would have to be developed for animals of different ages as the form of spinal anterior horn neurones obviously changes during development.

These difficulties and the errors associated with histochemical quantitation (Adams 1965 a) were avoided in the present study by the use of methods allowing direct measurement of the dry mass (reflecting the protein content) and absolute amounts of RNA *per* cell. Rosengren's direct X-ray absorption method (Rosengren 1950) was chosen for the mass determinations. This method allows a relatively large number of measurements to be done within reasonable time and with satisfactory accuracy (Rosengren 1959, Hallgrim 1969). Edström's microchemical methods were used for the measurements of neuronal RNA content and composition (Edström 1961). These methods necessitate the use of isolated neurones.

It was therefore necessary to evolve a procedure that would make it possible to isolate an anatomically well defined, comparable population of nerve cells from both newborn and adult rats under identical conditions. It was also imperative that the procedure should not affect the RNA or protein content of the nerve cells.

For the isolation of neurones free-hand dissection of fresh nerve cells (Hydén 1960) was first tried. These preliminary experiments showed, however, that it was very difficult or impossible to isolate the small and fragile spinal anterior

horn neurones in sufficient numbers from newborn and young rats by the free *hand dissection method*. On the other hand, such neurones were easily dissected out from fixed tissue sections with the aid of a pneumatic de Fonbrune micromanipulator (Edström 1961). This method also made it possible to select a three-dimensionally well defined anatomical area for dissection of nerve cells.

Since fixed tissue had to be used, a fixation procedure had to be chosen which would guarantee good preservation of nucleic acids and protein. Controlled immersion fixation with Carnoy's solution at room temperature up to 72 hours has been found to result in the quantitative preservation of the neuronal RNA (Edström 1961, Jarlstedt 1962).

The suitability of Carnoy-fixation for mass determination with methods based on X-ray absorption has been tested previously (Brattgård 1952) with special regard to the preservation of proteins. As the neurochemical analyses presented in Brattgård's study were made by Associate Professor Lars Swennerholm he was also consulted. He could confirm that fixation with Carnoy's solution and further histological preparation of nervous tissue resulted in a loss of 8.5 per cent of nitrogenous substances calculated as nitrogen, consisting mainly of low polymeric substances such as amino acids. Thus the protein content of the tissue was not essentially affected. In contrast, lipids were almost completely extracted with the exception of less than 5 per cent. These neurochemical observations were confirmed by X-ray microradiographical analysis; no significant difference in mass was found between nerve cells fixed in Carnoy's solution and cells frozen sectioned in the fresh state with subsequent extraction of the lipids with chloroform (Brattgård 1952). These observations appeared also to be valid for direct ray absorption analysis of isolated spinal anterior horn neurones, as no significant differences in the reduction in mass could be observed between cells dissected in the fresh state and subsequently treated with Carnoy's solution, or only with chloroform. The markedly reduced X ray absorption of nerve cell nuclei treated with Carnoy's solution (Brattgård and Hydén 1954) may thus be due to redisposition rather than actual loss of organic material other than lipids.

The effect of fixation on carbohydrates is of no importance as the amount of carbohydrates in nerve cell material is very low and can be neglected as a source of error in the determination of total cell mass. The same is true for a number of electrolytes which do not absorb X-rays at the wavelength employed (Brattgård & Hydén 1952, Brattgård 1952). The results of X-ray absorption analysis of material fixed in Carnoy's solution thus correspond to the mass of protein and nucleic acids added together. As the amount of nucleic acids in nerve cells is of the order of 10 per cent of the mass of protein, the values obtained reflect essentially the amount of protein per cell. Henceforth the mass of the isolated nerve cell body after Carnoy fixation and histological treatment will be referred to as neuronal dry mass.

2 Sampling

Large and small nerve cells occur intermingled in the anterior horns of the spinal cord (Rexed 1952, 1964, Schadé and van Harreveld 1961) The spinal anterior horn neurones are of varying size (Ngowyang 1930) and also have different RNA contents at various levels of the spinal cord (Edström 1956). It was thus essential that the nerve cells used for the cytochemical analyses always represented the same neurone population and were derived from the same precisely defined anatomical area. This was accomplished by using only large anterior horn neurones from the anterolateral nucleus (corresponding to Rexed s lamina IX) of the 7th cervical segment at the level of the attachment of the posterior root on the spinal cord.

The 7th cervical segment was chosen because some systematic developmental data were available on this segment (Donaldson & Nagasaka 1918). The most cranial attachment of the 7th posterior root on the spinal cord was regar ded as the cranial limit of the segment, according to Thomas and Combs (1962).

12 large anterior horn neurones from each of six subsequent sections, six cells from each side, altogether 72 cells per animal, were used for the cytochemical analyses. 36 cells from three sections were used for the RNA determinations, and 36 cells from alternating sections for mass determinations.

To compensate roughly for the changes in the size of the nerve cells and length of the spinal cord segments during postnatal development, 40 μ sections were used for newborn and up to 8-day-old rats, 60 μ sections for 10 to 15-day-old rats, and 80 μ sections for older rats.

3. Preparation of material for cytochemical analysis

On the basis of the reasoning explained above the following procedure was adopted.

The animals were sacrificed by decapitation without previous anaesthesia. The cervical and upper thoracic spinal cord with the bony coverings was rapidly removed and the cord and its nerve roots exposed by laminectomy The structures were fixed in Carnoy s solution (absolute ethanol, chloroform and glacial acetic acid in proportions 6.3.1) for two hours. The fixed spinal cord was cut by means of a razor blade under a dissecting microscope perpendicular to the long axis of the cord at the level of the most cranial attachment of the posterior root of the 7th cervical segment.

The caudal part of the cord containing the 7th cervical segment was dehydrated for two hours in absolute ethanol, cleared for 30 minutes in benzene, and embedded in soft paraffin (melting point 52—54 C) From the cranial end of the preparation thus corresponding to the level of attachment of the 7th posterior root on the cord sections 40, 60 or 80 μ in thickness were cut with an ordinary sliding microtome. The sections were mounted on cover glasses (12×30×0.17 mm) and flattened at 37 C without using water The cover glasses were fastened with two strips

of tape on to conventional glass slides on which identification data were applied. Prior to dissection with the micromanipulator the sections were deparaffinized with chloroform for 5 minutes and transferred to successive baths of absolute ethanol and 0 01 N acetic acid, 5 minutes in each To render the nerve cell bodies more easily distinguishable the hydrated sections were lightly stained with methylene blue in 0 01 N acetic acid (Edstrom 1959).

The isolation of spinal anterior horn neurones from the hydrated sections was carried out in an oil chamber with the aid of two dissecting glass needles directed by a de Fonbrune micromanipulator (Etablissements Beaudouin, Paris, France) as described by Edström (1964). The microscope used in combination with the micromanipulator was a Zeiss Standard microscope equipped with phase contrast optics.

4 Determination of neuronal dry mass

For mass determination the isolated nerve cell bodies (Fig. 2a & b) were individually transferred to a supporting membrane attached on an aluminium ring. The cells were allowed to dry overnight in the oil chamber and the preparation was passed through four chloroform baths, 5 minutes in each, to remove the paraffin oil completely The supporting 12 μ thick polyester membrane used (Mylar^{R} Du Pont De Nemours) has been found to be very suitable for X-ray absorption measurements because of its physical and chemical resistance and uniform absorption characteristics (Haljamae 1969)



Fig. 2 a. Isolated cell bodies of spinal anterior horn neurones from the anterolateral nuclei of the 7th cervical segment of a newborn rat. b Corresponding cell bodies of 180-day-old rat. Phase contrast 180.

As noted above, Rosengren s direct \ ray absorption technique (Rosengren 1959) was used for the mass determinations. The essential equipment consists of an \-ray tube of the sealed-off type (a 0 μ aluminum filter also serves as the window) fed from servo-regulated, stabilized anode and filament current supplies, a specimen holder and specimen stage with an aperture, and a proportional counter tube with a decatron counter and a display unit (Fig. 3)

The aluminium ring with the membrane was attached to the specimen holder with pressure sensitive tape and the specimen holder was placed on the specimen stage. A nerve cell was centered on the aperture with the aid of a light microscope provided with both incident and transmitted light. The \-ray transmission was then measured with the cell centered over the aperture followed by background reading of the foil close to the cell. The mass value was calculated from the observed difference in transmission.

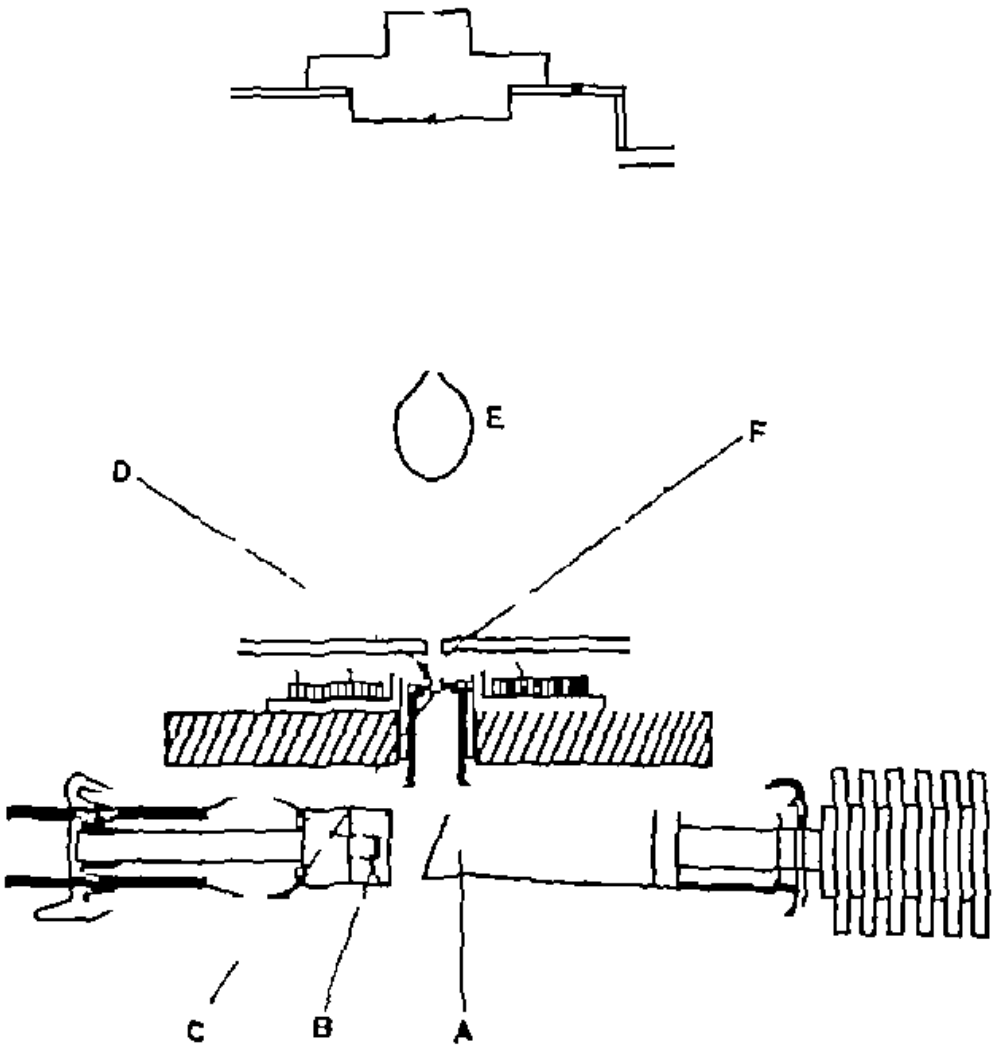


Fig 3. \-ray tube specimen mount and proportional detector \ — anode B — filament. C — 0 μ Al. window D — pecimen tape with aperture E — high voltage lectrode. F — specimen. (Reproduced by kind permission from B Rosengren: Acta Radiologica, Suppl 178 1959).

The standard curves used in the calculations were based on a nitrocellulose reference system covering a transmission range from 95 to 75 per cent. With the apertures used this corresponded to cellular mass values from 0.41 to 11.45 × 10^3 pg. The rather large size range of the neurones from animals of different ages necessitated the use of three different apertures, 30, 50 and 80 μ in diameter. The apertures were made of thin gold foil, as described by Rosengren (1959).

The accuracy of the transmission measurements is influenced by the instability of the apparatus (see Rosengren 1959) and by variations in the positioning of the cell over the aperture and in the placing of the specimen stage over the window of the X-ray tube. To analyse the random variation of the transmission measurements 36 successive measurements were made on the same cell, repositioned over the aperture after each measurement. The mean transmission value proved to be 90.3 per cent and the standard deviation 1.2 per cent. This corresponds to a mean mass value of 1.62 × 10^3 pg and a standard deviation of 0.51 × 10^3 pg or about 11 per cent. This figure is somewhat larger than that reported by Rosengren (1959) who states that the inaccuracy of the method is about 5 to 6 per cent.

b. Determination of neuronal RNA content and composition

For the determination of the RNA content of the isolated cell bodies of anterior horn neurones the method devised by Edström (1961) was used. The isolated nerve cell bodies were transferred in pairs through the oil chamber to a clean cover glass and left to dry. The cell pairs were then incubated for 1 hour at 37° C in droplets of buffered ribonuclease solution (Worthington Biochemical Corporation, Freehold, New Jersey; free of proteolytic activity). The individual extracts were transferred in a micropipette to a dry quartz slide where they were evaporated to dryness. The extraction and evaporation procedure was repeated three times. The spots, each containing all three extracts of a cell pair, were then dissolved in a buffer solution to form lens-shaped droplets. The droplets were photographed in ultraviolet light at 257 mμ together with a reference system at a magnification of × 130. The pictures of the ultraviolet absorbing droplets (Fig. 4a & b) were finally investigated by photometry at a further magnification of × 5. Two tracks were run through the centre and superimposed (Fig. 4c). A mean curve was drawn on transparent paper and the amounts of RNA per droplet determined with the aid of a device for integration of the absorption.

The accuracy of the method was checked with measured amounts of RNA. With amounts of 211 pg RNA the standard deviation was 13 and the coefficient of variation 6.2. These values agree well with those reported by Edström (1961). The completeness of extraction was checked at times by staining with methylene blue in 0.01 N acetic acid.

A tentative microelectrophoretic analysis was made of the base composition of the neuronal RNA after hydrolysis with hot 1N HCL, according to Edström (1961).



Fig. 4. a. Microdrops, each containing the pooled RNA extract of liver and rib bone marrow neurones from newborn rat photographed in ultraviolet light at 257 mμ together with the reference system (×130). b. Corresponding RNA extract from 180-day-old rat. c. Microphotodensitometric tracings of the photographs of the ultraviolet light absorbing RNA extract from newborn rat (left) and 180-day-old rat (right).

D Statistical methods

The standard deviation and the standard error of the mean (S.E.M.) of the neuronal dry mass and RNA content of each individual animal as well as of the different age groups were calculated according to the usual formula. The statistics obtained in this manner are given in connection with the various results.

In dealing with neuronal mass or RNA content as a function of age, it appeared desirable to make inference on the corresponding regression function in its entirety not only on individual values of the independent variable (age). Accordingly it was considered preferable to measure the precision of the estimation of the entire function rather than to analyze only the confidence limits for various individual values of the regression function. Thus, by using the principles of simultaneous statistical inference, to be more specific, the Bonferroni inequality (Miller 1966), and without any assumptions as to the shape of the regression function, confidence bands were constructed. Assuming some slight regularity of the curve describing the regression function such a band contains the entire regression function with a prescribed probability. An attempt was also made to describe the relation between neuronal mass or RNA content and the age or body weight of the animals in simple mathematical terms. For this purpose various equations have been tried on the material.

The nature of the correlation between neuronal dry mass and RNA content was investigated by means of single regression analysis. The product-moment correlation coefficient was also estimated.

The significance of the difference between the means of various experimental and control groups was analyzed by means of Student's t test.

III. DRY MASS AND RNA CONTENT OF RAT SPINAL ANTERIOR HORN NEURONES DURING NORMAL POSTNATAL DEVELOPMENT

A Review of the literature

While the literature contains a great deal of quantitative data on the morphologic development of the nerve cell (cf Brizzee and Jacobs 1959 Mellström and Skoglund 1969 Conradi and Skoglund 1969) quantitative information concerning the chemical maturation of the neurone is relatively sparse. This is also true as far as the RNA and protein metabolism of the nerve cell is concerned though the topographic distribution of the nucleic acids in developing nerve cells has been examined more frequently (cf Spector 1965, Friede 1966 Lodin *et al* 1968).

Ultraviolet spectrography was the first reliable method to be used for the quantitative analysis of nucleic acids in single cells (Caspersson 1940). This method was applied by Hydén (1943) to the study of nucleic acids and proteins in the spinal neuroblasts and young anterior horn cells of rat and other mammalian embryos. The protein content was reckoned approximately with the aid of the absorption due to the cyclic amino acids at 2800 Å. In order to estimate the total content of nucleic acids or protein *per cell*, the volume of the Carnoy-fixed cells was computed from measurements of cell diameter Roughly estimated by these means, the cytoplasmic proteins in the cell body were found to increase more than 2000 fold during the development of the neurone from an apolar neuroblast to an adult anterior horn cell. When the neurite was included it increased much more. Judging from the ultraviolet absorption spectra the proteins increased at a higher rate than the nucleic acids during embryonic development. Hydén concluded that instead of cessation of growth together with a reduction in the size of the nucleolar apparatus, as in most other embryonal cells, the young nerve cell passes through a second period of growth during which the nucleolar material as well as the cytoplasmic nucleotides and proteins rapidly increase.

Sourander (1953) investigated the dry mass and nucleic acid content of spinal anterior horn cells from the ventrolateral nuclei of the brachial swelling of chick embryos. The mass *per* unit of volume was measured using X-ray microradiography and changes in concentration of nucleic acids were estimated with the aid of ultraviolet microscopy and photography Cell body volume was approximately calculated by the method used by Hydén (1943). The amount of dry

substance or the bulk of the protein *per* unit of volume increased from the 7th day of incubation to the 9th day. After 9 days of incubation the values for the amount of dry substance in the cytoplasm remained at about the same level. Calculations of the mass *per* cellular cytoplasm showed a 12 fold increase in mass between the 9th and 21st day of incubation. The young nerve cells absorbed ultraviolet light intensely at 2570 Å, near the absorption maximum of the nucleic acids. No differences in the intensity of absorption were observed at different stages.

Hughes (1955) studied the relative density in ultraviolet light of developing nerve cells of embryonal chick spinal cord and spinal ganglia with a simple microdensitometer. He observed a peak in the cytoplasmic density of nucleic acids at 2—5 days of incubation. On the 6th day the level of nucleic acid was low, but thereafter there was a steady rise in the cytoplasmic concentration up to the 12th day. He emphasized the similarity between his own results and the biochemical findings of Novikoff and Potter (1948) on whole chick embryos and concluded that the density of RNA was an index of neuronal differentiation.

The only quantitative information available on postnatal changes in the nucleic acids of spinal anterior horn cells is the short comment by Hydén (1960) that the RNA content of these cells in the human spinal cord increases from 3 to 40 years of age, followed by a constant period up to 55—60 years. At higher ages there is a *decrease*.

The postnatal development of rabbit retinal ganglion cells was analysed by Brattgård (1952). Applying X-ray microradiography on Carnoy-fixed material from rabbits 11 days, 10 weeks and 8 months old, he found an increase in the total dry weight *per* unit of volume from 11 days to 8 months of age. The pentose nucleoprotein fraction (estimated after treatment with ribonuclease) already reached a high level at 10 weeks of age, after which there was no significant change. On the contrary, the protein fraction at 8 months was markedly higher than that at 10 weeks of age.

Ringborg (1960) investigated the content and composition of RNA in the pyramidal cells of rat hippocampus in foetal, newborn, adult and old rats. Using Edstrom's microchemical methods (Edström 1961) he observed an increase in the neuronal RNA content from 21 pg in the newborn to 110 pg in the adult rats. In the very old rats the neuronal RNA content was down to 53 pg. He also observed that *the guanine-cytosine/adenine-uracil-ratio increased with advancing age*.

Numerous quantitative studies have dealt with the biochemistry of nucleic acids and proteins of the developing nervous system at the organ level (for recent comprehensive reviews see Oja 1966, Dobbing 1968, Miller 1969, Talwar 1969, Rappoport *et al.* 1969, Roberts *et al.* 1970). Most of these studies have been concerned with the whole brain or its subdivisions, the extremely scanty information concerning the spinal cord has been reviewed by Levi (1969). Winick and collaborators (Winick and Noble 1965, Winick 1968, Fish and Winick 1969) have tried

to elucidate trends in the cellular growth of the brain on the basis of such gross biochemical analyses of the whole organ or its parts. Using a principle devised by Enesco and LeBlond (1962), they have calculated the mean cellular RNA and protein content of brain cells by determining the total amounts of DNA, RNA and protein *per* brain. As in other organs, Winick and collaborators found that three phases could be separated in the cellular growth of the brain During the first period, hyperplasia, DNA RNA and protein increased in proportion. This was followed by the period of hyperplasia and hypertrophy when DNA synthesis still continued but RNA and protein increased out of proportion to DNA increment. During the last period, that of hypertrophy alone, DNA synthesis stopped while RNA and protein continued to accumulate indicating increase in cell size.

Although the biochemical procedures mentioned above do give valuable information on the overall trends of cellular growth in many organs, they do not account for the extraordinary structural complexity of nervous tissue. The results of the macrochemical determinations merely reflect the sum of numerous simultaneous cellular processes following extremely varying time schedules in the different cell populations and anatomical regions of the nervous system. The neurones, the basic structural and functional units of the nervous system constitute only a fraction of the total number of cells, or total volume, of the nervous tissue (Pope 1955). Thus, conclusions reached on the basis of averaging biochemical determinations cannot be considered valid for nerve cells.

To sum up, it may be stated that previous quantitative cytochemical studies have mainly been concerned with the early embryonal period of neuronal development and that in most of these studies spinal anterior horn neurones have served as model. On the other hand, relatively little attention has been devoted to the postnatal growth and differentiation of the mammalian nerve cell. The available information is limited to very few ages and practically nothing is known about neuronal development during the immediate postnatal period.

B Results of the present investigations

1 Neuronal dry mass

Neuronal dry mass as function of age. Table IV gives the mean values, S.E.M., and frequency distributions in groups of 0.50×10^3 pg of the dry mass of the cell bodies of Carnoy fixed rat spinal anterior horn neurones from the anterolateral nucleus of the seventh cervical segment at different ages.

In Fig 5 the frequency distributions of the neuronal dry mass in some age groups are graphically shown in histograms. From the table and the histograms it is seen that both the median and the range of the frequency distributions of the neuronal dry mass change with advancing age. At birth the distribution of the neuronal mass is seen to fall within a relatively narrow range, i.e. the mass values

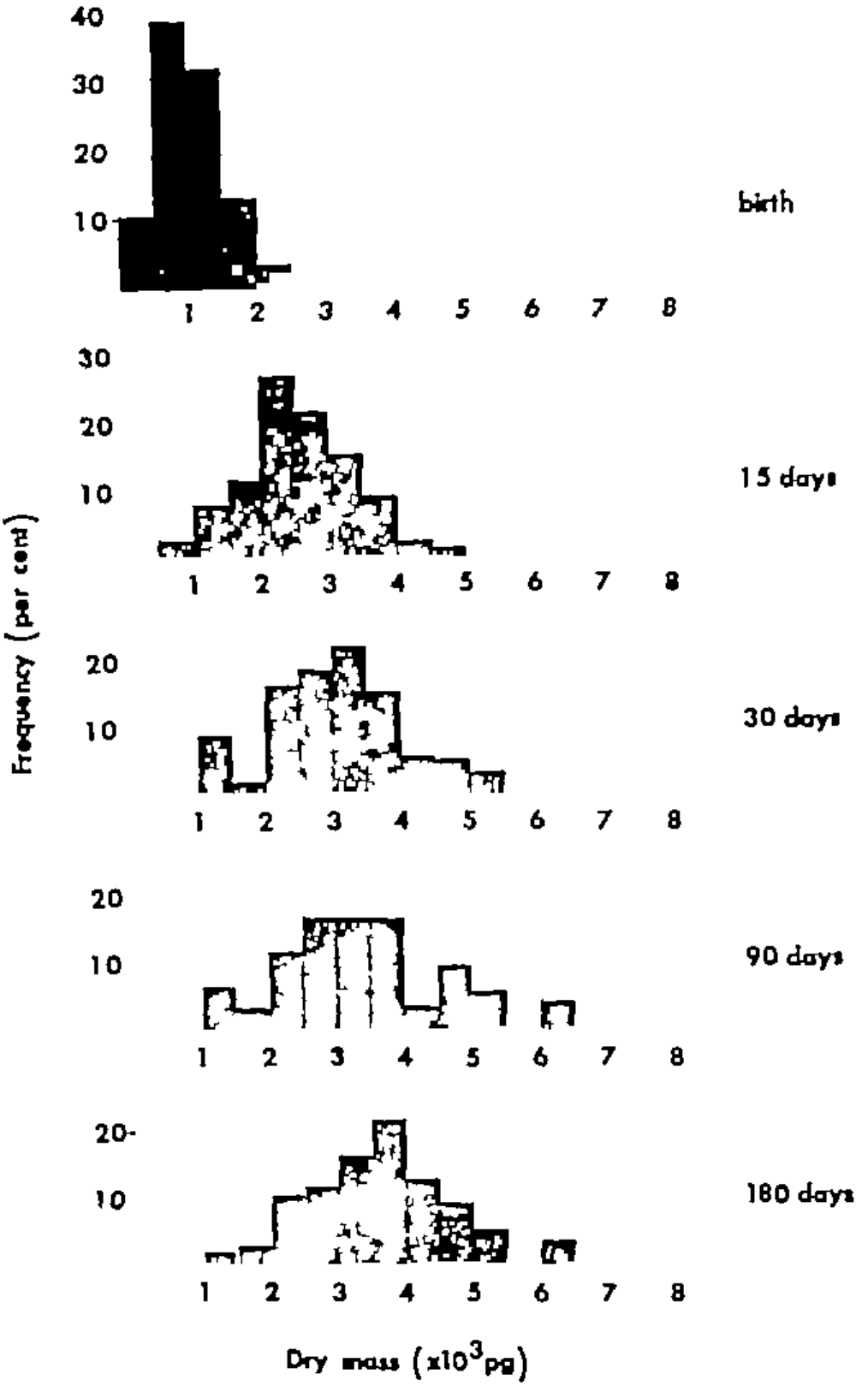


Fig. 3. Frequency distribution of the dry mass of the cell bodies of spinal anterior horn neurones from the 7th cervical segment of normal rats at various ages.

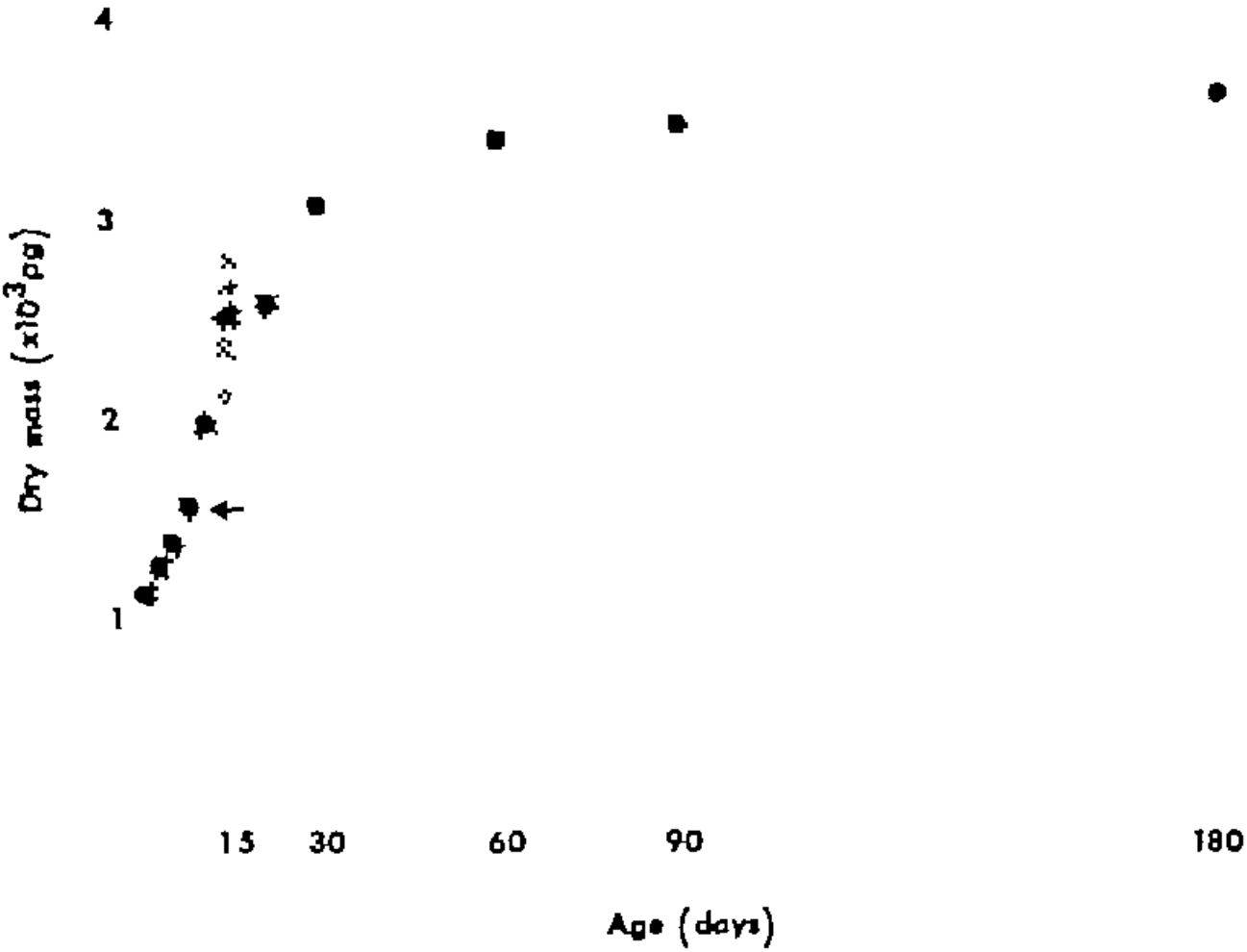


Fig. 6. Mean dry mass f th cell bodies f spinal anterior horn neurones from the 7th cervical segment f normal rats plotted against ge Each pol t represents the mean alue for 6 animals. The angular pol ts of the ppe and lower edges f the shadowed area give the observed ppe and low confidence limits, respectively on a confidence level exceeding 99 per cent, for the mean neuronal dry mass of the corresponding age. This implies that the simultaneous confidence level for all confidence interval is over 90 pe cent. N t the rapid increase f the mean neuronal dry mass aft the age f 3 days (plle).

are rather homogeneous. During the first days of postnatal life the changes are slight but from the age of 10 days onwards there is a marked shift of the entire distribution to the right and an increase in the distribution range At 30 days of age the distribution of the neuronal dry mass is already very much like that at 180 days.

From table IV it is seen that the mean dry mass of the anterior horn neurones increases from 1 10 $\pm$ 0.0) $\times$ 10^3 pg at birth to 3.60 $\pm$ 0 11 $\times$ 10^3 pg at the age of 180 days. The postnatal increase in mean neuronal mass is thus more than threefold (3.3 times)

The change in mean neuronal dry mass with advancing age is graphically shown in Fig. 6. The simultaneous confidence level for the confidence intervals in Fig. 6 was calculated without any assumptions as to the shape of the curve representing the neuronal mass as a function of age. Assuming some slight regu-

lurity of the curve, the shadowed area can be regarded as a confidence band for the curve on a confidence level exceeding 90 per cent. On the basis of these calculations it appears sound to assume that the curve representing the neuronal dry mass as a function of age is convex-concave (S-shaped) with a very steep rise between the ages of 8 and 16 days. Nearly adult values are already attained at 30 days of age after which there is only a very slow increase in neuronal dry mass up to the age of 180 days.

An attempt was also made to describe the neuronal dry mass as a function of age in simple mathematical terms. The simplest function, which describes the observed values satisfactorily was found to be a rational function

$$y = \frac{3290\ x^2 + 157000\ x + 1403000}{x^2 + 29\ x + 1280}$$

In this equation y is the neuronal dry mass in pg and x the age of the animal in days.

Neuronal dry mass in relation to body size. Table I and Fig 7 show the body weights of the rats used in the present study at the time of sacrifice The steepest

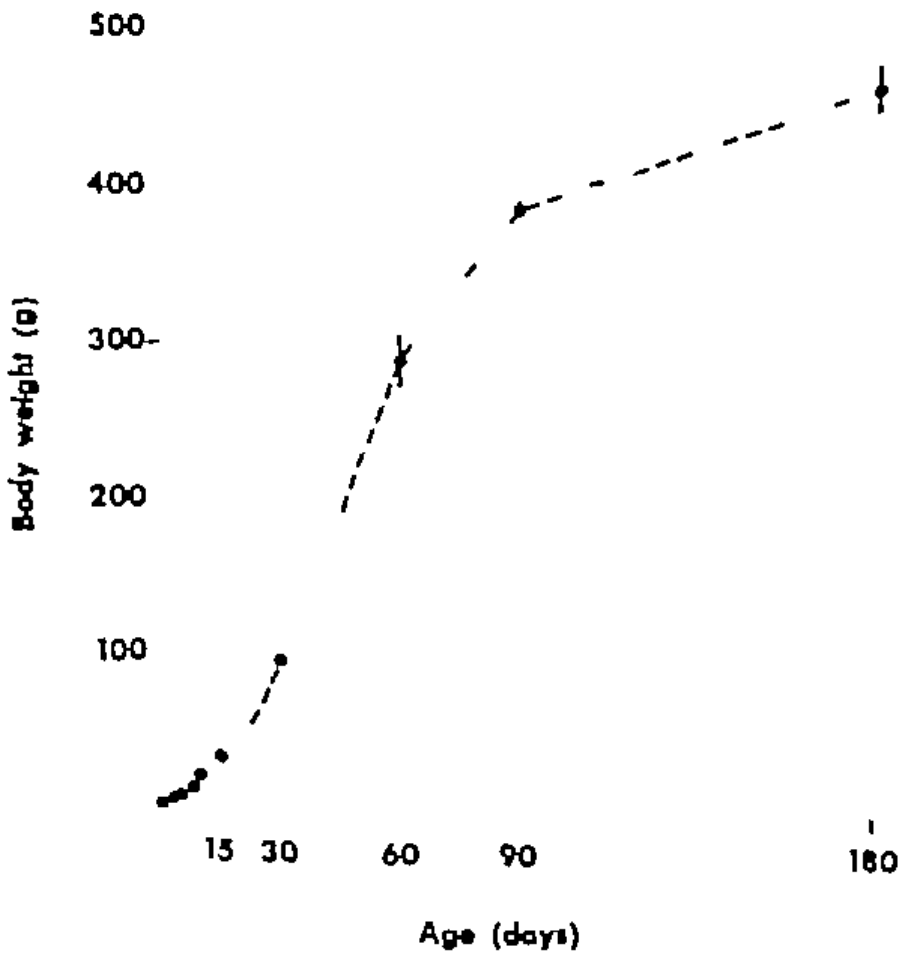


Fig 7 Mean body w ights ± S E M t th time f sacrifice f the normal rat plotted against ge

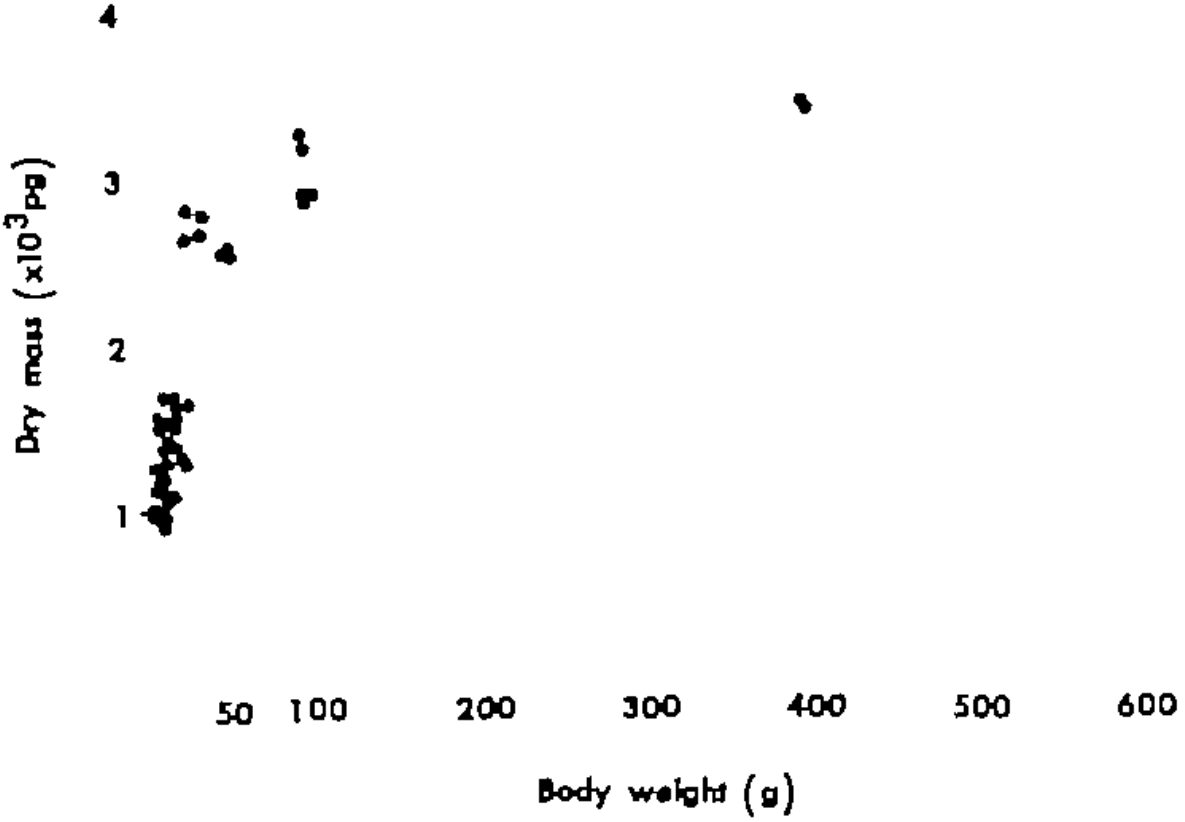


Fig. 8. Mean dry mass of the cell bodies of spinal anterior horn neurones from the 7th cervical segment of normal rats plotted against body weight.

rise of the body weight curve is seen between one and two months of age. It is thus evident, by comparison with Fig. 6 that the increase in mean neuronal dry mass principally occurs before the main increase in body weight.

In Fig. 8 the mean neuronal dry mass is plotted against the body weight. The scatter diagram shows a steep, nearly linear rise in neuronal dry mass from birth weight up to a body weight of about 50 g However increase of body weight beyond 100 g is accompanied with only a very slight increase in neuronal dry mass. It is thus obvious that no direct proportional relation exists between these variables. For the purpose of description a simple mathematical expression for the relation between neuronal dry mass and body weight was sought. The simplest function which describes the observed values well was found to be a rational function

$$y = \frac{3550\ x - 11100}{x + 10}$$

In this equation y is the neuronal dry mass value in pg and x is the body weight of the animal in g

To illustrate the relation between the neuronal dry mass and body weight with increasing age the mean neuronal dry mass/body weight ratio for each age

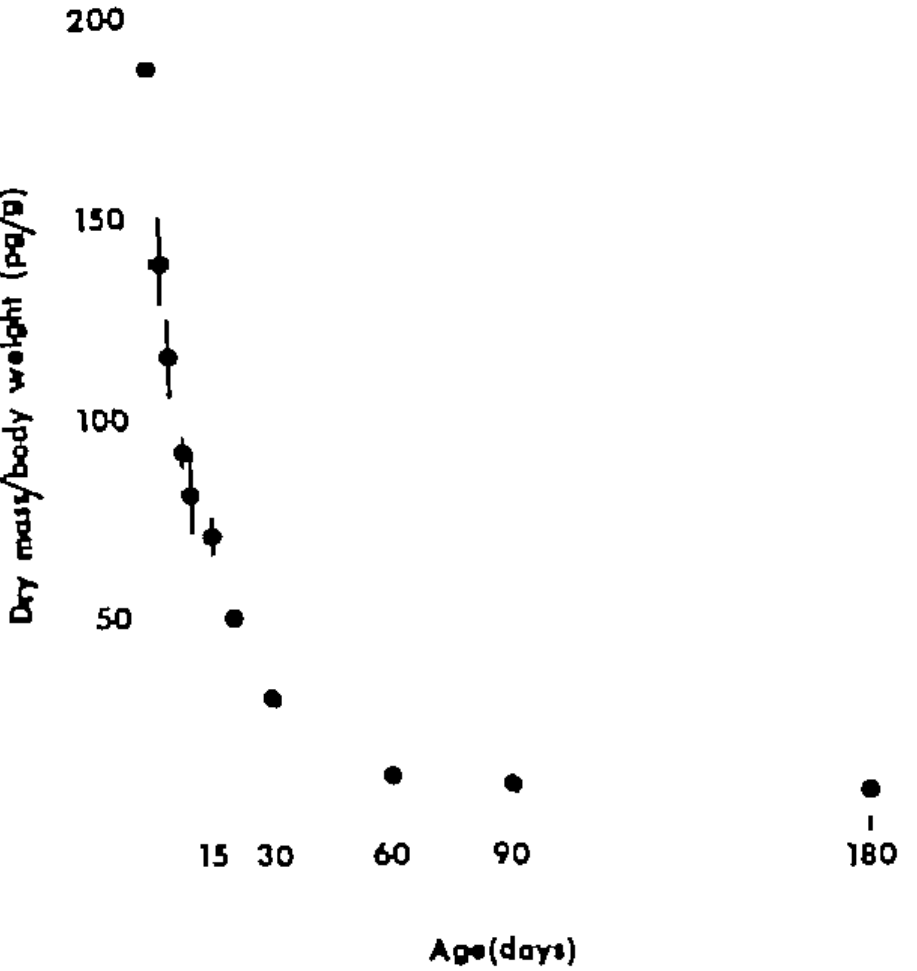


Fig. 9 Mean neuronal dry mass/body weight ratio ± S.E.M. in normal rats plotted against age. Each point represents the mean value for 6 animals.

group is calculated and plotted against age (Fig. 9). It will be seen that there is a continuous decrease in the neuronal dry mass/body weight ratio with advancing age. This decrease is very rapid during the first three postnatal weeks but then gradually slows down and the ratio reaches a nearly constant level at the age of three months.

As the growth characteristics of the anterior horn neurones and body weight thus obviously differed very markedly from each other, an attempt was made to plot the mean neuronal dry mass values against other parameters of somatic growth. A scatter diagram suggesting a roughly linear correlation (Fig. 10) was then obtained using the length of the humerus and ulna added together to denote extremity length. These values were obtained from Donaldson's tables (1924) giving the length of rat humerus and ulna as a function of body weight.

Neuronal RNA content and composition

Neuronal RNA content as function of age. Table V gives the mean values, S.E.M., and frequency distributions in groups of 25 pg of the RNA content of the cell

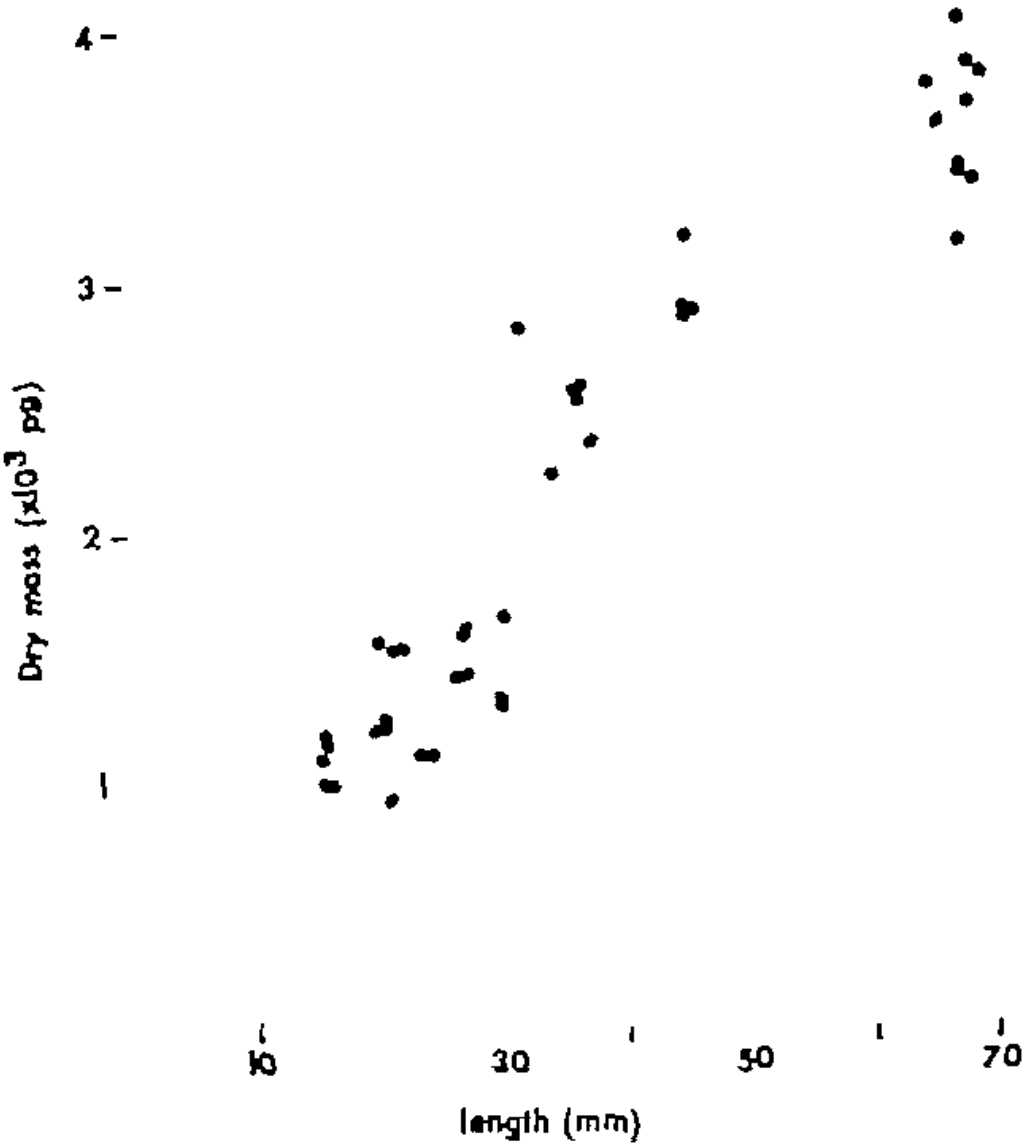


Fig. 10 Mean dry mass of the cell bodies of spinal anterior horn neurones from the 7th cervical segment of normal rat plotted against extremity length (length of humerus and ulna added together)

bodies of rat spinal anterior horn neurones from the anterolateral nucleus of the seventh cervical segment at different ages.

In Fig. 11 the distribution of the neuronal RNA content in some age groups is graphically shown in histograms. From the table and the histograms it will be seen that the median and the range of the frequency distribution of the neuronal RNA content change with advancing age. At birth the distribution of the neuronal RNA content is seen to be within a rather small range. From the age of 8 days onwards a rapid shift of the entire distribution to the right and a marked increase in the distribution range are observed. At the age of 30 days the distribution range is already very much like that at 180 days.

Table V shows that the mean neuronal RNA content increases from 120 ± 1 pg at birth to 500 ± 23 pg at 90 days of age to remain at approximately the

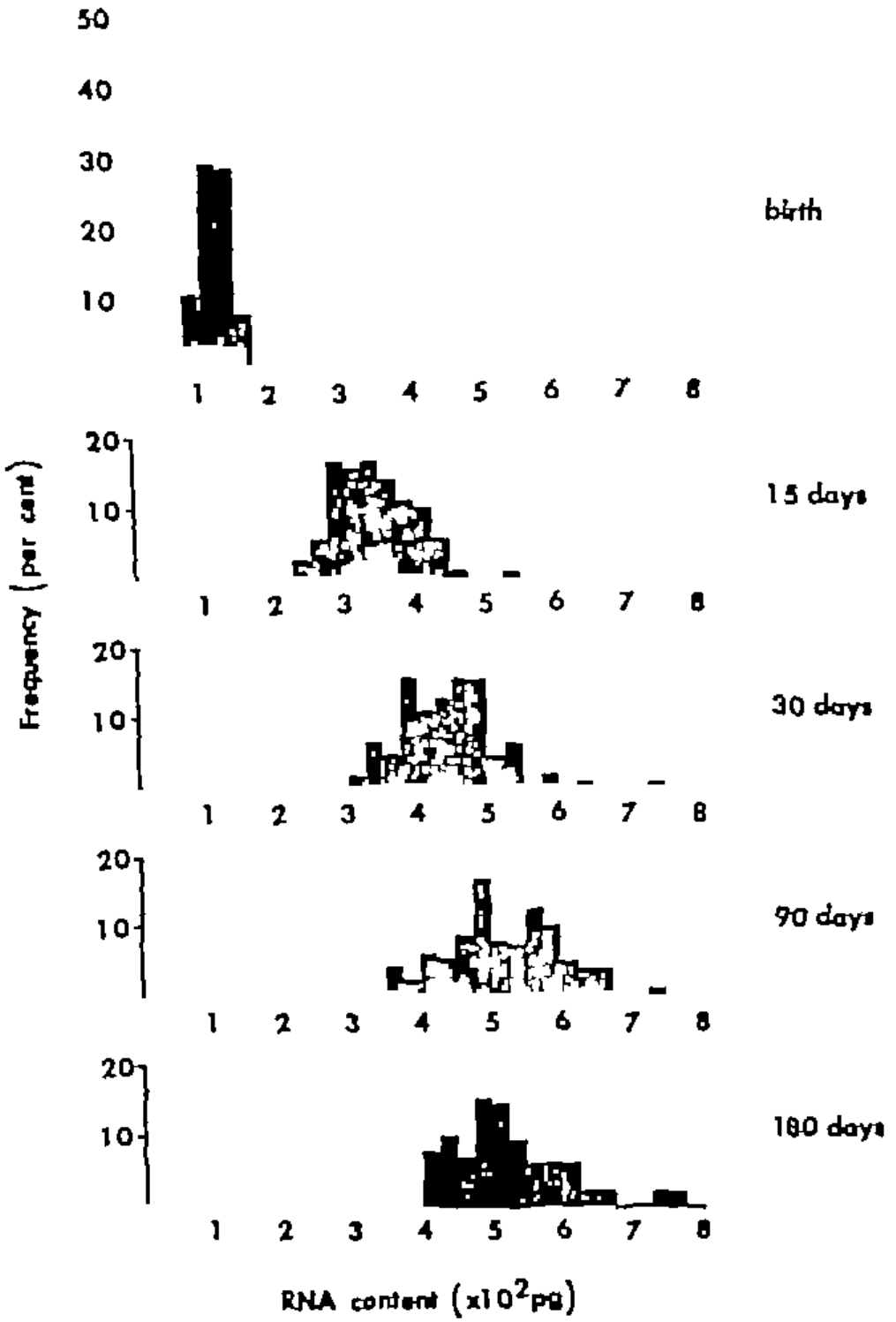


Fig. 11 Frequency distribution of the RNA content of the cell bodies of spinal anterior horn neurones from the 4th cervical segment of normal rat at various ages.

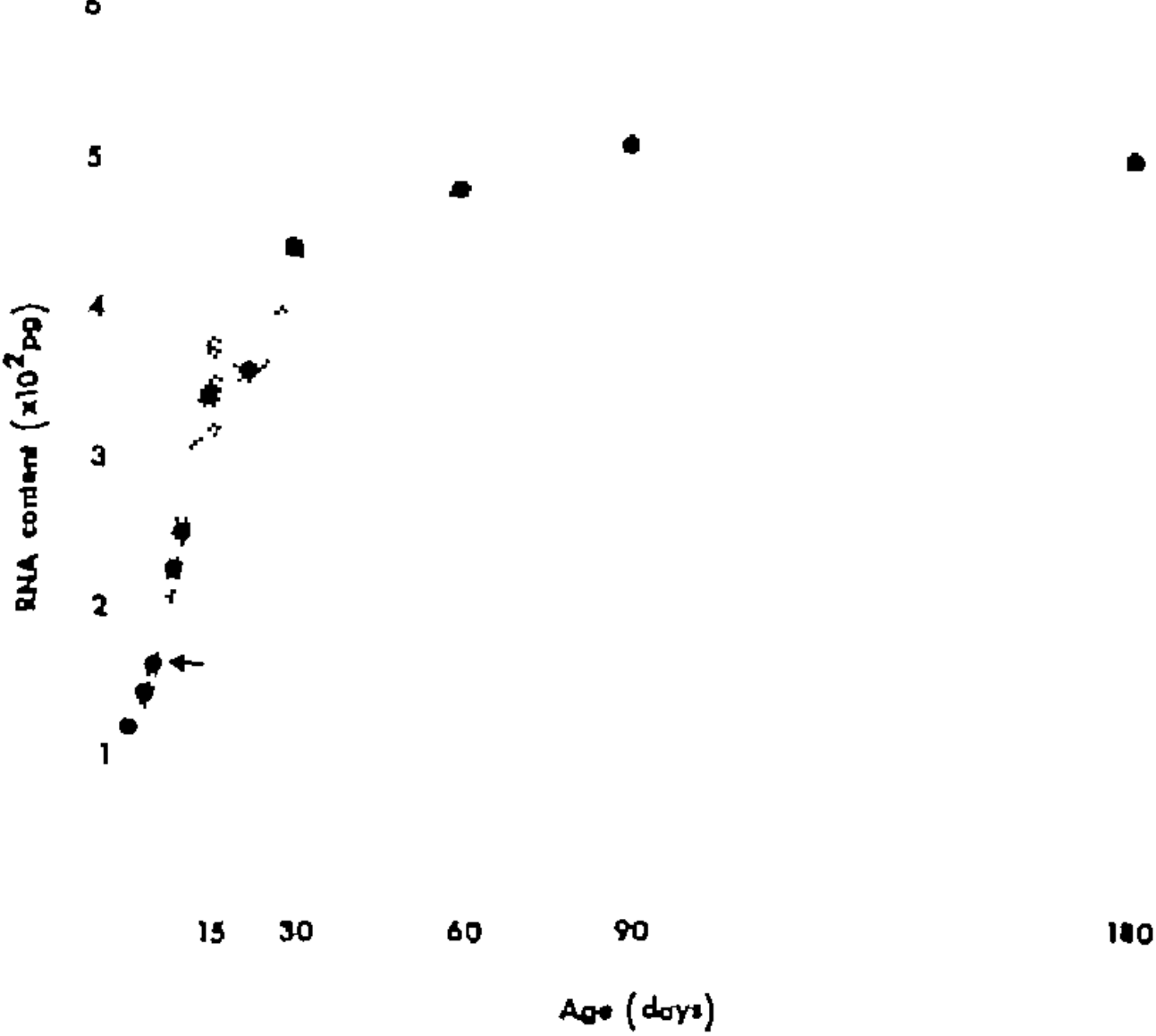


Fig. 12. Mean RNA content of the cell bodies of spinal anterior horn neurones from the 7th cervical segment of normal rats plotted against age. Each point represents the mean value for 6 animals. Statistical calculations as in Fig. 6. Note the rapid increase in the mean neuronal RNA content after the age of 5 days (pile).

same level at 180 days of age. The mean neuronal RNA content has thus increased postnatally more than four-fold (4.2 times).

The change in the mean neuronal RNA content with advancing age is graphically shown in Fig. 12. The simultaneous confidence level for the confidence intervals in Fig. 12 was calculated without any assumptions as to the shape of the curve representing the neuronal RNA content as a function of age. Assuming again some slight regularity of the curve, the shadowed area can be regarded as a confidence band for the curve on a confidence level exceeding 90 per cent. On the basis of these calculations it appears sound to assume that also the curve representing the neuronal RNA content as a function of age is convex-concave (S-shaped) with a very steep rise between the ages of 5 and 15 days. Nearly adult values are

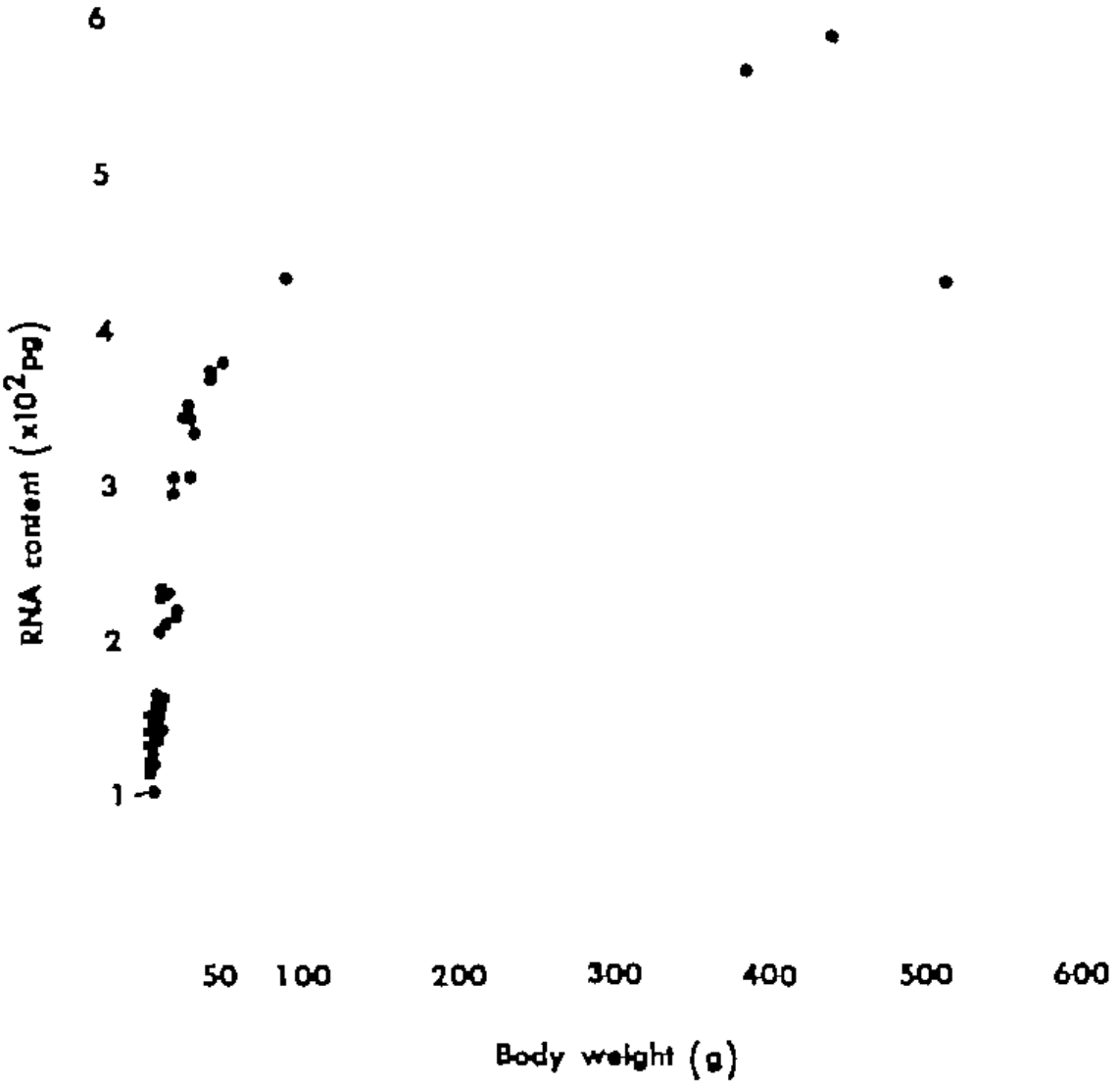


Fig. 13 Mean RNA content of the cell bodies of spinal anterior horn neurones from the 7th cervical segment of normal rats plotted against body weight.

already attained at the age of 30 days after which the increase is very slow. A plateau is reached at 90 days of age. The rational function

$$y = \frac{185\ x^3 + 15000\ x + 122800}{x^3 + 19\ x + 1000}$$

was found to describe the observed values well. In this equation y is the neuronal RNA content in pg and x the age of the animal in days.

Neuronal RNA content in relation to body size. It is evident by comparing Figs. 7 and 12 that the increase in neuronal RNA content principally occurs before the main growth spurt of the whole body.

In Fig. 13 the neuronal RNA content is plotted against total body weight. The scatter diagram shows a steep, nearly linear rise in the neuronal RNA content up to a body weight of approximately 50 g. Above this body weight there is only

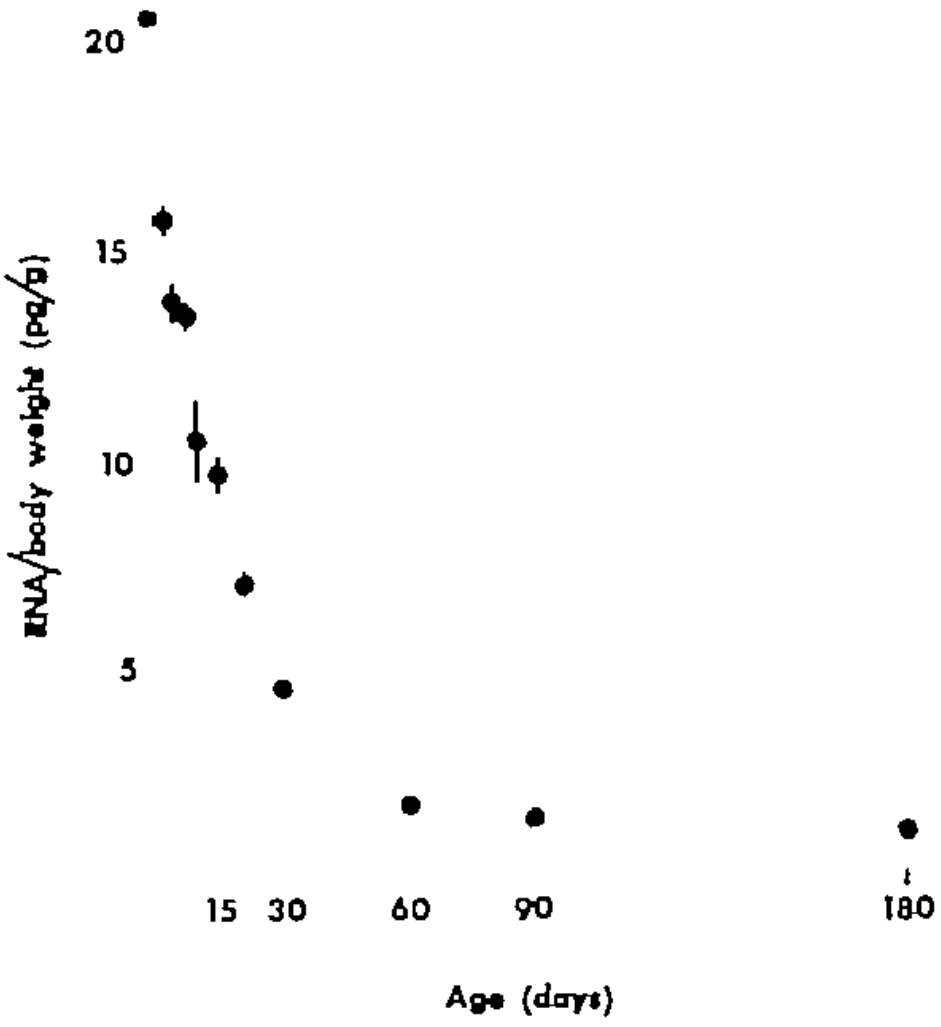


Fig. 14 Mean neuronal RNA content/body weight ratio ± S.E.M. in normal rats plotted against age. Each point represents the mean value for 6 animals.

a very slight increase in neuronal RNA content. An attempt has been made to describe the neuronal RNA content in relation to body weight with a single equation. The simplest function which describes the values actually observed satisfactorily was found to be a rational function

$$y = \frac{520\,x - 2100}{x + 14}$$

In this equation y is the neuronal RNA content in pg and x is the body weight of the animal in g. There was a rapid decrease in the neuronal RNA content/body weight ratio after birth (Fig 14)

A scatter diagram (Fig. 15) suggesting a roughly linear correlation was again obtained when the mean values for the neuronal RNA content were plotted against sextremity lengths (see p. 26).

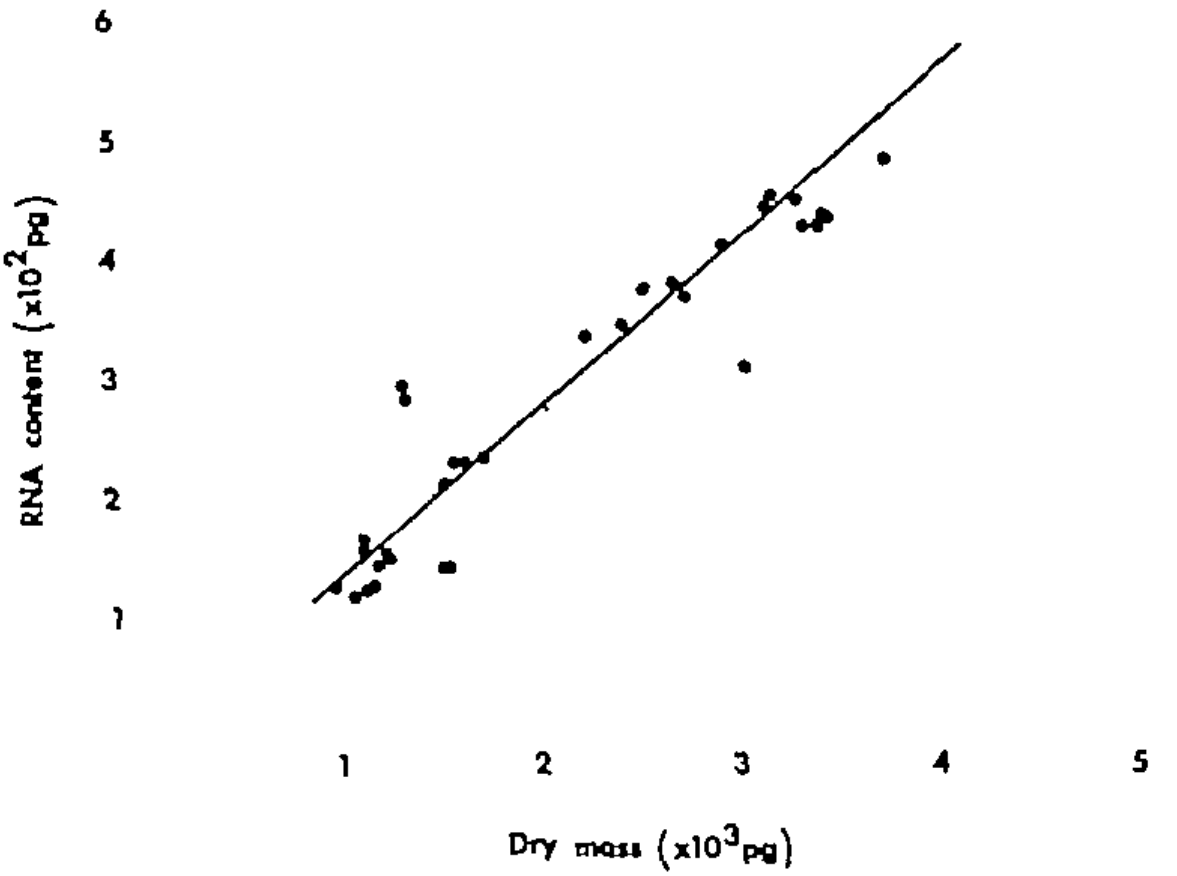


Fig. 17 Mean neuronal RNA content of normal rats of various ages plotted against corresponding mean neuronal dry mass. The regression line has been calculated by using the method of least squares.

reaches a high level at about 8 days of age. This level is then maintained until adult age

C. Discussion

The entire population of large spinal anterior horn neurones studied appears to follow a rather uniform developmental pattern. There is a shift of the whole frequency distribution of both neuronal dry mass and RNA content indicating an increase in the dry mass and RNA content of all cells of the population studied. On the other hand, the distribution range of both of these parameters is widened with advancing age reflecting differences in the rate of accumulation of cell constituents between individual anterior horn neurones. This agrees with the volumetric observations of Mellstrom and Skoglund (1980) of a differential growth rate of cat anterior horn neurones during postnatal development. At all ages they also noted a persistent peak, corresponding to the volume groups with the highest frequencies during the immediate postnatal period. Such a peak was not observed in the present study obviously because of the different principles of sampling.

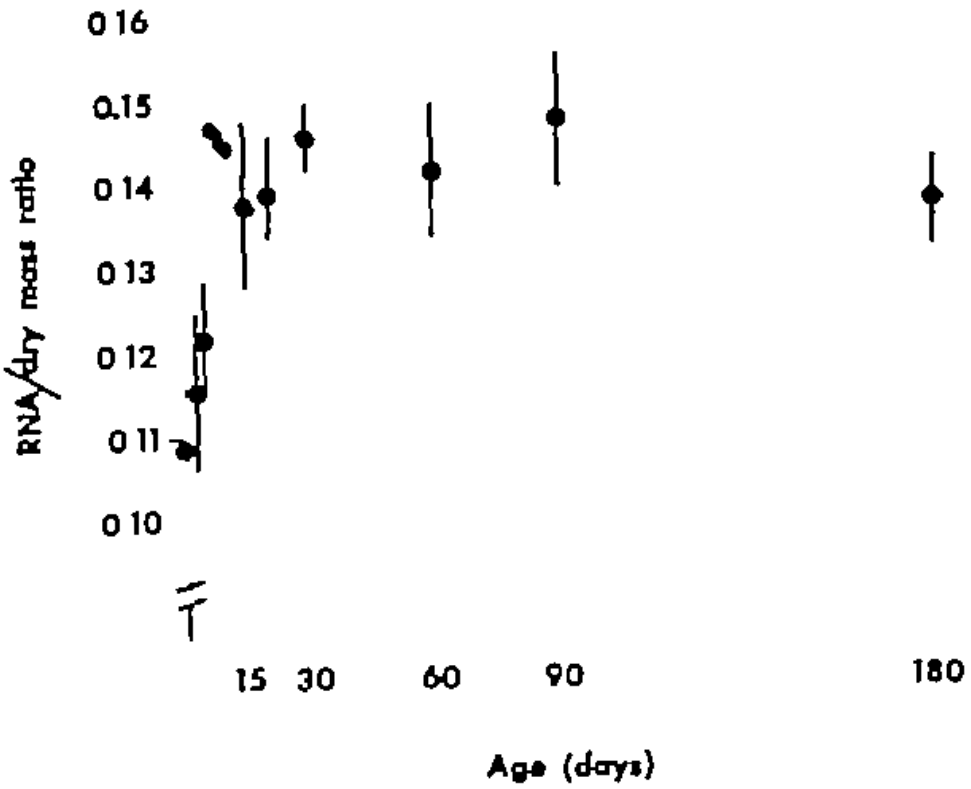


Fig. 18. Mean neuronal RNA content/dry mass ratio ± S.E.M. in normal rats plotted against age. Each point represents the mean value for 6 animals.

The variation in the neuronal dry mass values appears to be considerably higher than in RNA values. This is obviously partly due to the larger methodological error of the mass determinations. A further explanation is that the RNA determinations were performed in pairs, which reduces the biological variation by a factor of $\frac{1}{\sqrt{2}}$. A true difference in the biological variation of neuronal dry mass and RNA content cannot be excluded, however.

Treatment of the experimental results according to the principles of simultaneous statistical inference (Miller 1966) provides a sound basis for the assumption that the curve representing the mean neuronal dry mass as a function of age is convex-concave (S-shaped). The same is true for the mean neuronal RNA content as a function of age. This means that there is relatively little change in the mean dry mass and RNA content of anterior horn neurones during the first three to five days of postnatal life. Between days 5 and 8 an abrupt increase in mean neuronal RNA content is observed, followed slightly later between days 8 and 10, by a rapid augmentation of neuronal dry mass. A similar acceleration of the growth rate of cat anterior horn neurones at about 14 days of age was noted by Mellström and Skoglund (1969) in their volumetric study. They suggested that this

change in growth rate might be associated with simultaneous qualitative alterations in the metabolism of anterior horn neurones (see below)

The rapid increase in neuronal dry mass and RNA content continues up to 15 days of age and then slows down slightly Nearly adult values are reached at 30 days of age after which only relatively small increases in dry mass and RNA content occur in the anterior horn neurones. An increase in diameter (Donaldson and Nagasaka 1918 and volume (Ford and Cohan 1968) of the cell body of rat spinal anterior horn neurones during the later phases of postnatal development has been reported to follow a similar course. If the differences in the time scale are taken into consideration, the results of Mellström and Skoglund (1960) concerning the postnatal increase in the volume of cat spinal anterior horn cells also fit into this general pattern Sakla (1959) found that the volume of mouse anterior horn cells decreased after the age of 20 days, but his method is open to criticism (Mellström and Skoglund 1969).

It would be tempting to compare the postnatal changes reported to occur in neuronal volume with those in dry mass or RNA content in order to obtain information about possible changes in the concentration of protein or RNA. Unfortunately such comparisons prove to be rather fallacious. Mellström and Skoglund (1969) found a 3.8-fold increase in the mean volume of cervical anterior horn neurones of cats from birth to maturity This figure is close to the 3.3-fold augmentation of neuronal dry mass in rats found in the present study However even if relative volume changes of the same order were measured in rats, this would not justify the conclusion that the protein concentration of anterior horn neurones remains constant during development. As pointed out by Mellström and Skoglund (1969) figures for relative changes in cellular volume during development obtained by measurements on fixed tissue, are probably too high. The X-ray microradiographic observations of Brattgård (1952) and Sourander (1953) suggest, in fact that the neuronal protein concentration actually increases during development

The observations made during the present study on the dry mass and RNA content of mature rat anterior horn neurones appear to be consistent with data provided by previous investigations on other species if due allowance is made for the differences in sampling and the level of the spinal cord. The mean neuronal dry mass after lipid extraction of large spinal anterior horn neurones from the antero-lateral nucleus of the 7th cervical segment of the 180-day-old rats was 3.60×10^3 pg Assuming that lipids constitute about 20 per cent of the total dry mass of the neuronal cell body (Hydén 1960) the total dry mass of these cells would be of the order of 4.5×10^3 pg. This figure is somewhat lower than that reported by Embree *et al* (1967) for rabbit cervical anterior horn neurones (6.7×10^3 pg) They used the same method as was used in the present study for mass determination. Lowry (1957), in an example, quotes the figure 5.8×10^3 pg for the mass of spinal anterior horn cells dissected free hand from freeze-dried material and

weighed with quartz fiber balance. The largest values obtained in the present study correspond to a total mass of 7—8 × 10^3 pg which agrees well with the figures given by Hydén (1960) for anterior horn cells investigated by x ray microradiography

It is hardly possible to compare the dry mass values obtained in the present study with those recently published by Ford and Cohan (1968) They weighed formalin-fixed lumbar anterior horn neurones, dissected free hand from rats aged 15 days to 180 days, with a Sauter micro balance. The mass values obtained by them are about ten times larger than those reported by other workers and also quite inconsistent with the neuronal volumes obtained by themselves. In an earlier investigation (Ford *et al.* 1965) they explain this as possibly being due to their dissection method, by which varying, considerable lengths of cell processes are taken along with the cell bodies.

The values obtained in the present study for the mean neuronal RNA content of spinal anterior horn cells were 500 pg for the 90-day-old rats and 497 pg for the 180-day-old rats. These figures agree well with those given by Edström (1956) for rabbit motor anterior horn cells from the anterolateral nucleus at the thickest part of the cervical enlargement. The values given by Embree *et al.* (1967) for corresponding cells are somewhat higher

As noted above, an acceleration in the increase in the RNA content and dry mass of the anterior horn neurones was noted between 5 and 10 days of age. During this period there is a very distinct peak in the rate of net accumulation of RNA and dry mass, suggesting an increased rate of synthesis of RNA and protein, although the degradation rate might also change during development, as observed at the whole brain level (Oja 1967). The regulation mechanisms controlling the RNA synthesis (transcription) and protein synthesis (translation) in developing mammalian cells are poorly understood, especially as regards such factors as developmental changes in the activity of DNA-dependent RNA polymerase or precursor pools (Mandel and Jacob 1970) Therefore only some tentative suggestions can be made to explain this sudden increase in the rate of accumulation of RNA and protein in the anterior horn neurones.

At the neuroblast stage the beginning of the rapid elaboration of the Nissl substance and of RNA in the cytoplasm seems to depend on the maturation of the nucleolus (e.g. Hydén 1943, Flexner 1955, Sensenbrenner and Mandel 1967 Lodin *et al* 1968). A well developed nucleolus, however is already present in the spinal anterior horn neurones of the rat before birth (Hydén 1943) indicating that other explanations must be sought for the sudden postnatal increase in the rate of RNA accumulation.

Mellstrom and Skoglund (1960) suggested that the postnatal change in the growth rate of cat anterior horn neurones might be associated with alterations in the energy metabolism of these cells. A great increase in the number of mitochondria was demonstrated in cat motoneurones during the first 20 days after

birth. A coincident change in the NAD-diaphorase activity was also observed (Skoglund 1967). These and other simultaneous alterations (Mellström and Skoglund 1969) indicate an increase in aerobic metabolic processes. The accompanying increase in the efficiency of energy production might lead to an intensified synthesis of both RNA and protein. In the rat, as well, the oxygen consumption of the nervous tissue, activity of enzymes linked to the oxidative metabolism, and number of mitochondria increase rather abruptly around the 10th day postnatally (cf. Flexner 1955 Richter 1967 Seller 1969) It is assumed that most of the increase in energy turnover in this period of development is correlated with an increase of the adenosinetriphosphatase system. Thus it may be significant that adenosinetriphosphatase activities start to increase around the 6th day after birth (cf. Seller 1969), coincident with the abrupt acceleration of the rate of RNA accumulation in the anterior horn neurones.

The bulk of the cellular RNA is contained in the cytoplasmic ribosomes (Datta 1966) which constitute an essential part of the protein-synthesizing apparatus of the cell (cf De Man and Noorduyn 1969) Not only an increase in the number of ribosomes (e.g Eschner and Glees 1963, Wechsler 1966) which may thus be interpreted as the morphological counterpart of the increase in the neuronal RNA content, but also a change in the ratio free/membrane-bound ribosomes in maturing nerve cells may influence the rate of protein synthesis during cytodifferentiation (Wechsler and Kleihues 1968, Murthy 1970) Recent evidence suggests that hormones may also influence the rate of neuronal protein synthesis during development, operating primarily through the regulation of polyribosome formation and translation (Tata 1968, Geel and Timiras 1970).

The RNA/dry mass ratio appears to rise during the first 8 days of postnatal life in the cell bodies of the anterior horn neurones, to remain thereafter at an extremely high level. This is in clear contrast to most other somatic cells where the amount of RNA diminishes rapidly in relation to the cellular protein content during the later phases of development (Winick and Noble 1965) The exceptional characteristics of the postnatal RNA and protein metabolism of the neurone may be illustrated by comparing the present cytochemical data with biochemical findings at the organ level. In the whole rat brain the RNA/DNA ratio reflecting the mean cellular RNA content in the brain, was found to rise only up to about 14 days of age, and then to level off On the other hand, there was a consistent, pronounced rise in the protein/DNA ratio up to adult age (e.g Winick and Noble 1965 Oja 1966, Balazs et al. 1968) This results in a rapid decrease in the RNA/protein ratio of brain cells after birth This striking difference between the development of the RNA/protein ratio in the anterior horn cells and the average brain cell probably reflects profound differences in the RNA and protein metabolism of the neurones and glial cells, which are known to differ in their metabolism also in many other respects (Hamberger 1963, Hydén 1968).

The increasing RNA/protein ratio in the cell bodies of the anterior horn neurones

is followed by an increase in the rate of protein accumulation, as evidenced by the sudden increase in the dry mass of the cell body But in spite of the nearly constant dry mass of the cell body after the age of 30 days the RNA/protein ratio remains at an extremely high level until adult age, reflected morphologically by the tremendous number of ribosomes contained in the Nissl substance of adult neurones (Palay and Palade 1955, Peters *et al* 1970) This suggests continued active protein synthesis in the neuronal cell bodies, direct evidence for which has been obtained from studies of incorporation of labeled amino acids (Fisher *et al.* 1956 Altman 1963, Ford and Rhines 1967 Ford *et al* 1967). A possible explanation, for the persistence of this extensive protein-synthesizing apparatus is the dependence of the neuronal processes on the cell body The cell body remains the major site of macromolecular synthesis in the neurone and apparently has to cover the protein needs for the growth and maintenance of the increasingly long cellular processes (cf Weiss 1969 Drox and Koenig 1969 1970) although evidence for some local protein synthesis in the Mauthner axon of fish and in the nerve endings has been presented (cf A Edström 1969 Barondes 1969 Austin *et al* 1970) The persistence of the extremely high RNA/protein ratio in the neuronal cell body may thus reflect the unique cytological organization of the neurone.

It appears reasonable to suppose that the size of the central protein-synthesizing apparatus, reflected by the RNA content of the cell body is at least partly determined by the extent of the neuronal processes. In the large anterior horn neurones innervating the musculature of the extremities (like in those of the 7th cervical segment) the axon is by far the dominating cellular process because of its huge length. If it is assumed that the length of the axon changes in proportion to the length of the extremity then the observation that the RNA content of the cell body increases at roughly the same rate as the length of the extremity suggests, in fact, that a close relationship exists during development between the RNA content of the cell body and axon length.

Previous volumetric studies on the relation between neuronal and somatic growth gave conflicting results. Donaldson and Nagasaka (1918) and Levi (1925) concluded that the volume of spinal ganglion cells increased in proportion to the body area (square of the cube root of body weight) Other investigators found that the rate of growth of the spinal ganglion cells was proportional to the body weight (Olivo *et al.* 1932, Rondinini 1936) and that the growth of the spinal ganglion cells in man continued during the entire life (Pilati 1938). Donaldson and Nagasaka (1918) also noted that the growth in volume of the spinal anterior horn neurones did not follow the same pattern as that of spinal ganglion cells.

If the increase in dry mass is used as an indicator of neuronal growth it is evident that no linear correlation exists between the dry mass of the cell body of the anterior horn neurones and the body weight or body area. The same is true for the neuronal RNA content There is a consistent, rapid drop in the neuronal

dry mass/ body weight ratio as well as in the neuronal RNA content/body weight ratio during postnatal development, indicating that the growth rates of the muscle and the nerve cell bodies are quite different. The real differences in the growth rates are actually still more pronounced as the percentage of muscle of the body weight increases markedly during development (Miller 1960). On the other hand, the dry mass of the nerve cell body appears to increase roughly at the same rate as the length of the extremity. This might indicate that the size of the cell body of an anterior horn neurone is more related to the length of its axon than to the size of its peripheral field of innervation.

Although there was a remarkable increase in the content of RNA of the anterior horn neurones during postnatal development, no significant changes were observed in its base composition. Ringborg (1966) offered as a possible explanation for the increase in the guanine-cytosine/adenine-uracil-ratio observed by him in the pyramidal cells of rat hippocampus that this change might be due to the relative increase in the ribosomal RNA (which has a higher content of guanine and cytosine; Hydén and Egyházi 1962) as opposed to nuclear RNA. This explanation seems quite tenable for the cells of the Ammon's horn which are very immature at birth with extremely scanty cytoplasm in relation to the relatively large nucleus. This is not the case with the anterior horn cells which already have a relatively well developed cytoplasm at birth and in which, consequently the ratio of ribosomal to nuclear RNA need not markedly change.

IV NEURONAL DRY MASS AND RNA CONTENT IN RATS SUBJECTED TO POSTNATAL UNDERNUTRITION

A. Review of the literature

In the early years of this century Donaldson and his collaborators carried out extensive studies, thoroughly reviewed by Jackson (1925), on the effect of under nutrition on the nervous system of adult and growing rats. They found that brain weight was little affected by undernutrition instituted after weaning (Hatai 1908, Donaldson 1911 Jackson 1915). On the other hand, undernutrition from birth to weaning resulted in brain weights below normal for corresponding age but above normal for corresponding body weight (Stewart 1918 a, b Sugita 1918). These early observations on the susceptibility of the developing nervous system to undernutrition were confirmed and extended during recent years by a number of workers using mainly neurochemical methods (cf Dobbing 1968, Counan 1968, Miller 1969 Winick 1969).

Dobbing studied the process of myelination in undernourished pigs and rats, as reflected by changes in cholesterol concentration. He found that undernutrition instituted during the period of active myelination resulted in a permanently lowered brain cholesterol concentration. On the basis of these studies he put forward the general hypothesis that the period of fastest developmental change might be one of heightened vulnerability (Dobbing 1966, 1968). The studies of Winick and collaborators (Winick and Noble 1966, Winick and Rosso 1969) concerning the cellular growth of the brain lend further support to this hypothesis. They demon strated that the total DNA content of the brain in rats and children subjected to undernutrition during the early postnatal period was markedly lower than that of normal controls. This indicates that undernutrition curtails cell division in the brain during the period of active cellular proliferation.

The neurochemical observations cited above, however do not give any infor mation concerning the effect of undernutrition on the nerve cells. The macro-neurones of the rat brain are formed prenatally (Altman and Das 1966) and it is thus not probable that postnatal undernutrition would reduce their number through inhibition of the proliferation of neuroblasts. On the other hand, there is some histological evidence indicating that early undernutrition might affect the growth and maturation of nerve cells. Sugita (1918) reported that the size of cortical neurones was diminished in rats undernourished from birth. Eayrs and

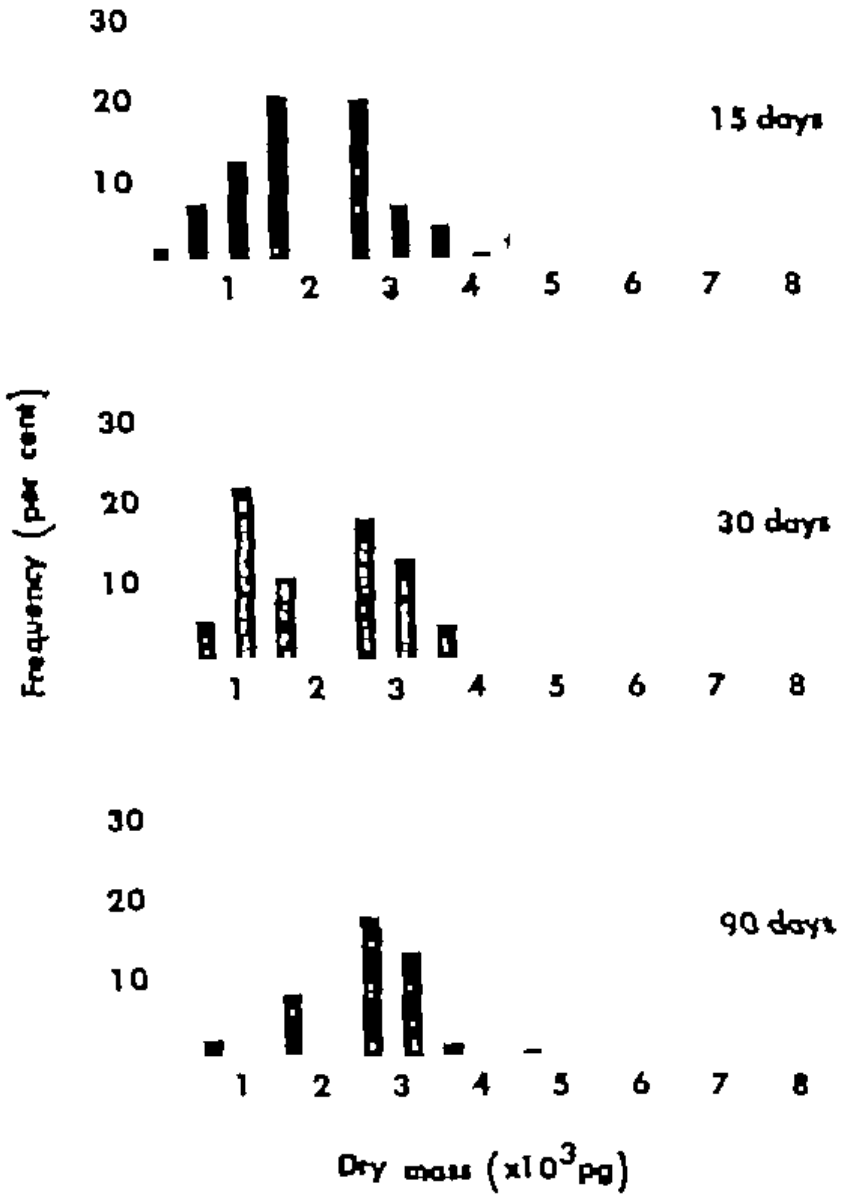


Fig. 19 Frequency distribution of the dry mass of the cell bodies of spinal anterior horn neurones of rat undernourished from the age of 1 day and sacrificed at 15, 30 or 90 days of age (dark stippled) compared with the corresponding frequency distribution for normal rats of the same age (light stippled).

Horn (1955) noted a decrease in the amount of cortical neuropil in such rats. Platt and collaborators observed consistent abnormalities in the spinal anterior horn cells of protein-calorie deficient pigs and dogs. The nerve cells exhibited an apparent loss of Nissl substance (Stewart *et al* 1968).

B. Results of the present investigations

1 Neuronal dry mass

Table VI gives the mean values, S.E.M., and frequency distributions in groups of 0.50×10^3 pg of the neuronal dry mass after lipid extraction of the spinal

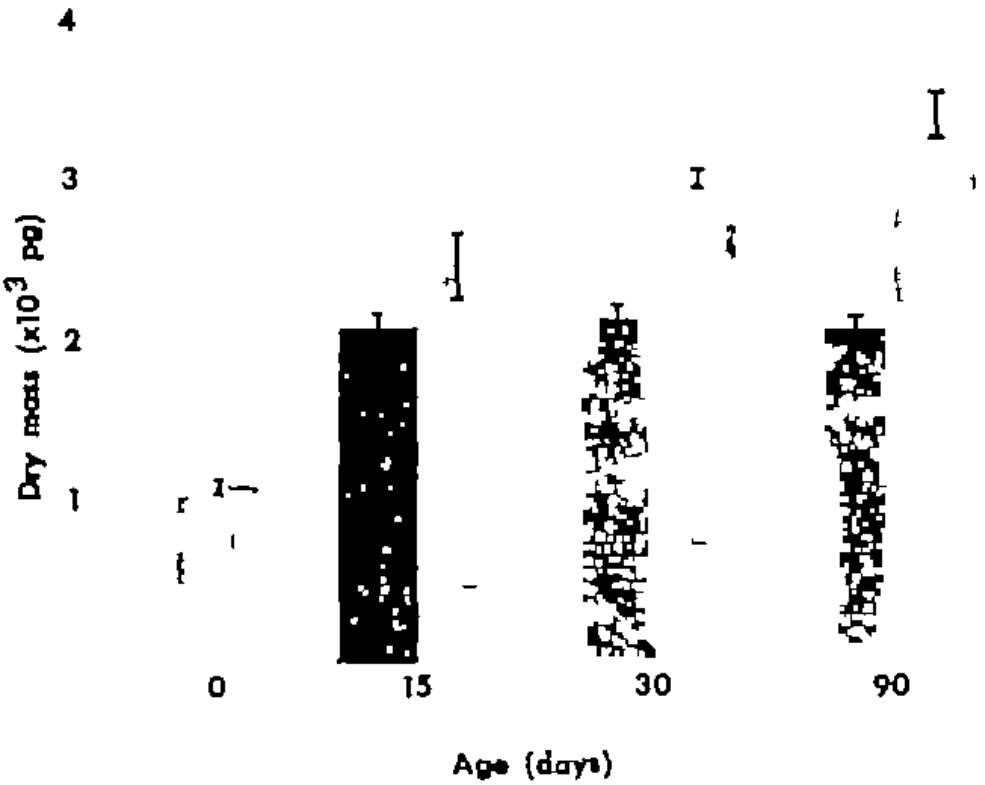


Fig. 20. Mean dry mass of the cell bodies of spinal anterior horn neurones in the various age groups of undernourished rats (dark staples) compared with the corresponding values for normal rats of the same age (light staples).

anterior horn neurones from the 7th cervical segment of rats undernourished from the age of 1 day and sacrificed at the ages of 15 30 or 90 days.

As the body weight, neuronal dry mass and neuronal RNA content of the control animals (table V) did not differ significantly from those of normal animals of the same age the values for the undernourished animals are in the following compared directly with corresponding figures for normal rats. In Fig. 19 the frequency distribution of the neuronal dry mass in undernourished rats is compared with that of normal rats of corresponding age. It is evident that the distributions differ from each other In the normal rats there is a marked shift of the median to the right and an increase in the distribution range with advancing age, i e. the number of cells with high mass increases. In the undernourished rats, on the contrary the distribution does not essentially change after the age of 15 days.

In a comparison of the mean neuronal dry mass values of the undernourished rats (Table VI) and those of normal rats of corresponding age (Table IV) it will be seen that in all age groups the values for the undernourished rats are markedly lower (Fig 20). There is already a statistically significant difference ($p<0.05$) between the experimental and the normal group at the age of 15 days. This difference becomes more accentuated at 30 and 90 days of age ($p < 0.001$) as the mean neuronal dry mass of the undernourished rats remains at a practically constant

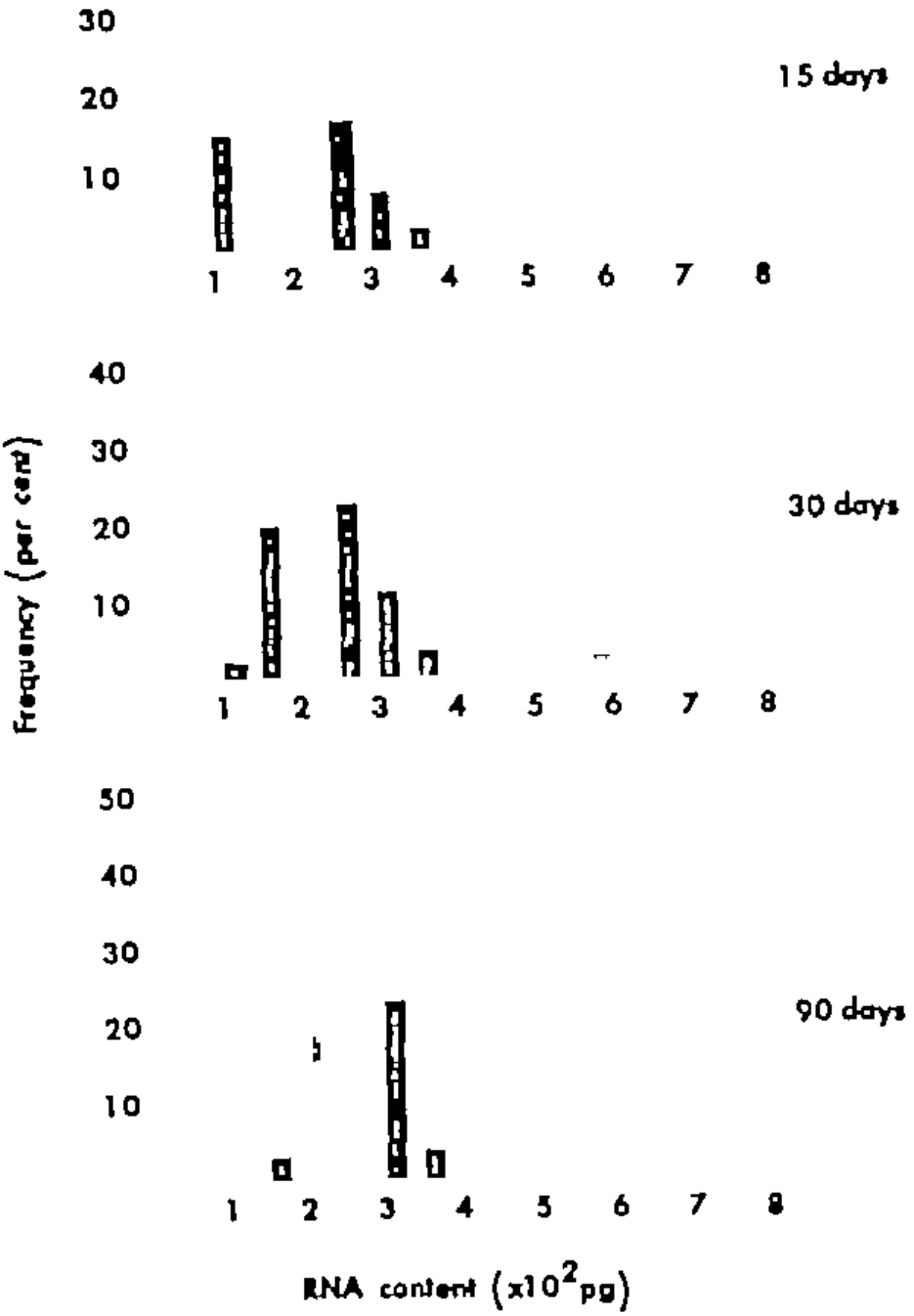


Fig. 21 Frequency distribution of the RNA content f the cell bodies f spinal anterior horn neurones of rats undernourished from the age of 1 day and sacrificed t 15, 30 or 90 days f age (dark staples) compared with the corresponding frequency distribution for normal rats of the same age (light staples).

level (between 2.10 and 2.10 $\times$ 10^3 pg) whereas a marked increase is noted in the neuronal dry mass values of the normal animals (from 2.51 to 3.15 $\times$ 10^3 pg).

It may be noted that the mean neuronal dry mass value (2.10 $\times$ 10^3 pg) of the 15-day-old undernourished rats (mean body weight 9.8 g) is markedly and significantly ($p < 0.001$) higher than the mean neuronal mass values of 3-day-old

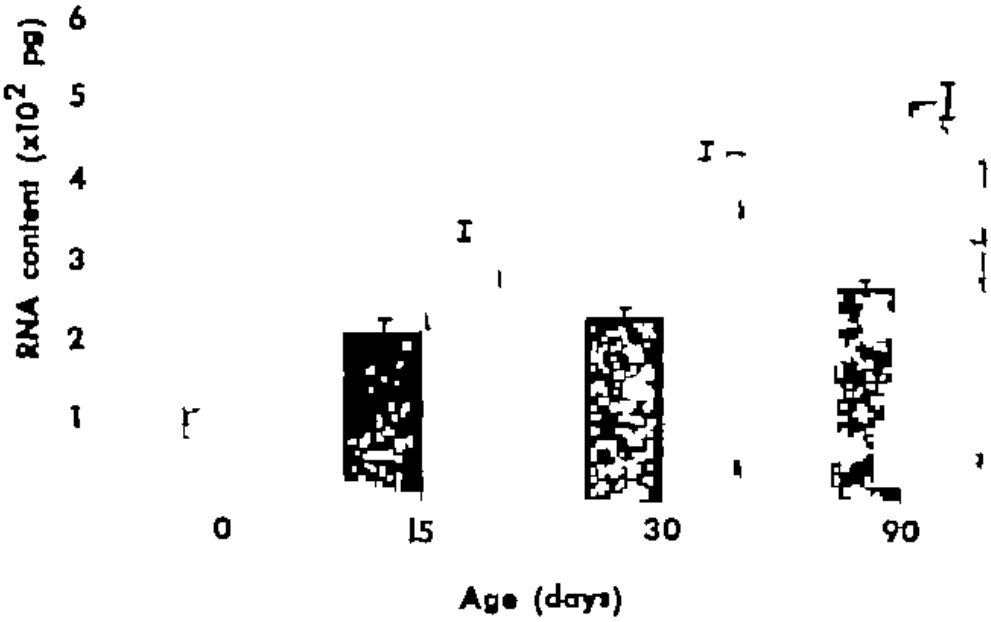


Fig. 22. Mean RNA content of th cell bodies of pinal anterior horn neurones in the different age groups of undernourished rats (dark taples)compared with the corresponding values for normal rats f the same age (light taples).

(1.27×10^3 pg) or 5-day-old (1.37×10^3 pg) normal rats of approximately similar body weight (mean body weight 9 1 g and 11.8 g, respectively). It is thus evident that the neuronal dry mass/body weight ratio increases after birth in the under nourished animals in contrast to the rapid decrease of the ratio in normal rats.

2. *Neuronal RNA content*

The mean values, S.E.M., and frequency distributions in groups of 25 pg of the neuronal RNA content of the undernourished rats are shown in Table VII.

The frequency distribution of the neuronal RNA content in the various experimental groups, as compared with that of normal animals of corresponding age, is shown in Fig 21 The distributions of the experimental and normal groups differ very markedly from each other at all ages studied. In the experimental animals there is practically no shift of the distribution to the right or increase in the distribution range with advancing age, such as is observed in the correspond ing normal rats. Whereas more and more cells with high RNA content occur in the normal groups with advancing age, such differentiation of spinal anterior horn neurones is not observed in the undernourished animals.

The mean values for the neuronal RNA content of the undernourished animals are markedly lower ($p < 0.001$) than normal values (Fig. 22) in all age groups. The differences increase with advancing age as the neuronal RNA content of normal animals becomes larger whereas the values for the undernourished animals remain at a low level. At 90 days of age the mean neuronal RNA content of the undernourished group (275 pg) is 16 per cent lower than the corresponding normal value (509 pg).

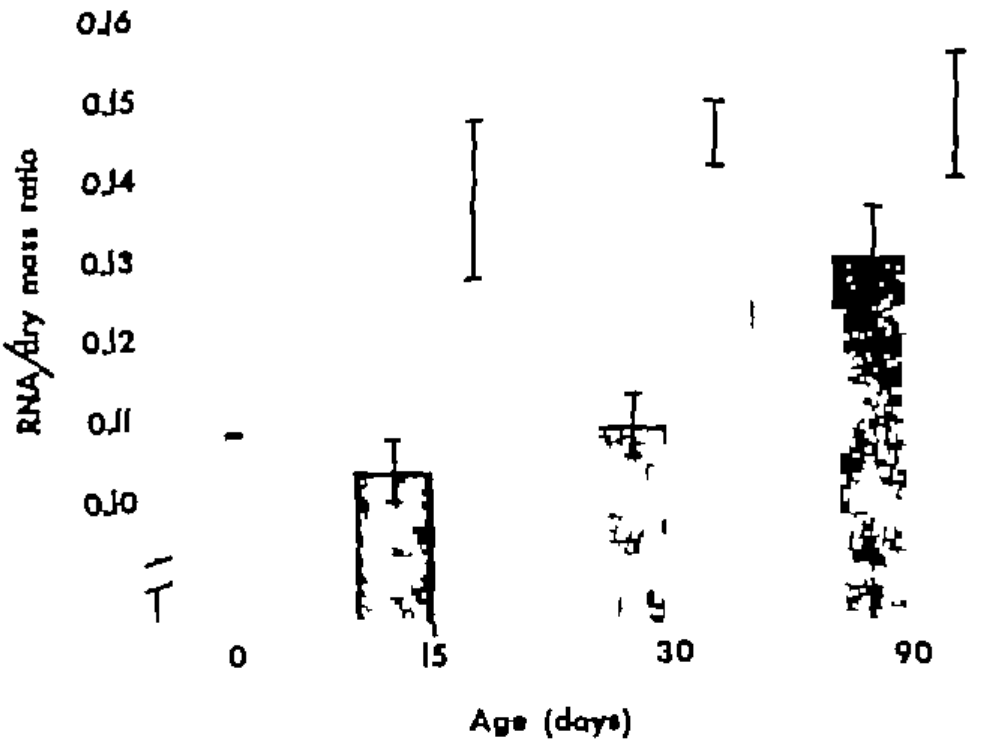


Fig. 23. Mean neuronal RNA/dry mass ratios ± S.E.M. in the different age groups. I under nourished rats (dark staples) compared with those for normal rats of the same age (light staples).

The mean neuronal RNA content of 15-day-old undernourished rats is markedly higher ($p < 0.01$) than that of 3 or 5-day-old normal animals of similar body weight. The neuronal RNA content/body weight ratio of the undernourished animals thus seems to increase postnatally unlike that in normal rats.

The mean neuronal RNA content/dry mass ratios are consistently lower in the undernourished animals than in the control animals of corresponding age (Fig. 23). These differences are statistically significant in the case of the 15 and 30-day-old animals ($p < 0.02$ and $p < 0.001$ respectively).

C. Discussion

At all ages studied both the mean neuronal dry mass and mean neuronal RNA content of the undernourished animals were significantly lower than the corresponding values for normal rats of the same age. This effect was already evident in the 15-day-old animals. It is interesting to note that further periods of undernutrition up to 90 days of age did not essentially change the neuronal dry mass and RNA values established during the first 15 days after birth i.e. during the period of most intense accumulation of RNA and dry mass in the anterior horn neurones in normal rats. These observations emphasize once more the importance of the immediate postnatal period for the chemical maturation of the neurone and demonstrate the sensitivity of the maturational processes to nutritional deprivation.

It may be noted that the distributions of both the neuronal dry mass and RNA content of the undernourished animals remain within a very narrow range, indicating that the differential growth of the neurones is impaired The neuronal RNA content appears to be more affected than the neuronal dry mass, as reflected by the low neuronal RNA/dry mass ratios, recalling the histological observations of a loss of Nissl granules in the anterior horn neurones of undernourished pigs and dogs (Stewart *et al* 1968) This might have unfavorable consequences for the neuronal protein synthesis.

The precise mechanism of the effect of undernutrition on neuronal maturation can only be a matter of speculation. Miller (1969) has stressed the important and central role of diet in the regulation and maintenance of development. It is obvious that diet provides the necessary precursor pools for macromolecular synthesis and that a reduction in pool size may affect the synthetic rate. But the explanation for the reduced neuronal RNA content and dry mass of the undernourished rats may be more complicated, and disturbances in enzyme patterns, in the energy metabolism or in hormonal regulation may be involved.

The mean neuronal dry mass and RNA content of the 15-day-old undernourished rats was much higher than in normal animals of similar body weight. This dissociation of neuronal and somatic growth in young animals illustrates the relative independence of neuronal development from increase in body mass. This observation also suggests the existence of efficient protection mechanisms which allow the nerve cells to grow at the expense of most other cells of the body

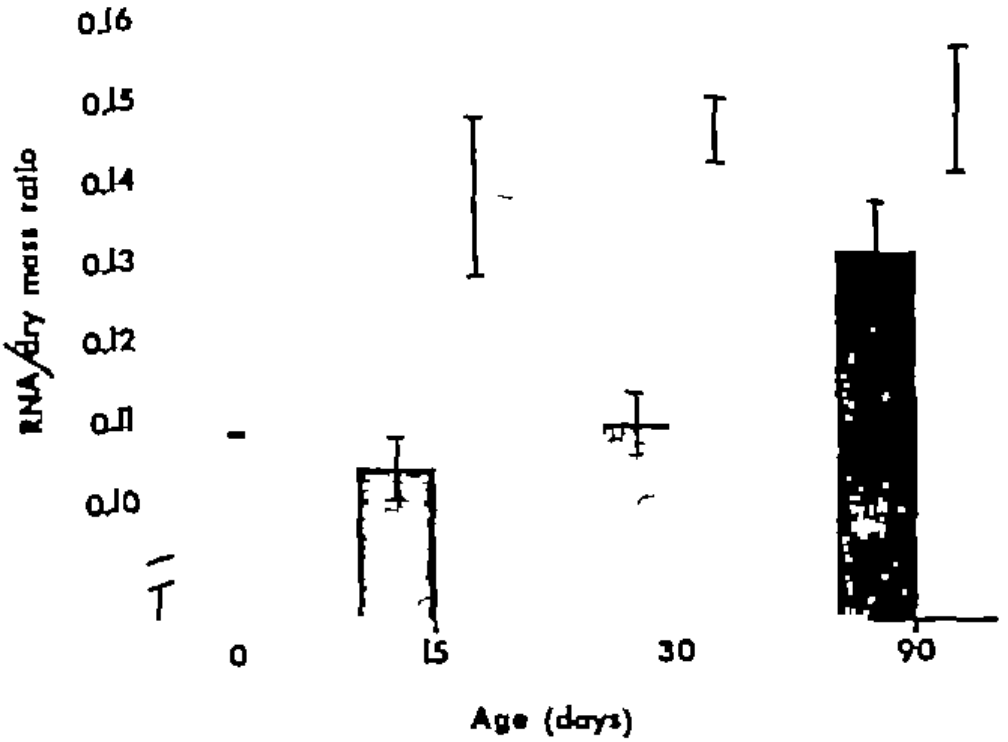


Fig. 23. Mean neuronal RNA/dry mass ratios ± S.E.M. in the different age groups of undernourished rats (dark staples) compared with those for normal rats of the same age (light staples).

The mean neuronal RNA content of 15-day-old undernourished rats is markedly higher ($p < 0.01$) than that of 3 or 5-day-old normal animals of similar body weight. The neuronal RNA content/body weight ratio of the undernourished animals thus seems to increase postnatally unlike that in normal rats.

The mean neuronal RNA content/dry mass ratios are consistently lower in the undernourished animals than in the control animals of corresponding age (Fig. 23). These differences are statistically significant in the case of the 15 and 30-day-old animals ($p < 0.02$ and $p < 0.001$ respectively).

C. Discussion

At all ages studied both the mean neuronal dry mass and mean neuronal RNA content of the undernourished animals were significantly lower than the corresponding values for normal rats of the same age. This effect was already evident in the 15-day-old animals. It is interesting to note that further periods of undernutrition up to 30 days of age did not essentially change the neuronal dry mass and RNA values established during the first 15 days after birth, i.e. during the period of most intense accumulation of RNA and dry mass in the anterior horn neurones in normal rats. These observations emphasize once more the importance of the immediate postnatal period for the chemical maturation of the neurone and demonstrate the sensitivity of the maturational processes to nutritional deprivation.

VI. SUMMARY AND CONCLUSIONS

Changes in the dry mass after lipid extraction (reflecting the protein content) and RNA content of isolated cell bodies of large spinal anterior horn neurones from the anterolateral nuclei of the 7th cervical segment were studied during normal postnatal development of the rat, in animals subjected to controlled undernutrition from birth for various periods, and in animals subsequently rehabilitated.

The cell bodies of the anterior horn neurones were isolated by micromanipulation from Carnoy-fixed tissue sections. The neuronal dry mass was determined with the aid of Rosengren's direct X ray absorption method. Edström's microchemical methods were used for determinations of the neuronal RNA content and base composition. A state of severe undernutrition was produced by daily isolation of the young rats from the mother. After weaning they were given restricted amounts of a defined balanced diet.

The main observations may be summarized under the following points:

1. The neuronal dry mass was found to increase 3.3 times, and the neuronal RNA content 4.2 times from birth to the age of 180 days.

2. A marked acceleration of the rate of increase of the neuronal RNA content was observed between 5 and 8 days of age and a corresponding distinct peak in the rate of increase of the neuronal dry mass between 8 and 10 days of age.

3. The rapid increase in both neuronal dry mass and RNA content slowed down after the age of 15 days and nearly adult values were reached at 30 days of age. No significant changes were noted after the age of 90 days.

4. The RNA/dry mass ratio of the neuronal cell bodies appeared to increase from birth up to 8 days of age to remain thereafter at approximately the same very high level until adult age.

5. A consistent rapid decrease was found postnatally in the neuronal dry mass/body weight ratio and the neuronal RNA content/body weight ratio. On the other hand, the neuronal parameters studied appeared to increase roughly in proportion with the length of the extremity assumed to reflect axon length.

6. At all ages studied both the neuronal dry mass and RNA content of the undernourished animals were significantly lower than the corresponding values for normal rats of the same age. Further periods of undernutrition did not essentially change the neuronal dry mass and RNA values established during the first 15 days after birth.

7 The neuronal RNA content was more affected than the dry mass resulting in a lowered neuronal RNA/dry mass ratio in the undernourished animals.

8. The neuronal dry mass values of the rehabilitated animals were only slightly lower than those of normal rats of the same age. The neuronal RNA values of the rehabilitated rats, on the other hand, remained at a remarkably low level.

These results show that the intense accumulation of RNA and protein, previously demonstrated in the neuroblasts of embryonal rat spinal cord, not only *continues after birth but that by far the major part of this accumulation actually* occurs postnatally During this »third period of growth» of the neurone the RNA/protein ratio appears to increase in the cell body in contrast to other somatic cells.

These maturational changes take place during an early and limited period, mainly between 5 and 15 days of age. Caloric restriction imposed during this period results in marked disturbances in the growth and chemical maturation of the neurone which are only partly reversed by rehabilitation.

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TABLES

Table I Age and body weight of the normal rats at the time of sacrifice. Age group mean values are given in boldface.

Rat no.	Age (days)	Body weight (g)	Rat no.	Age (days)	Body weight (g)
1	0	5.8	34	15	37.1
2	0	5.8	35	15	34.7
3	0	5.8	36	15	36.4
4	0	6.1		**15**	**35.4**
5	0	6.0			
6	0	5.6	37	21	49.3
	0	**5.9**	38	21	47.4
			39	21	57.2
7	3	9.1	40	21	48.4
8	3	9.3	41	21	50.7
9	3	9.4	42	21	53.2
10	3	9.1		**21**	**51.9**
11	3	8.7			
12	3	8.8	43	30	97
	3	**9.1**	44	30	96
			45	30	105
13	5	11.4	46	30	101
14	5	11.4	47	30	94
15	5	10.5	48	30	97
16	5	12.5		**30**	**98**
17	5	12.9			
18	5	12.3	49	60	228
	5	**11.8**	50	60	266
			51	60	311
19	8	17.7	52	60	315
20	8	17.5	53	60	319
21	8	17.2	54	60	315
22	8	16.5		**60**	**292**
23	8	15.9			
24	8	15.8	55	90	394
	8	**16.8**	56	90	384
			57	90	390
25	10	23.5	58	90	393
26	10	22.9	59	90	358
27	10	22.9	60	90	394
28	10	26.2		**90**	**386**
29	10	24.0			
30	10	23.8	61	180	422
	10	**23.9**	62	180	440
			63	180	493
31	15	35.7	64	180	445
32	15	34.3	65	180	515
33	15	34.4	66	180	464
				180	**463**

Table *II* Age and body weight of the undernourished and rehabilitated rats at the time of sacrifice. Group mean values are given in boldface.

Rat no.	Age (days)	Body weight (g)	Treatment
67	15	9.8	undernourished 15 days
68	15	8.3	
69	15	9.7	
70	15	10.4	
71	15	9.8	
72	15	10.7	
	15	9.8	
73	30	22.2	undernourished 30 days
74	30	21.3	
75	30	21.2	
76	30	21 0	
77	30	20.8	
78	30	20.4	
	30	21.2	
79	90	30.0	undernourished 90 days
80	90	29.9	
81	90	30.1	
82	90	29.9	
83	90	29.7	
84	90	30.8	
	90	30.1	
85	180	294	undernourished 90 days and rehabilitated 90 days
86	180	300	
87	180	304	
88	180	305	
89	180	304	
	180	301	
90	180	325	undernourished 30 days and rehabilitated 150 days
91	180	339	
92	180	312	
	180	325	

Table *III* Composition of the experimental diet.

	per cent
Protein[1]	21.3
Sucrose	59.2
Arachid oil	10.0
Cellulose »340«	3.0
Salt mixture	4.5
Vitamin mixture	1.0

[1] Purified fish protein (Astra Nutrition AB, Mölndal, Sweden), crude protein content 89.5 per cent, protein efficiency ratio (Osborne *et al* 1919) 3.34.

Table 1 The mean values, S.E.M. and frequency distribution of neuronal RNA content of normal rats at various ages (values for the cell bodies of large spinal anterior horn neurones from the anterolateral area of the seventh cervical segment). Age group mean values, S.E.M. and number of animals per group are given in boldface

Rat no.	Age (days)	Mean neuronal RNA content ± S.E.M. (pg)	Number of analyses	Frequency distribution of neuronal RNA content (class interval 25 pg)																											
				50–75	75–100	100–125	125–150	150–175	175–200	200–225	225–250	250–275	275–300	300–325	325–350	350–375	375–400	400–425	425–450	450–475	475–500	500–525	525–550	550–575	575–600	600–625	625–650	650–675	675–700	700–725	725–750	750–775	775–800
1	0	122 ± 4	18		1	9	7	1																							
2	0	121 ± 4	15		1	7	7																								
3	0	121 ± 4	17		1	10	4	2																							
4	0	102 ± 4	18	1	6	9	2																								
5	0	134 ± 6	18	1	—	7	5	4	1																						
6	0	115 ± 3	18		2	13	2	1																							
	0	120 ± 4	6																												
7	3	117 ± 5	18	1	1	2	4	7	3																						
8	3	137 ± 6	18		1	6	6	4	1																						
9	3	141 ± 7	18		1	2	10	3	1	1																					
10	3	151 ± 6	18			5	4	6	2	1																					
11	3	142 ± 5	18		1	1	11	3	2																						
12	3	140 ± 5	18			6	5	6	1																						
	3	143 ± 2	6																												
13	5	154 ± 7	18		1	3	4	6	3	1																					
14	5	150 ± 6	18			2	9	5	1	1																					
15	5	160 ± 5	18			1	5	8	3	1																					
16	5	161 ± 9	18			5	1	5	3	3	1																				
17	5	162 ± 7	18			3	5	4	3	3																					
18	5	187 ± 8	18			3	—	4	5	3	4																				
	5	163 ± 5	6																												
19	8	255 ± 9	18						1	2	6	7	—	1	—	1															
20	8	229 ± 6	18						4	3	7	3	1																		
21	8	228 ± 7	18						4	6	1	6	1																		
22	8	209 ± 7	18					3	4	5	4	2																			
23	8	207 ± 10	18			1	1	1	6	3	3	2	1																		
24	8	231 ± 11	18					2	4	1	5	2	3	—	1																
	8	227 ± 7	6																												
25	10	303 ± 9	18								3	—	5	5	4	—	1														
26	10	293 ± 7	18								2	2	7	3	4																
27	10	282 ± 9	18							1	3	5	2	5	1	—	1														
28	10	196 ± 8	18					6	5	4	1	2																			
29	10	214 ± 5	18					1	4	8	4	1																			
30	10	216 ± 9	18			1	—	1	3	5	6	1	1																		
	10	231 ± 19	6																												

31	15	342 ± 10	18
32	15	393 ± 10	18
33	15	304 ± 10	18
34	15	331 ± 18	18
35	15	349 ± 13	18
36	15	340 ± 10	18
	15	343 ± 13	6
37	21	308 ± 12	17
38	21	335 ± 7	18
39	21	375 ± 13	18
40	21	371 ± 10	18
41	21	311 ± 10	18
42	21	306 ± 13	18
	21	337 ± 13	6
43	30	458 ± 12	18
44	30	430 ± 9	18
45	30	438 ± 17	18
46	30	403 ± 13	18
47	30	415 ± 15	18
48	30	469 ± 23	18
	30	444 ± 13	6
49	60	423 ± 20	32
50	60	432 ± 13	36
51	60	452 ± 23	18
52	60	483 ± 15	18
53	60	574 ± 21	18
54	60	509 ± 30	18
	60	479 ± 23	6
55	90	542 ± 11	36
56	90	446 ± 14	36
57	90	559 ± 13	36
58	90	518 ± 13	17
59	90	557 ± 17	18
60	90	431 ± 12	16
	90	509 ± 23	6
61	180	513 ± 14	27
62	180	510 ± 11	18
63	180	479 ± 21	17
64	180	578 ± 25	16
3	180	423 ± 21	18
	180	479 ± 19	18
)	180	497 ± 21	6

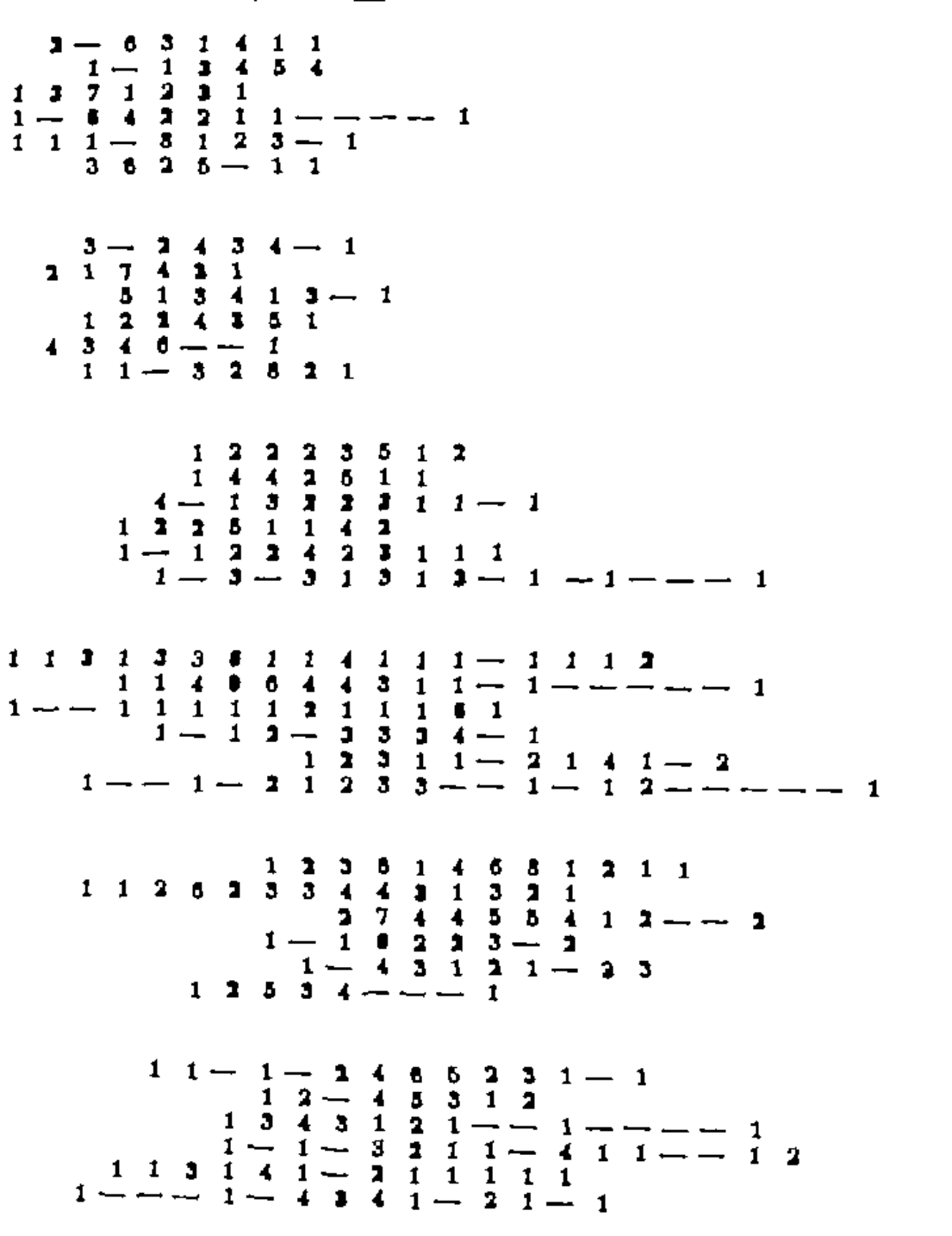


Table VI The mean values, S.E.M. and frequency distributions of the neuronal dry mass of undernourished rats at various ages (values for the cell bodies of large spinal anterior horn neurones from the anterolateral nucleus of the seventh cervical segment). Age group mean values, S.E.M. and number of animals per group are given in boldface.

Rat no.	Age (days)	Mean neuronal dry mass ± S.E.M ($\times 10^{-8}$ pg)	Number of analyses	Frequency distribution of neuronal dry mass values (class interval 0.50×10^{-8} pg)													
				0.00–0.50	0.50–1.00	1.00–1.50	1.50–2.00	2.00–2.50	2.50–3.00	3.00–3.50	3.50–4.00	4.00–4.50	4.50–5.00	5.00–5.50	5.50–6.00	6.00–6.50	6.50–7.00
67	15	1.61 ± 0.17	27	2	8	3	6	2	4	1	1						
68	15	2.10 ± 0.06	34		1	4	8	11	10								
69	15	2.02 ± 0.14	29	1	2	4	6	9	4	3							
70	15	2.36 ± 0.16	36		1	0	2	8	7	3	5	1					
71	15	2.20 ± 0.10	35			3	11	11	5	3	2						
72	15	2.32 ± 0.12	25		1	—	5	9	7	3							
	15	**2.10 ± 0.11**	**6**														
73	30	2.37 ± 0.18	31			6	4	10	3	5	2	—	—	—	1		
74	30	2.35 ± 0.14	36			8	3	7	12	3	2	—	1				
75	30	2.14 ± 0.13	36		3	7	4	11	2	8	1						
6	30	2.17 ± 0.15	34		1	9	2	10	4	6	2						
77	30	2.20 ± 0.13	36		1	9	2	8	10	4	2						
78	30	1.71 ± 0.12	30		5	5	6	9	5								
	30	**2.16 ± 0.10**	**6**														
79	90	2.34 ± 0.11	33			4	3	13	5	8							
80	90	2.14 ± 0.14	29			9	5	3	6	6							
81	90	1.99 ± 0.11	30			11	3	8	6	2							
82	90	2.24 ± 0.15	32		1	6	3	10	7	2	2	—	1				
83	90	2.16 ± 0.16	31		1	8	—	13	3	4	1	1					
84	90	1.79 ± 0.13	35		2	16	1	6	7	3							
	90	**2.11 ± 0.08**	**6**														

Table VII The mean values, S.E.M. and frequency distributions of the neuronal RNA content of undernourished rats at various ages (values for the cell bodies of large spinal anterior horn neurones from the anterolateral nucleus of the seventh cervical segment). Age group mean values, S.E.M. and number of animals per group are given in boldface.

Rat no.	Age (days)	Mean neuronal RNA content (pg)	Number of analyses	Frequency distribution of neuronal RNA content (class interval 25 pg)												
				50–75	75–100	100–125	125–150	150–175	175–200	200–225	225–250	250–275	275–300	300–325	325–350	350–375	375–400
67	15	188 ± 11	15			1	4	4	3	—	4	2					
68	15	174 ± 8	15			1	3	7	2	4	1						
69	15	193 ± 11	15			1	1	7	1	3	1	3	1				
70	15	218 ± 14	15				3	2	4	1	4	—	2	1	1		
71	15	264 ± 14	15				1	1	—	—	7	1	3	2	2	1	
72	15	261 ± 16	15				1	—	2	5	—	1	5	1	1	—	2
	15	217 ± 15	6														
73	30	206 ± 7	17				1	1	5	5	4	1					
74	30	265 ± 9	36						3	6	8	7	4	2	3	2	1
75	30	234 ± 11	15					2	—	7	4	1	1	2	1		
76	30	264 ± 11	35					4	5	4	5	—	7	6	2	2	
77	30	253 ± 6	15							3	5	6	4				
78	30	198 ± 8	15				2	3	5	3	4	1					
	30	235 ± 11	6														
79	90	287 ± 11	15						1	—	1	8	2	2	2	1	1
80	90	251 ± 7	15						1	1	8	4	3	1			
81	90	294 ± 9	15									7	3	6	—	1	1
82	90	299 ± 8	17								2	1	4	6	4		
83	90	249 ± 7	15						1	3	4	5	5				
84	90	269 ± 8	15								7	3	4	4			
	90	275 ± 9	6														

Table VIII. The mean values, S.E.M. and frequency distributions of the neuronal dry mass of rehabilitated rats at various ages (values for the cell bodies of large spinal anterior horn neurones from the anterolateral nucleus of the seventh cervical segment). Age group mean values, S.E.M. and number of animals per group are given in boldface.

Rat. no.	Age (days)	Mean neuronal dry mass ± S.E.M. ($\times 10^6$ pg)	Number of analyses	Frequency distribution of neuronal dry mass values (class interval 0.50 $\times 10^6$ pg)												
				0.00–0.50	0.50–1.00	1.00–1.50	1.50–2.00	2.00–2.50	2.50–3.00	3.00–3.50	3.50–4.00	4.00–4.50	4.50–5.00	5.00–5.50	5.50–6.00	6.00–6.50	6.50–7.00
85	180	2.90 ± 0.25	35			9	1	6	2	4	8	—	3	—	—	1	1
86	180	3.45 ± 0.25	33			2	1	3	8	4	8	1	1	—	—	2	1
87	180	3.35 ± 0.21	30				2	5	5	5	8	—	2	1	1	1	
88	180	3.41 ± 0.15	31				1	1	12	6	8	1	3	1	1		
89	180	3.39 ± 0.20	34			3	1	3	7	5	5	3	3	3	1		
	180	3.30 ± 0.10	5														
90	180	3.33 ± 0.20	33			3	1	2	8	5	4	5	3	1	—	1	
91	180	3.25 ± 0.21	37			2	1	3	6	4	7	—	3	2			
92	180	2.85 ± 0.13	33			1	—	6	13	5	6	1	1				
	180	3.17 ± 0.11	3														

Table IV The mean values, S.E.M. and frequency distributions of the neuronal RNA content of rehabilitated rats at various ages (values for the cell bodies of large spinal anterior horn neurones from the anterolateral nucleus of the seventh cervical segment). Age group mean values, S.E.M. and number of animals per group are given in boldface.

Rat no.	Age (days)	Mean neuronal RNA content (pg)	Number of analyses	Frequency distribution of neuronal RNA content (class interval 25 pg)														
				150	175	200	225	250	275	300	325	350	375	400	425	450	475	500	525
85	180	370 ± 14	18					1	1	3	2	3	3	—	4	1			
86	180	368 ± 9	18						2	1	2	1	11	—	1				
87	180	377 ± 10	18						1	2	1	3	7	2	1	1			
88	180	389 ± 11	18							2	2	3	3	4	2	1	1		
89	180	389 ± 11	18						1	—	2	4	4	1	5	—	1		
	180	**379 ± 5**	**5**																
90	180	352 ± 10	18						2	4	3	3	3	2	1				
91	180	354 ± 13	18					2	2	2	3	2	2	3	2				
92	180	360 ± 13	18						1	2	5	6	1	—	2	—	—	1	
	180	**355 ± 2**	**3**																

Table V. Body weight neuronal dry mass and neuronal RNA content of 30 and 90-day-old control rats.

Rat no.	Age (days)	Body weight (g)	Mean neuronal dry mass ± S.E.M. ($\times 10^6$ pg)	Mean neuronal RNA content ± S.E.M. (pg)
93	30	98	3,04 ± 0,18	455 ± 12
94	30	90	2,77 ± 0 19	396 ± 14
95	30	101	3,13 ± 0,19	417 ± 10
	30	**96**	**2,98 ± 0,11**	**423 ± 17**
96	90	383	3,28 ± 0,21	535 ± 12
97	90	393	3,19 ± 0,23	459 ± 10
98	90	384	3,50 ± 0 19	526 ± 10
	90	**387**	**3,33 ± 0,09**	**507 ± 24**

Contractility

Influence of

Parameters and

ACTA PHYSIOLOGICA SCANDINAVICA
SUPPLEMENTUM 353

From the Department of Pharmacology and from the Department of Obstetrics and Gynaecology at the General Hospital of Malmö, University of Lund Sweden

K E Andersson G Gennser and E Nilsson

Contractility of Isolated Human Foetal Hearts Influence of Contraction Rate, Acid Base Parameters and a Local Anaesthetic

Malmö 19 0

Contents

Influence of Contraction Rate, Coronary Perfusion Pressure and Diastolic Pressure on the Mechanical Performance of Isolated Human Foetal Hearts 5

Influence of Acid-Base Changes on Isolated Human Foetal Hearts 20

Influence of Mepivacaine on Isolated Human Foetal Hearts at Normal and Low pH 34

Influence of Contraction Rate, Coronary Perfusion Pressure and Diastolic Pressure on the Mechanical Performance of Isolated Human Foetal Hearts

By

K. E. Andersson G Gennser and E. Nilsson

Abstract

Andersson K. E., G Gennser and E. Nilsson *Influence of contraction rate coronary perfusion pressure and diastolic pressure on the mechanical performance of isolated human foetal hearts.* Acta physiol. scand. 1970. *80* Suppl. 353 5—19.

Left intraventricular pressure and electrogram were recorded in isolated hearts, perfused *ad modum* Langendorff, from human foetuses at midgestation. The Frank-Starling mechanism could be demonstrated in these preparations. An increase in coronary perfusion pressure increased both the active systolic pressure and the end diastolic pressure. Active systolic pressure declined at contraction rates above the spontaneous one both during perfusion with a Ringer's solution and with an erythrocyte suspension. A lowering of the contraction rate, induced by excision of the sinus node region and/or addition of acetylcholine to the perfusion solution, increased the active systolic pressure. The rate of rise of intraventricular pressure decreased both at contraction rates above and below the spontaneous one, whereas the duration of the contractile cycle measured at 50 % level decreased with increasing contraction rates at all frequencies studied. The frequency dependence of the active systolic pressure in isolated human foetal hearts differs from that of the isometric tension of excised papillary muscles of these hearts (Gennser and Nilsson 1968). This discrepancy is most likely explained by the asynchronous activation of the whole heart. It is concluded that foetal bradycardia *per se* does probably not reduce the mechanical output of the individual foetal heart beat.

Introduction

The isometric twitch tension of isolated ventricle myocardial preparations such as papillary muscles of various mammalian species is dependent upon contraction rate (for references, see Koch Weser and Blinks 1963). In most species the contractile force of excised ventricle preparations is diminished when the contraction rate is reduced below the frequency range normally existing *in vivo*. This has been demonstrated also for papillary muscles from human foetal hearts (Gennser and Nilsson 1968). It was shown that at 30° C the isometric tension of the papillary muscles decreased markedly with decreasing contraction rates below approximately 70 beats per min. The influence of contraction rate upon the mechanical output of the individual foetal heart beat might be considered of great clinical interest, as marked alterations in heart rate are often related to "foetal distress" during delivery

(Quilligan, Katigbak and Hofschild 1965 Caldeyro-Barcia *et al.* 1966, Hon 1968) The present study was undertaken in order to elucidate to what extent the mechanical output of the individual beat of the isolated human foetal heart was influenced by changes in contraction rate. The mechanical parameter measured was the isovolumetrically developed left intraventricular pressure. It was considered of interest to find out how far results obtained in animal experiments might be applicable also to the preparation used in the present study and therefore the influence of coronary perfusion pressure and end diastolic pressure was also analysed.

Methods

Material

This and the following two reports (Andersson, Gennser and Nilsson 1970 a, b) are based upon investigations on 30 human foetal heart preparations performed during the period December 1968—November 1969 The foetal hearts were obtained at legal interruptions of pregnancy performed by hysterotomy Hearts from foetuses with no signs of malformation or intrauterine disorder were used. The estimated gestational age ranged from 14 to 24 weeks and the foetal crown-heel length from 14 to 30 cm. The weight of the heart preparations ranged 0.5—3 g. Due to the technical difficulties in mounting the preparations (depending on the small size and the friability of the hearts) and to the time-consuming experimental procedures used, only a limited number of observations could be obtained from each heart.

Preparation and mounting of the hearts

After the uterine evacuation the hearts were removed from the foetuses as soon as possible Within a few minutes perfusion was started *ad modum* Langendorff at room temperature with an oxygenated Ringer's solution of the composition described below Care was taken to tie the perfusion cannula to the superior part of the ascending aorta to avoid interference with the closure of the aortic valve. During preparation the heart was immersed in Ringer solution and the perfusion pressure was kept at about mm Hg. The preparation was then mounted in a thermostatically controlled bath (volume 25 ml) containing Ringer's solution flowing from the coronary circulation. The temperature of the bath and the perfusing solution was kept at 37±0.5 C During each experiment the hydrostatic perfusion pressure was kept constant. Depending on the size of the hearts, the perfusion pressure was set within the range of 29 to 35 mm Hg This range was chosen on the basis of arterial pressure measurements on human foetuses at midgestation (Enhörning and Westin 1954)

In some experiments, the left part of the insertion of the superior vena cava into the right atrium (the sinus node region) was excised in order to reduce the contraction rate of the heart

Recording techniques

Pressure was recorded within the isovolumetrically contracting left ventricle by means of a Statham pressure transducer. Three different arrangements were used. In two of these a polyethylene catheter (external diameter 1·0 or 1·5 mm, internal diameter 0·5 or 1·0 mm) whose one end was connected to the pressure transducer was inserted into the left ventricle *via* the mitral ostium. The catheter ended either (1) directly in the ventricular cavity or (2) in a thin, nonelastic balloon firmly tied to the catheter. When an intraventricular balloon was used, the catheter connected to the balloon was fixed at the mitral ring by means of a silk suture. The volume of the balloon was chosen larger than the estimated volume of the ventricular cavity. With this type of registration the diastolic volume and hence the diastolic pressure could be altered by changing the volume of the balloon. In a few preparations the catheter connected to the pressure transducer was placed (3) within the aortic perfusion cannula, running into the left ventricle *via* the aortic ostium. In all the cases the pressure recording system was filled with Ringer's solution. In none of the experiments using the open catheter types of registration did the total systolic pressure (see Terminology) exceed perfusion pressure. Left ventricular contractions were therefore considered as isovolumetric also in these preparations.

Before starting an experiment the end diastolic pressure was adjusted to approximately 7 mm Hg, range 3—12 mm Hg. However when an open catheter through the mitral ostium was used for pressure registration, the diastolic pressure could not be altered by changing the volume of the left ventricle probably owing to opening of the mitral valve during diastole. End diastolic pressure in these preparations did not exceed 5 mm Hg at spontaneous contraction rates (95—120 beats per min).

The maximal rate of rise of intraventricular pressure, $(dp/dt)_{max}$, was determined graphically from the curves. The electrogram of the heart was obtained by means of glass electrodes with external tip diameter of 0·5 mm containing Ag/AgCl-wires in 3-M NH_4Cl-agar. One electrode was placed above the superior part of the right atrium, the other near the apex.

Coronary inflow was measured with a photoelectric drop counter. Intraventricular pressure, electrogram and coronary inflow were recorded on a Grass polygraph. The frequency response of the electrical recording system was 75 Hz, the frequency response of the pressure recording device exceeded 40 Hz irrespective of the size of the catheter used.

Electrical stimulation

The heart preparations were stimulated electrically at all frequencies above the spontaneous contraction rate by passing a current through a bipolar platinum electrode placed in the region of the sinus node. The strength of the stimulus was approximately 50 per cent above threshold and the duration was 2 msec.

Perfusing solution

A Ringer's solution of the following composition was used (mM) NaCl 117 $NaHCO_3$ 20 $MgSO_4$ 1.5 NaH_2PO_4 1.5 KCl 5 $CaCl_2$ 2 and glucose 3.3. A low molecular weight dextran, Rheomacrodex® was added to the Ringer's solution to a final concentration of 2.5 %. The perfusing solution was equilibrated with a gas mixture containing 80 vol. % O_2, 15 vol. % N_2 and 5 vol. % CO_2. pH of the perfusion solution was 7.35 (range 7.29–7.41). pCO_2 of the solutions was calculated as $\frac{A \cdot (P-B)}{100}$ where A is % (vol./vol.) CO_2 in gas mixture, P atmospheric pressure (760 mm Hg) and B vapour pressure of water at 37°C (47 mm Hg). Double distilled water was used. Chemicals used were of analytical grade. Acetylcholine was used in the form of acetylcholine chloride (British Drug House Ltd).

Erythrocyte suspensions

were prepared by adding Ringer's solution to freshly obtained human adult erythrocytes, which had been washed twice in Ringer's solution. The haematocrit was adjusted to 20 % and the pH of the suspension was set to 7.35. Samples from the erythrocyte suspension were taken at various times during the experiment for analysis of potassium in the supernatant after separation of the erythrocytes. The potassium concentration in this supernatant did not exceed the concentration in the ordinary Ringer's solution by more than 10 %.

The temperature of the Ringer's solution and the erythrocyte suspension was kept constant at 37 ± 0.5° C.

Terminology

The term *end diastolic pressure* is used in its usual sense. *Active systolic pressure* denotes the maximal pressure developed above the end diastolic pressure during the contraction cycle studied. *Total systolic pressure* is the sum of the end diastolic and the active systolic pressure.

In the electrogram the distance between the peak of the atrial wave (or when electrical stimulation was used the beginning of the stimulation artefact) and the beginning of the ventricular complex was taken as a measure of atrio-ventricular conduction time. The duration of the ventricular complex was measured at the 50 % level above the isoelectric level.

Results

Influence of contraction rate on active systolic pressure

After an equilibration period of approximately 30 min, the spontaneous contraction rates of the preparations were 108 ± 11 beats per min (mean ± SD, n = 8). At contraction rates above the spontaneous rate active systolic pressure decreased to a varying extent with increasing contraction rates. The active systolic pressure devel-

TABLE I Activ systolic pressure at contraction rates 100 125 and 150 beats per min.

Date of experiment	Relativ active systolic pressure at contraction rate (beats per min)			Type of pressu registration
	100	125	150	
9 4	1.0	0.88	0.61	Intraventricular balloon
19.5	1.0	0.86	0.63	Intraventricular balloon
27.5	1.0	0.89	0 71	Intra entricular balloon
1.8	1.0	0.94	0.87	Mitral catheter
1 10	1.0	0.87	0.67	Aortic catheter

oped during steady state conditions at contraction rates of 100 125 and 150 beats per min is shown in Table I. The negative inotropic effect of an increase in contraction rate was associated with a reduction in the maximal rate of rise of intraventricular pressure $(dp/dt)_{max}$. Both the rise time and the duration of the systolic contraction, measured at the 50 % level, decreased with increasing contraction rate. The results from one typical experiment are shown in Fig 1

A lowering of the contraction rate below the spontaneous level, induced by excision of the sinus node region and/or by addition of acetylcholine to the perfusion medium, had a positive inotropic effect. In preparations with pressure registration by means of an aortic catheter or an intraventricular balloon a slow and constant increase in intraventricular pressure occurred during diastole. This was not seen in preparations in which the pressure was recorded with a mitral catheter In these latter preparations active systolic pressure could therefore be studied at low contrac tion rates without interference by changes in end diastolic pressure. The results of two such experiments are shown in Fig 2. In order to reduce the contraction rates of the hearts, they were perfused with acetylcholine chloride ($1—2\times10^{-7}$ g/ml) preparation A during the whole experiment, preparation B only when measuring the active systolic pressure at the lowest contraction rate studied. (In concentration 10^{-7} g/ml acetylcholine chloride itself caused a minor negative inotropic effect in the human foetal heart preparations.) In preparation A the sinus node was also excised. In this way steady state conditions could be achieved during contraction rates as low as 44 and 23 beats per min. Active systolic pressure increased with decreasing contraction rates within the range studied (Fig. ?) This positive inotropic effect of a reduction in contraction rate was attributable only to a prolongation of the contrac tion cycle, as $(dp/dt)_{max}$ decreased with decreasing contraction rates below approximately 90 beats per min.

Influence of oxygen supply and co nary perfusion pressure on intraventricular pressure

In order to ascertain whether the reduction of the active systolic pressure at high stimulation frequencies could be ascribed to inadequate oxygen supply to the

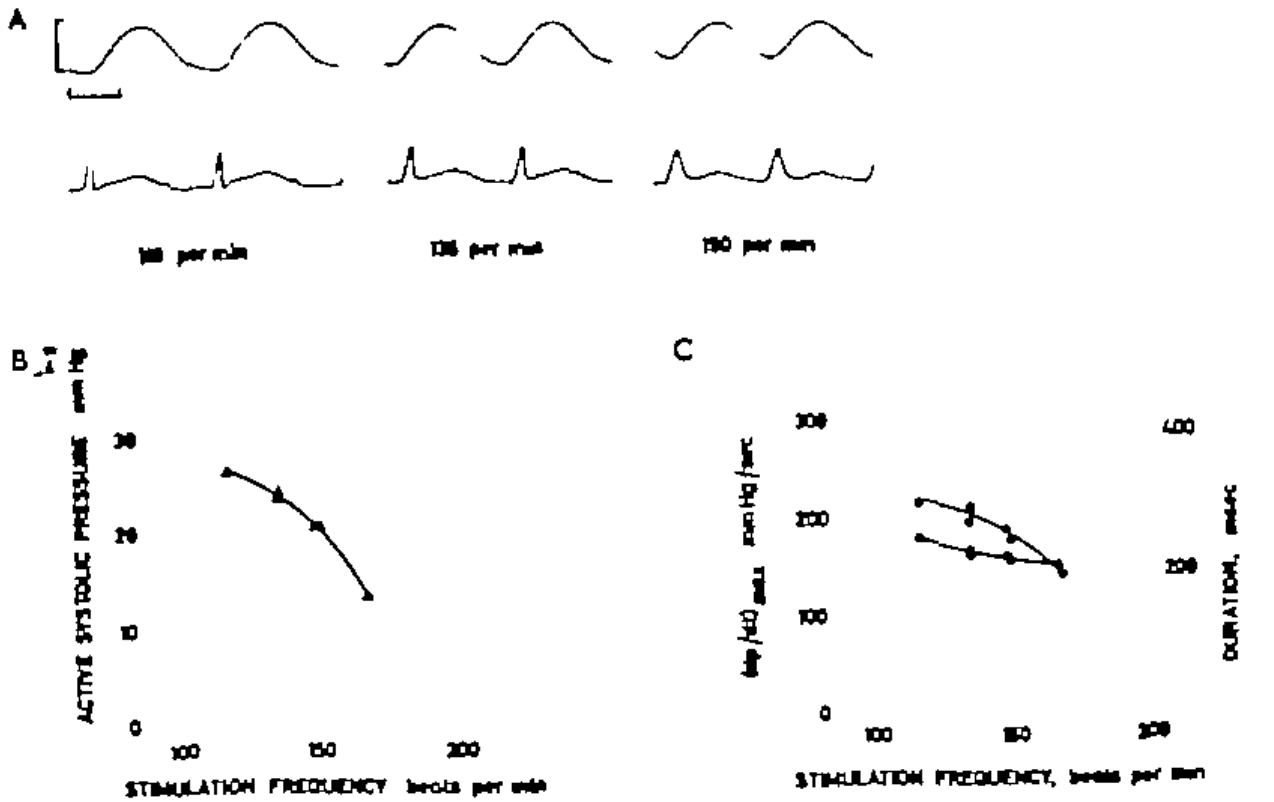


Fig. 1 A. Intra entricular pressure and elect ogram recordings of foetal heart preparation at different contraction rates. Pressure recorded by means of an intra entricular balloon. Vertical bar 20 mm Hg, horizontal bar 500 msec.

B. Activ systolic pressure (▲) as function of contraction rate.

C. $(dp/dt)_{max}$ ( ) and duration of systolic contraction ( ) as function of contraction rate. Fig. 1 B and C give results obtained with same preparation as Fig. 1 A. (Lines in this and following graphs are drawn by the eye.)

myocardium, two preparations were perfused with an erythrocyte suspension (hematocrit 0 %) of the same pH and aerated with the same gas mixture as the preceding Ringer's solution (see Methods). Fig. 3 shows that replacement of the Ringer's solution by the erythrocyte suspension did not alter the relation between contraction rate and active systolic pressure although the oxygen content of the erythrocyte suspension was 5.5 times that of the Ringer's solution.

The amount of physically dissolved oxygen i 100 ml Ringer sol tion in equilibration w th gas mixture containing 80 % oxygen is

$$\frac{80}{100}(760 - 47)\,0.0239\,\frac{100}{760} = 1.79 \text{ ml}$$

using a alue for xygen solubility of 0.0239 ml O per ml fl id t pressure of 1 tmospheric and 37 C (Umbreit Burris and Stauffer 1964) and w ter apour pressure value of 47 mm Hg. The hemoglobin-bound oxygen in 100 ml of the rythroc te suspension (hematocrit 20 %) containing 6 g% hemoglobin would be 6 1.36 ml O_2 8.16 ml O_2 and the physically dissol ed oxygen in 80 ml Ringer solution ad in 13 ml rythrocyt w te in 100 ml suspension would be 1.69 ml, gi ing total of 9.85 ml oxygen per 100 ml erythrocyt suspension. This ncrease n the amount of oxygen available to the myoc rdium induced by replacement of the

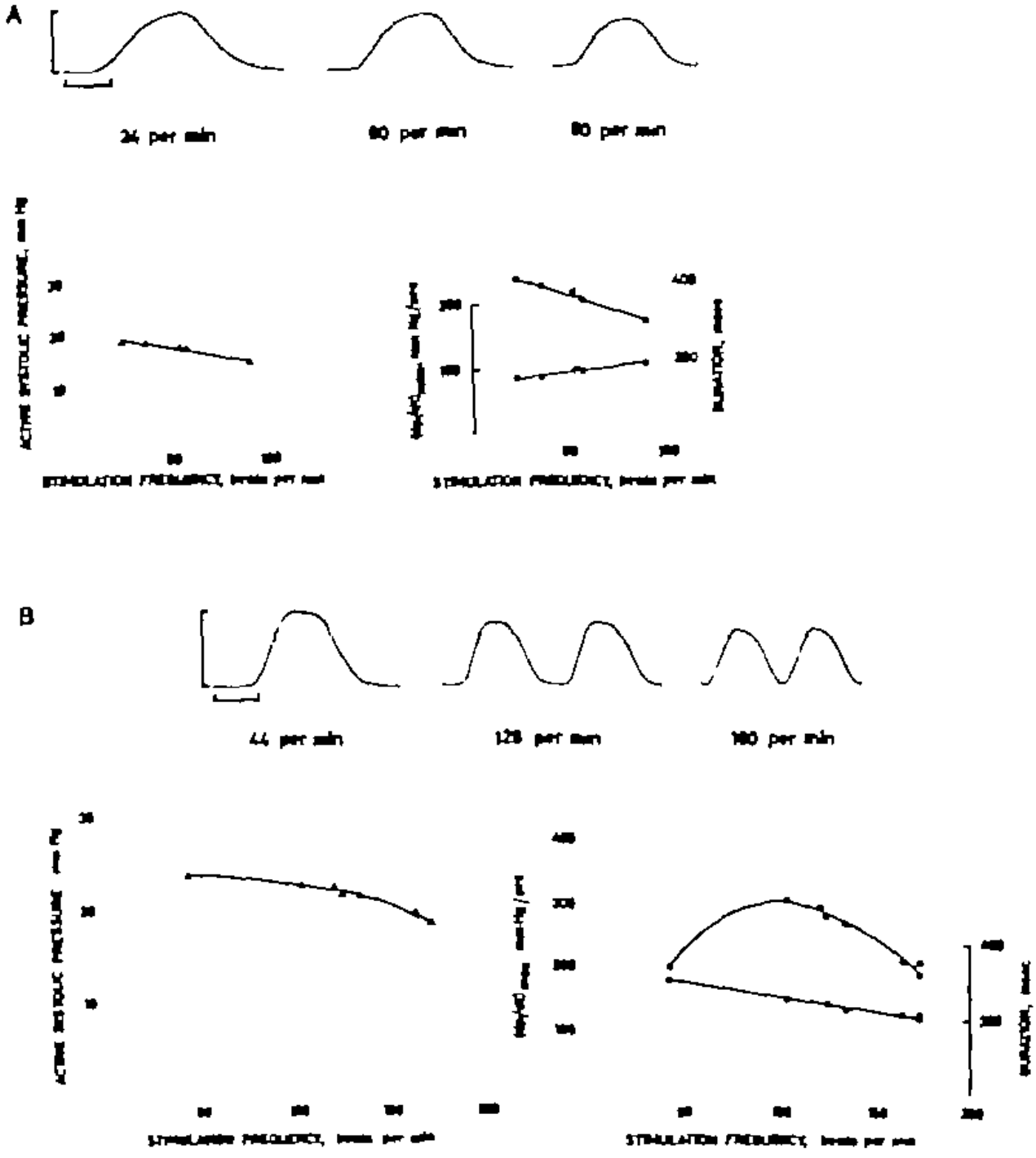


Fig. 2. Intra ventricular pressure curves of two heart preparations (A) and (B) recorded by means of open mitral cathe ers. The sinus node of preparation (A) was excised, and this preparation was perf sed with Ringer' solution containing 2×10^{-7} g/ml acetylcholine chloride. Preparation (B) was perfused with a Ringer solution containing 10^{-7} g/ml acetylcholine chloride during measurements t contraction rate of 44 beats per min. Calibration: ertical bar 20 mm Hg in (A) and 5 mm Hg in (B) horizontal bar 200 msec. Graphs show relations between stimulation frequency and activ systolic pressure (A) $(dp/dt)_{max}$ ( ) and duration of ystolic contr ction measured on 50 % level ( )

Ringer solution by the erythrocyt suspension was in part offset by reduction of coronary flow in response to alteration of the perfusion medium. However this coronary flow reduction was relativ ly small, 5 % and 22 % in the two experiments performed.

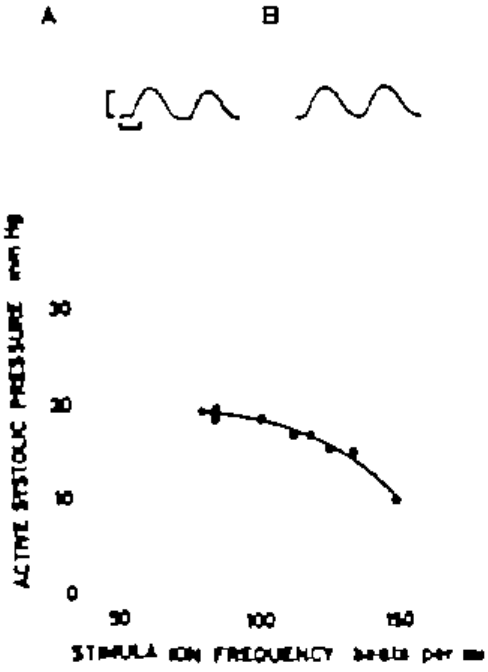


Fig. 3 Intraventricular pressure recordings of heart preparation perfused with Ringer solution (A) and erythrocyte suspension (B). Diagram shows relation between active systolic pressure and contraction rate during perfusion with the Ringer's solution ( ) and the erythrocyte suspension ( ). Pressure recorded by means of an intraventricular balloon. Vertical bar 0 mm Hg, horizontal bar 200 msec.

Changes in contraction rate of the heart preparations were not accompanied by any consistent alterations in coronary blood flow. It was, however considered of interest to study the effect of coronary perfusion pressure and coronary flow upon the mechanical performance of the isolated foetal hearts. In four heart preparations with a balloon in the isovolumetrically working left ventricle, the coronary perfusion pressure was varied from 14 to 37 mm Hg. The results are summarized in Fig. 4 A and show that an increase in perfusion pressure within the range studied raised both the active systolic pressure and the end diastolic pressure. As changes in coronary blood flow always accompanied alterations in perfusion pressure, it was not possible to distinguish between the individual effects of these two variables. A linear relation was found between coronary blood flow and perfusion pressure as shown in Fig. 4 B. Thus no autoregulatory mechanisms in the myocardial vascular bed occurred in response to changes in perfusion pressure.

End diastolic pressure as a determinant of active systolic pressure

The influence of the end diastolic pressure upon active systolic pressure was studied in two preparations; the results obtained were similar in both. Data from one experiment are shown in Fig. 5. It can be seen that active systolic pressure increased with increasing end diastolic pressure up to approximately 10 mm Hg, but remained constant at higher diastolic pressures.

Influence of stimulation frequency on foetal electrogram

This is illustrated by the sequence shown in Fig. 6 obtained from one preparation without a balloon in the left ventricle. The atrio-ventricular conduction time

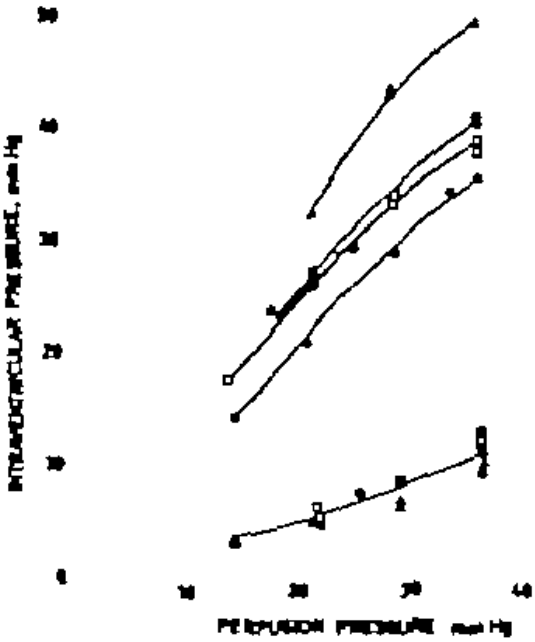


Fig. 4 A. Relation between applied coronary perfusion pressure and intraventricular pressure in four preparations. The four upper curves denote activ systolic pressure, the lower curve denotes end diastolic pressure. Contraction rate 106 (△) 111 ( ) 75 (□) and 110 ( ) beats per min. Pressure recorded by means of an intraventricular balloon.

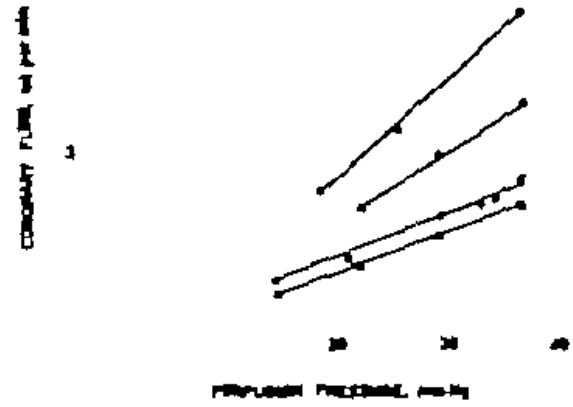


Fig. 4 B. Relation between applied coronary perfusion pressure and coronary flow in four hearts (*symbols indicate same preparations as in* Fig. 4 A)

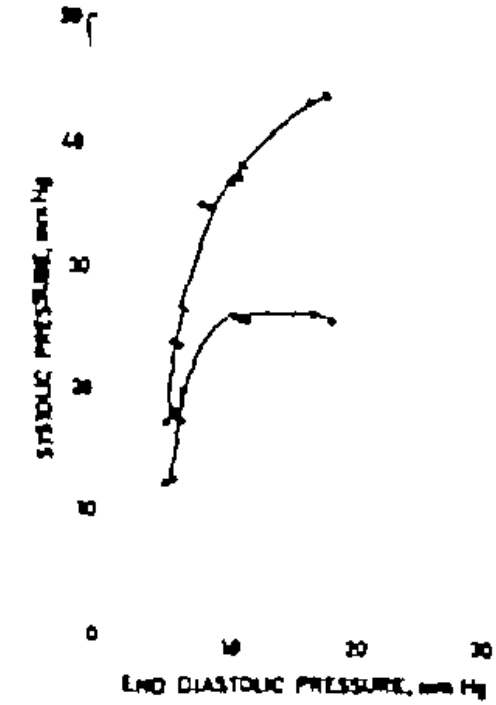


Fig. 5 Relation between *systolic pressure* and end diastolic pressure in one heart preparation. Upper curve denotes total systolic pressure lower curv activ systolic pressure. Contraction rate 109 beats per min. Pressure recorded by means of an intra ventricular balloon.

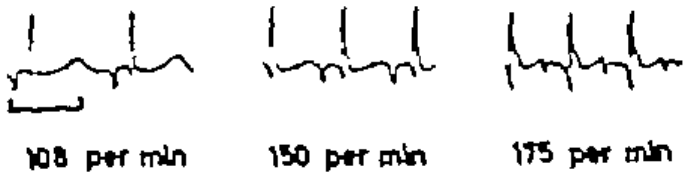


Fig 6. Electrogram recordings of one heart preparation without pressure recording. Stimulation frequencies as indicated. First (downward) deflection indicates trial wa e. Note increase in trio- entricular conduction time and broadening of entricular complex at high stimulation frequencies. Horizontal bar 200 msec.

increased with increasing stimulation frequency In three preparations, the mean increase was 4 msec for an increase in contraction rate of 10 beats per min within the frequency range of 105—18° beats per min. An increase in stimulation frequency also caused a broadening of the ventricular complex and at stimulation frequencies above 180 per min distortions of the ventricular complex appeared. These effects of stimulation frequency upon the ventricular complex of the electrogram were most pronounced in preparations with a balloon type of pressure recording (Fig 7)

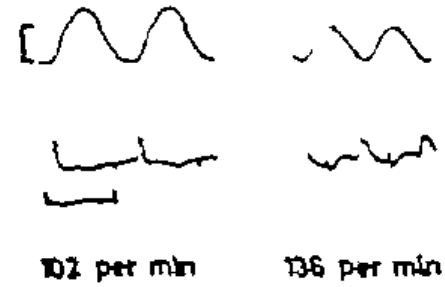


Fig. 7 Simultaneous recordings of electrogram and intra entricular pressure t stimulation frequency of 102 and 136 per min. Note broadening of entricular complex. Pressure ecorded by means of a intra entricular balloon. Vertical bar 20 mm Hg, horizontal bar 200 msec

Discussion

The main purpose of the present study was to assess the influence of contraction rate upon the mechanical output of the individual heart beat of isolated human foetal hearts. In view of the clinical significance of foetal bradycardia, effects induced by a reduction in contraction rate were considered particularly important. Three different recording arrangements were used, all giving similar results except for the fact that intra ntricular pressure alterations occurred during diastole in two of these types but not in preparations in which an open mitral catheter was used for recording the pressure

When comparing the results obtained with these heart preparations perfused via the coronary arteries with findings in isolated tissue preparations such as papillary muscles, the following factors should be considered The spread of excitation in perfused hearts resembles that seen i tiro in contrast with the situation in excised preparations where this factor has largely been excluded by the use of small preparations and/or multielectrode stimulation Therefore ontr ction of the myocardial cells in the whole heart is not compl t ly synchronous i e some muscle fibres are

still (or already) relaxed, while other fibres contract. A different type of inhomogeneity of myocardial contraction is due to the thickness of the walls of the cardiac chambers. Tissue pressure gradients exist within the ventricle walls. At a given pressure in the ventricle cavity tissue pressure is greater close to the endocardium than in the epicardial cell layers (Kirk and Honig 1964 Streeter *et al.* 1970). It might also be added that perfusion of an isolated heart *via* the coronary arteries is quite different from the perfusion of an excised tissue preparation the former reflecting physiological conditions more closely than the latter.

The general conditions of the heart preparations used in the present study are perhaps best judged by comparing the developed intraventricular pressure with the applied coronary perfusion pressure. In heart preparations with the pressure recorded by means of an intraventricular balloon, the total systolic pressure generally exceeded the coronary perfusion pressure when the diastolic pressure was increased above approximately 5 mm Hg these preparations would therefore have been able to perform external work. In preparations with a catheter type of pressure registration, however diastolic pressure was kept only at a few mm Hg. Hence total systolic pressure was lower than the coronary perfusion pressure, which allowed isovolumetric determination of intraventricular pressure also in these preparations.

Contraction rate

Abundant evidence support the view that a reduction in contraction rate below the frequency range existing *in vivo* causes a decrease in isometric twitch tension of excised ventricle preparations from various mammalian species (Koch Weser and Blinks 1963). On the other hand the effects of changes in contraction rate upon the whole heart are less clear. In the isolated dog ventricle a decrease in contraction rate reduces peak intraventricular pressure (Monroe and French 1961) and stroke volume (Rosenblueth *et al.* 1959) to a moderate extent, whereas experiments on dog hearts *in situ* have shown stroke work measured at constant end diastolic volume to remain unchanged over a wide range of contraction rates (Mitchell Wallace and Skinner 1963). By means of strain gauges applied to heart ventricle segments of patients during heart surgery peak contractile force was found to be uninfluenced by a change in contraction rate from 145 to 75 beats per min (Sonnenblick, Morrow and Williams 1966). In adult patients with a complete heart block, it has been shown that a lowering of the contraction rate from 117 to 47 beats per min approximately doubled stroke work index (Rowe *et al.* 1969). However a change in end diastolic volume might have contributed to this effect as intraventricular pressure was not measured in this investigation.

The present study shows that an increase in contraction rate above the spontaneous level (95—120 beats per min) reduced the active systolic pressure, whereas a decrease in rate induced a moderate increase in active systolic pressure. When the diastolic pressure and hence the diastolic volume of the heart was kept constant, such a positive inotropic effect of a reduction in contraction rate could be demon-

strated down to a contraction rate around 90 beats per min. The rate of rise of intraventricular pressure, $(dp/dt)_{max}$ has been taken as an index of the inotropic state or "contractility" of the myocardium (Wallace Skinner and Mitchell 1963 Mason 1969 Morgenstern *et al* 1970) It is of interest to note that in the present study $(dp/dt)_{max}$ decreased at contraction rates below as well as above the spontaneous rate of approximately 100 beats per min. Judging from the active systolic pressure developed, such a reduction in contractility at low contraction rates was fully compensated by an increased duration of the contraction cycle. The frequency dependence of the active systolic pressure of the isolated foetal heart appears to be in contrast with an earlier observation on isolated papillary muscles from human foetuses (Gennser and Nilsson 1968) It was shown that a reduction in contraction rate from 96 to 24 beats per min reduced the isometric tension of the preparations to about half of its original value. These results were obtained at 30 C, whereas the present study was performed at 37°C. The temperature difference probably causes an underestimation of the apparent discrepancy between the results obtained in the two studies, because at temperatures below 37 C the isometric twitch tension is enhanced at low contraction rates owing to a prolongation of the action potential (Kaufmann and Fleckenstein 1965) The present study offers no certain explanation for the difference in frequency-dependence between the active systolic pressure in the whole heart and the isometric tension of the excised papillary muscles. This might however be ascribed to a difference between the two preparations in modes of stimulation. Complete excitation of the foetal ventricles takes an appreciable time, approximately 40 msec at a physiological contraction rate (Durrer *et al.* 1961) whereas excitation of an isolated papillary muscle is complete within a few msec. (The latter figure would be 9 msec, assuming a conduction velocity of 0.5 m/sec (Gennser and Nilsson 1970) and a conduction length of 1 mm using a multielectrode assembly) This lightly asynchronous activation of the whole heart will tend to reduce the developed intra-ventricular pressure by increasing the extension of elastic elements in series and in parallel with the contractile system of conventional models for myocardial contraction (for discussion of these models see Brady 1968 Jewell and Blinks 1968) This effect of the asynchronous excitation of the heart will increase at high contraction rates due to shortening of the duration of the contractile process in myocardial cells (Gennser and Nilsson 1968) The decreased conduction velocity of the action potential at high stimulation frequencies might further increase the physiological asynchrony (Gennser and Nilsson 1970)

In the present study the contraction rates of the isolated human foetal hearts could be reduced to values below those usually encountered during foetal bradycardia. The positive inotropic effect of a decrease in contraction rate observed in isolated hearts, suggests that foetal bradycardia *in vivo* does not in itself diminish the capacity of the individual foetal heart beat to perform external work.

End diastolic pressure

As pointed out by Downing, Talner and Gardner (1965) the Frank-Starling mechanism might be considered of special importance in the adjustment of the foetal circulation from intra to extrauterine life with the accompanying marked reduction in pulmonary vascular resistance and increase in aortic pressure and systemic blood flow (Cross, Dawes and Mott 1959 Dawes 1961 Dawes 1966) The present results indicate that also in the midterm foetus, the left ventricle can change its mechanical output considerably in response to alterations in end diastolic pressure. It is of interest to note that in the experiment shown in Fig 5 active systolic pressure reached a plateau at approximately the same end diastolic pressure as that at which maximal stroke volume is approached in the adult mammalian left ventricle (Sarnoff and Mitchell 1961) This seems to be in agreement with the fact that end diastolic pressures are of the same magnitude in the foetus and the adult (Assali, Bekey and Morrison 1968) although systolic pressure is much greater in the adult than in the foetal left ventricle (Dawes 1961)

Coronary perfusion pressure and coronary flow

In isolated human foetal hearts studied under the present experimental conditions, coronary flow did not set an upper limit for the mechanical output of the ventricles by restricting metabolic supply to the myocardium. This conclusion is based upon the fact that several positive inotropic responses (induced by an increase in calcium concentration an increase in pH an increase in pCO_2 of the perfusion solution or addition of adrenaline (Andersson, Gennser Nilsson 1970 a)) could be evoked without alterations in coronary perfusion flow Furthermore, when the oxygen content of the perfusing medium was increased by the addition of erythrocytes, the mechanical performance of the entricles did not change.

In the present study an increase in coronary perfusion pressure was found to enhance the active systolic pressure within the range studied. This is in accordance with previous results (Arnold *et al.* 1968) obtained in isolated guinea pig hearts. The authors reached the conclusion that changes in myocardial contractility following alterations in perfusion pressure are due to effects of perfusion pressure *per se* (an increase in perfusion pressure causing stretching of arteries and hence of muscle fibres) and not to variations in coronary blood flow In the present experiments with a balloon type of pressure recording, an increase in perfusion pressure enhanced also the end diastolic pressure. Such changes in diastolic pressure probably reflect alterations in myocardial intravascular pressure and/or an accumulation of fluid in the ventricular cavity outside the pressure recording balloon due to a flow through the Thebesian vessels. The diastolic pressure changes were most evident at low contraction rates owing to the relatively slow and constant increase in intraventricular pressure during diastole. It is not possible to state to what extent the changes observed in end diastolic pressure might have contributed to the inotropic

effects of alterations in coronary perfusion pressure. The relation between coronary flow and perfusion pressure was linear. Thus no autoregulatory mechanisms in the myocardial vascular bed occurred in response to changes in perfusion pressure.

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Influence of Acid Base Changes on Isolated Human Foetal Hearts

By

K. E. Andersson G Gennser and E. Nilsson

Abstract

Andersson K. E., G Gennser and E. Nilsson *Influence of acid base changes on isolated human foetal hearts* Acta physiol. scand. 1970. *80* Suppl. 353 20—33.

Left intraventricular pressure and electrogram were recorded in isolated human foetal heart preparations during perfusion with solutions of different [$NaHCO_3$] and pCO_2. In spontaneously beating hearts a decrease in pH within the range 7.75—6.62, induced by an increase in pCO_2 and/or decrease in [$NaHCO_3$] reduced spontaneous contraction rate, active systolic pressure and $(dp/dt)_{max}$. Within the pH range studied contraction rate was reduced more than active systolic pressure. In electrically stimulated preparations lowering of external pH, caused by a decrease of [$NaHCO_3$] or by increase of pCO_2 reduced active systolic pressure. This reduction was more pronounced at high than at low contraction rates. Electrogram changes indicated that reduction in pH of the perfusion solution reduced the intraventricular conduction velocity. A parallel increase in both [$NaHCO_3$] and pCO_2 at a constant external pH of 7.04 or 7.33 increased active systolic pressure and $(dp/dt)_{max}$. Judging from electrogram recordings, such parallel increase in [$NaHCO_3$] and pCO_2 was accompanied by an increased intraventricular conduction velocity. The mechanisms of inotropic changes induced by alterations of acid-base parameters are discussed.

Introduction

This report is concerned with the effect of pH and pCO_2 changes on the electrogram and the intraventricular pressure of isolated human foetal hearts. In a preceding investigation (Andersson Gennser and Nilsson 1970) it was shown that the mechanical output of the individual heart beat of isolated foetal heart preparations was markedly affected by changes in contraction rate. The role of the contraction rate in modifying effects induced by acid-base changes will therefore be considered in this study.

Material and methods

15 foetal hearts were used (estimated gestational age of the foetuses ranged from 16 to 20 weeks and their crown-heel length 16—24 cm). Some of the hearts were identical with those reported in a previous paper (Andersson Gennser and Nilsson 1970). That report also described the mounting and recording techniques used in the present study.

Solutions

A Ringer's solution of the following composition was used as standard solution (mM) NaCl 117 $NaHCO_3$ 20 $MgSO_4$ 1.5 NaH_2PO_4 1.5 KCl 4 $CaCl_2$ 2 and glucose 3.3. In other perfusion solutions used the sum of [NaCl] and [$NaHCO_3$] was kept constant at 137 mM other constituents being as indicated for the standard solution. Solutions of different [$NaHCO_3$] were equilibrated with gas mixtures containing 5.0 (4.9—5.1) or 13.5 (13.4—13.6) vol. % CO_2, 80 vol. % O_2 and N_2 to 100 vol. %. The composition and pH of the various solutions are given below

[$NaHCO_3$] mM	10	20	54	10	27	54
CO_2, vol. %	5	5	5	13.5	13.5	13.5
pCO_2, mm Hg	35	35	35	96	96	96
pH	7.04	7.33	7.75	6.62	7.04	7.33
range	6.98— 7.10	7.28— 7.38	7.70— 7.80	6.55— 6.69	6.98— 7.10	7.28— 7.38

Rheomacrodex® was added to all solutions to a final concentration of 2.5 %.

As bicarbonate- and Ca^{2+} ions form diffusable calcium complexes (Moore 1970) changes in [HCO_3^-] might be expected to alter the ionized calcium concentration, [Ca^{2+}], of a solution. Therefore the [Ca^{2+}] was measured by means of an Orion "flow through" ion-exchange calcium electrode (No. 90—20) in the solutions with 20 and 54 mM $NaHCO_3$ (both containing 2 mM $CaCl_2$). Measurements were made in these solutions at a pCO_2 of 35 and 96 mm Hg. The [Ca^{2+}] in 20 and in 54 mM $NaHCO_3$ solutions was found to be equal to [Ca^{2+}] in 150 mM NaCl solutions containing 0.90 and 0.71 mM Ca^{2+} respectively. Changes in pCO_2 and pH were not found to influence [Ca^{2+}]. Thus at any pH replacement of 10 mM NaCl by 10 mM $NaHCO_3$ in the Ringer's solution decreases the [Ca^{2+}] by 6.2 %.

The temperature of the perfusion solutions was kept constant at 37° C±0.5.

Results

Influence of acid-base changes on contraction rate and active systolic pressure in spontaneously beating hearts

The effects of acid-base changes were studied by perfusing heart preparations with buffer solutions of pH 7.04, 7.33 and 7.75 equilibrated at pCO_2 35 mm Hg, and with solutions of pH 6.6., 7.04 and 7.33 equilibrated at pCO_2 96 mm Hg. The preparations were perfused with the standard solution of pH 7.33 (20 mM $NaHCO_3$) and pCO_2 35 mm Hg between perfusions with the other solutions. Data from the two most complete experiments are given in Fig. 1 A and B, a third experiment yielding similar results. The values in Fig. 1 are steady state determinations after at least ten min perfusion in the respective solutions. All points in Fig. 1 and 2 are given as percentages of the mean value of corresponding measurements in the preceding and

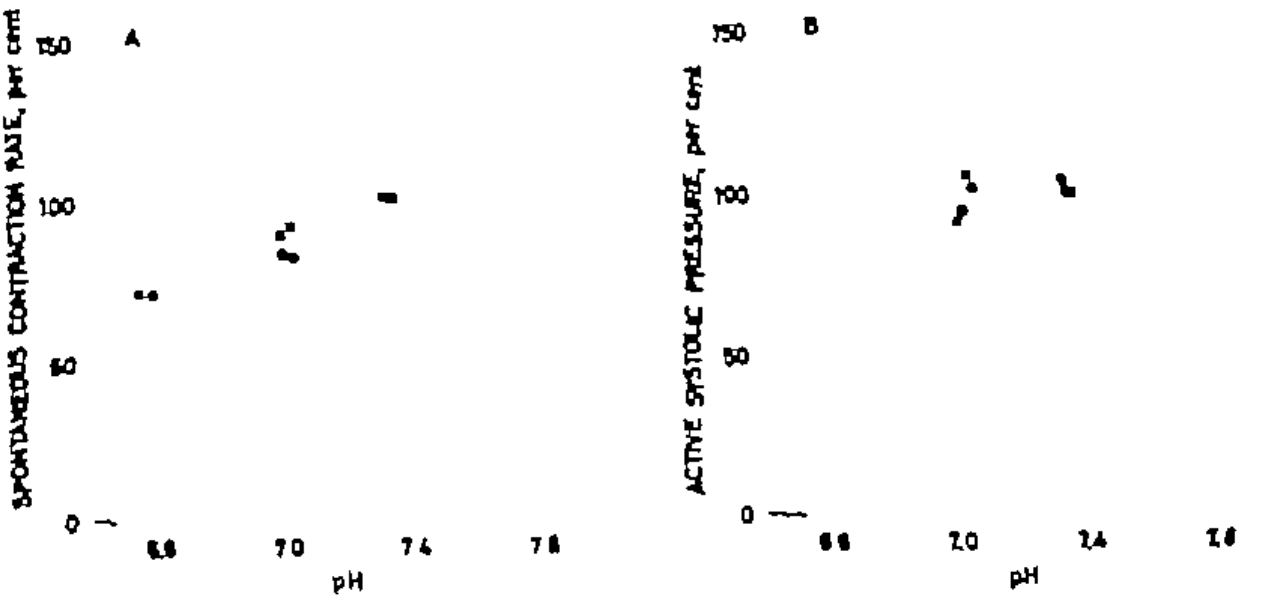


Fig. 1 A. Change in spontaneous contraction rate as a function of external pH. Squares and circles denote different preparations. Filled symbols indicate pCO_2 96 mm Hg, unfilled symbols pCO_2 35 mm Hg. Spontaneous contraction rate at $[NaHCO_3]$ 20 mM and pCO_2 35 mm Hg is taken as 100 %. Pressure recorded by means of an intraventricular balloon.

B. Change in active systolic pressure as a function of external pH. Same preparations and symbols as in Fig. 1 A. Note that at pH 7.0 and 7.3 active systolic pressure is slightly higher at pCO_2 96 mm Hg than at pCO_2 35 mm Hg.

following standard solution. This reduced the influence of deterioration of the heart preparations with time. (The experiments shown in Fig. 1 lasted approximately two hours. During this time the active systolic pressure fell in one experiment from 37.5 to 30.3 mm Hg, in the other from 33.7 to 27.2 mm Hg, while the spontaneous contraction rate changed from 101 to 104 and from 96 to 90 beats per min, respectively.) In the two experiments shown in Fig. 1 A and B a decrease in pH from 7.33 to 6.62 caused a 10 % decrease in active systolic pressure whereas the spontaneous contraction rate was reduced by 70 %. An increase in pH from 7.33 to 7.75 enhanced the active systolic pressure and the contraction rate by 5 and 15 % respectively. The effect of pH-changes upon $(dp/dt)_{max}$ and the duration of the active systolic contraction measured at the 50 % level is given in Fig. 2 A and B. As can be seen, a decrease in pH prolonged the systolic contraction, and $(dp/dt)_{max}$ was reduced to a greater extent than the active systolic pressure by this acid-base change. At a given pH the spontaneous contraction rate does not seem to be dependent upon the concentration of $NaHCO_3$ and pCO_2 (Fig. 1 A). On the other hand Fig. 1 B suggests that a parallel increase in both $[NaHCO_3]$ and pCO_2 at a constant external pH enhances active systolic pressure. The effect of pCO_2 and $[NaHCO_3]$ is also illustrated in Table I which shows that a decrease in pH from 7.33 to 7.01 at a constant pCO_2 of 35 mm Hg reduced the active systolic pressure and $(dp/dt)_{max}$ more mar-

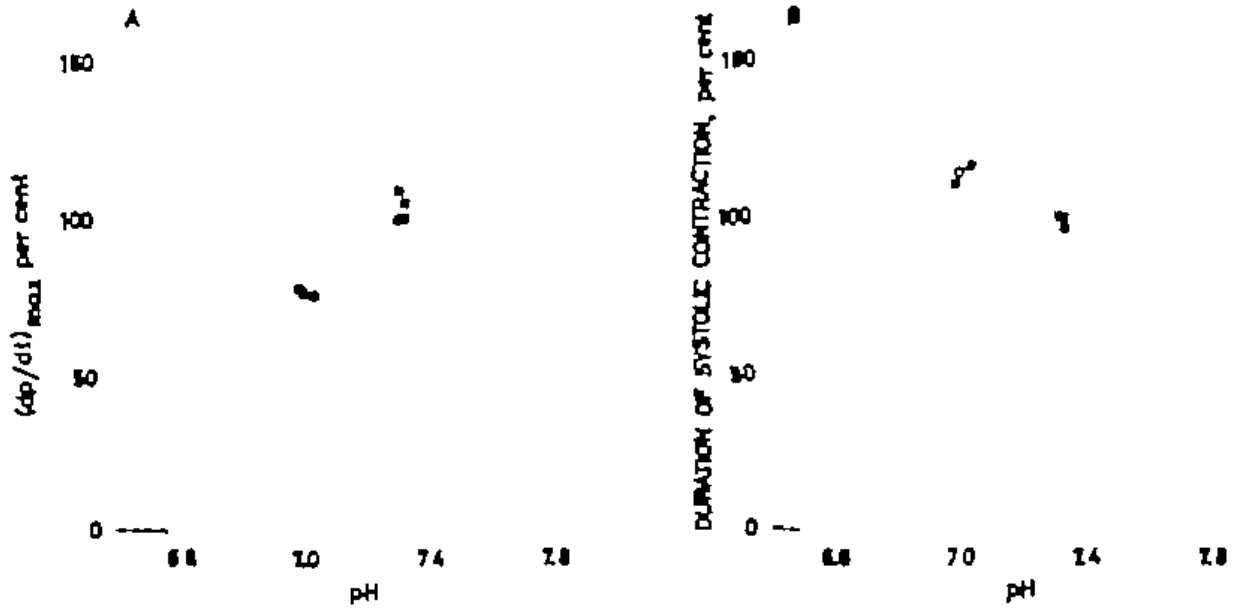


Fig. 4. $(dp/dt)_{max}$ (A) and duration of systolic contraction measured at the 50 % level (B) as function of external pH. Same preparations and symbols as in Fig. 1. Determinations at $[NaHCO_3]$ 20 mM and pCO_2 35 mm Hg is taken as 100 %.

kedly than when the same pH change was induced by an increase in $[NaHCO_3]$ and pCO_2 (from 35 to 96 mm Hg).

Influence of pH and pCO_2 on active systolic pressure in electrically stimulated heart preparations

It was considered desirable to eliminate the effect of changes in contraction rate in the analysis of inotropic effects of alterations in external pH and pCO_2. Intraventricular pressure was therefore recorded in heart preparations stimulated at selected

TABLE I. Changes in spontaneous contraction rate, active systolic pressure (ASP) and $(dp/dt)_{max}$ induced by acidosis. Decrease of pH of perfusion fluid from 7.33 to 7.04 produced by reduction of $[NaHCO_3]$ (II) or by simultaneous increase in $[NaHCO_3]$ and pCO_2 (I).

	I	II
$[NaHCO_3]$ mM	20 → 27	20 → 10
pCO_2, mm Hg	35 → 96	35 → 35
pH	7.33 → 7.04	7.33 → 7.04
Δ Rate	−13 % (−8, −18)	1 % (range −7 – −17)
Δ ASP	+ 3 % (+1 + 5)	−16 % (range −6 – −31)
Δ $(dp/dt)_{max}$	−15 % (−6, − 4)	−16 % (range −13 – −18)
Number of preparations	5	

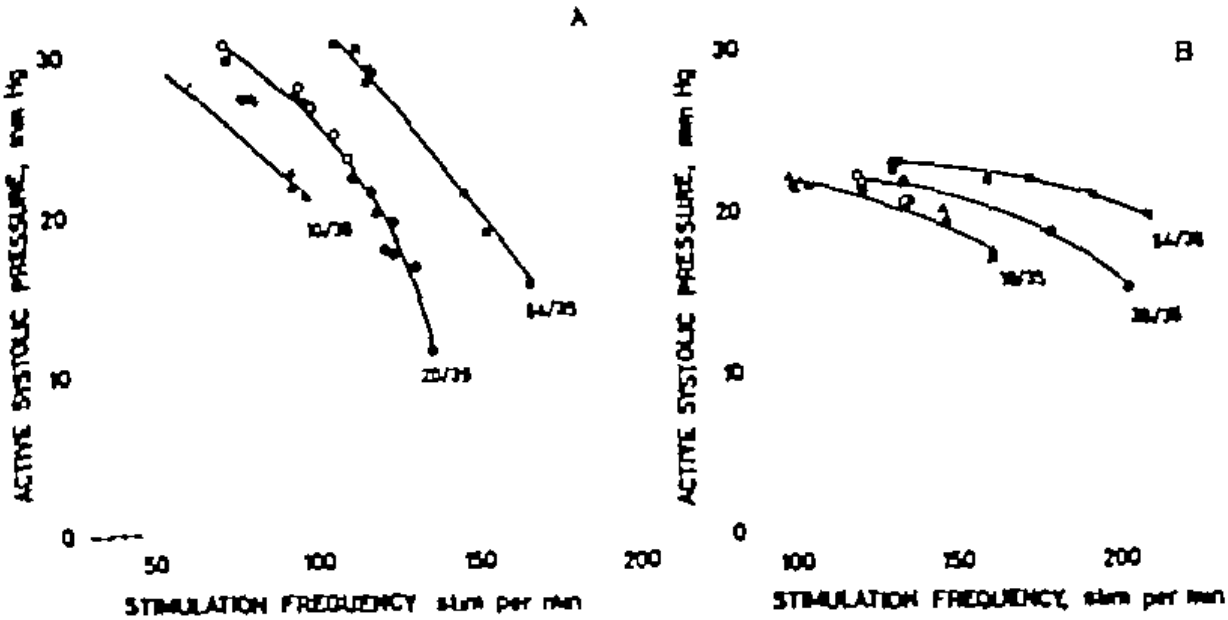


Fig. 3. Active systolic pressure at pH 7.75, 7.33 and 7.04 (right to left) as a function of stimulation frequency in two heart preparations, (A) and (B). Numbers before the oblique lines indicate [NaHCO$_3$] in mM; numbers after the oblique lines indicate pCO_2 in mm Hg in the perfusing solution.

A. Measurements were performed in the following order: ○ ▲ ○ ■ ●. Pressure recorded by means of an intraventricular balloon. In order to reduce spontaneous contraction rate the sinus node of the preparation was excised. The lines in this and following figures are drawn by the eye.

B. Measurements were performed in the following order: ○ ▲ ○ ■. Pressure recorded by means of an open catheter in the left ventricle.

frequencies during perfusion with solutions of different [$NaHCO_3$] and pCO_2. Solutions with pH 7.04, 7.33 and 7.75 equilibrated at a pCO_2 of 35 mm Hg were used. The maximum frequency at which the heart preparations could be paced without the appearance of an atrio-ventricular block decreased with decreasing pH in the perfusion solution. The influence of external pH upon active systolic pressure is illustrated in Fig. 3 A and B. The difference in active systolic pressure at the beginning and the end of the experiment measured in the standard solution at any contraction rate within the range studied was less than ten per cent. The values given were recorded under steady state conditions after five to ten minutes perfusion of the preparations with the respective solutions. As can be seen a *decrease in pH* from 7.33 to 7.04 caused a shift of the contraction frequency pressure curve to the left. At a contraction rate of 120 beats per min the decrease in active systolic pressure induced by such a pH change from 7.33 to 7.04 was 16 % (range 5—28 % in three preparations). In most experiments, the inotropic change induced by a reduction in pH was greater at contraction rates above 120 beats per min. The response of the active systolic pressure to an *increase in pH* from 7.33 to 7.75 at a constant pCO_2 of 35 mm Hg is also shown in Fig. 3. At a contraction rate of 120 beats per min this pH change caused a positive inotropic change of 20 % (range 8—36 % in three

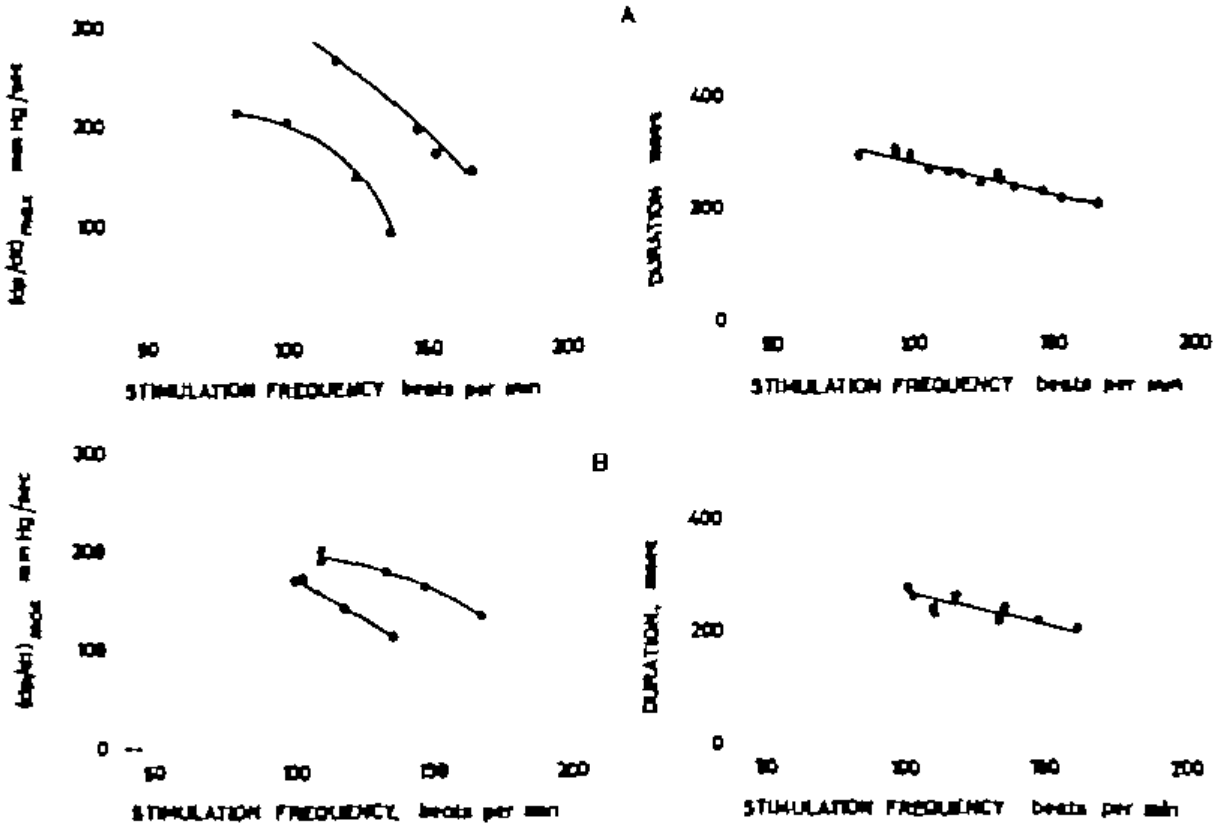


Fig. 4 $(dp/dt)_{max}$ and duration of systolic contraction measured at the 50 % level in two preparations (A) and (B) as a function of external pH. Pressure recorded by means of an intraventricular balloon in both hearts. Symbols (figures within brackets denote [$NaHCO_3$]/pCO_2) A: ● pH 7.33 (20/35) ○ pH 7.75 (54/35) B ● pH 7.33 (20/35) ○ pH 7.04 (10/35)

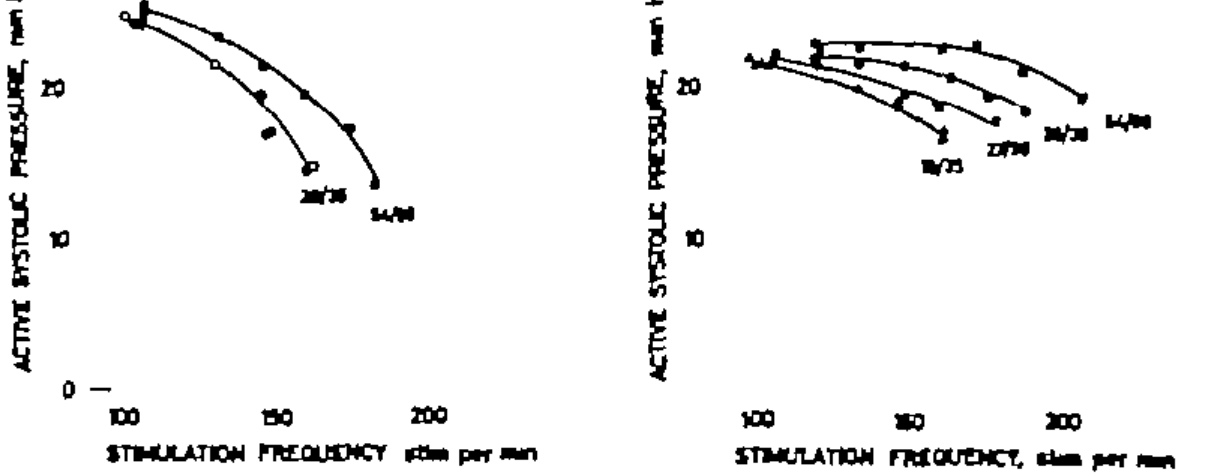


Fig. 5. Active systolic pressure at pH 7.04 and 7.33 as function of stimulation frequency in two heart preparations. Numbers indicate [$NaHCO_3$] and pCO_2 as in Fig. 3. Note that both 54/96 and 20/35 represent pH 7.33 both 7/96 and 10/35 pH 7.04

A. Measurements were performed in the following order: ○, □ ● Pressure recorded by means of an intraventricular balloon.

B. Measurements were performed in the following order □, ● Δ ■ Pressure recorded by means of catheter in the left ventricle. Same preparation as in Fig. 3 B.

preparations) Neither the interval between the beginning and the peak of the contraction nor the duration of the contractile cycle measured at the 50 % level at any given contraction rate varied with external pH, and the inotropic alterations were attributable only to changes in the rate of development of intraventricular pressure (Fig 4 A and B) At any given external pH the duration of the systolic contraction decreased with increasing contraction rate (cf Andersson, Gennser and Nilsson 1970)

In five experiments both [$NaHCO_3$] and pCO_2 in the perfusion solution were increased 2.7 fold, keeping the external pH constant at 7.33 or 7 04 respectively (see Methods) These experiments showed that after steady state conditions had been established (after 3—10 min) the active systolic pressure was augmented by such a parallel increase in both [$NaHCO_3$] and pCO_2 within the frequency range studied At a contraction rate of 120 beats per min the increase in active systolic pressure induced by this inotropic intervention was 6.4 % (range 2.6—11.6 % in four heart preparations) In three of the four preparations this positive inotropic effect was greater at high than at low contraction rates Two of these experiments are shown in Fig 5 A and B. The rate of intraventricular pressure development increased at high contraction frequencies, (Fig. 6) whereas the duration of the systolic contraction was unaffected by this inotropic intervention.

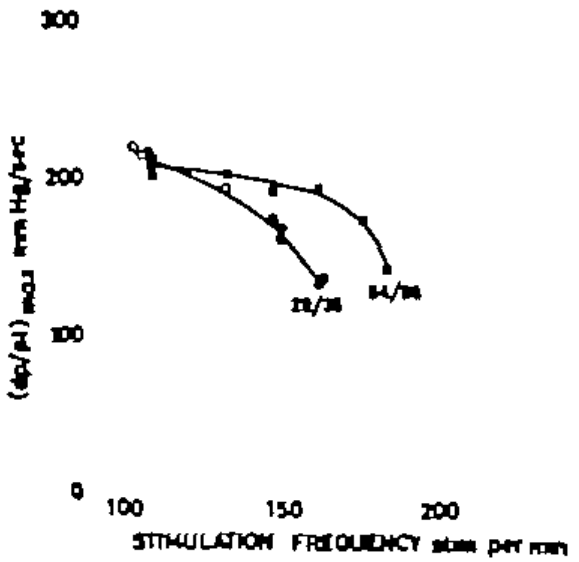


Fig 6. $(dp/dt)_{max}$ t different [$NaHCO_3$] and pCO_2 as f nction of stimulation frequency Numbers indicate [N HCO_3] and pCO_2 as in Fig. 5 pH is 7.33 in both solution 20/35 and solution 54/36. Measurements wer performed in the following order ○ ■ ● Pressure recorded by means of an intraventricular balloon.

Inotropic effect of adrenalin e a d calcium at n rmal and low pH

Adrenaline in concentrations 1×10^{-8} to 5×10^{-7} g/ml had a positive chronotropic and inotropic effect on isolated human foetal heart preparations. The effect of adrenaline (1×10^{-7} g ml) upon intraventricular pressure in one electrically stimulated heart preparat n perfused with a solution of pH 7.33 (20 mM $NaHCO_3$, pCO_2 35 mm Hg) is shown n Fig 7 A. The positive inotropic ffect of an increase in

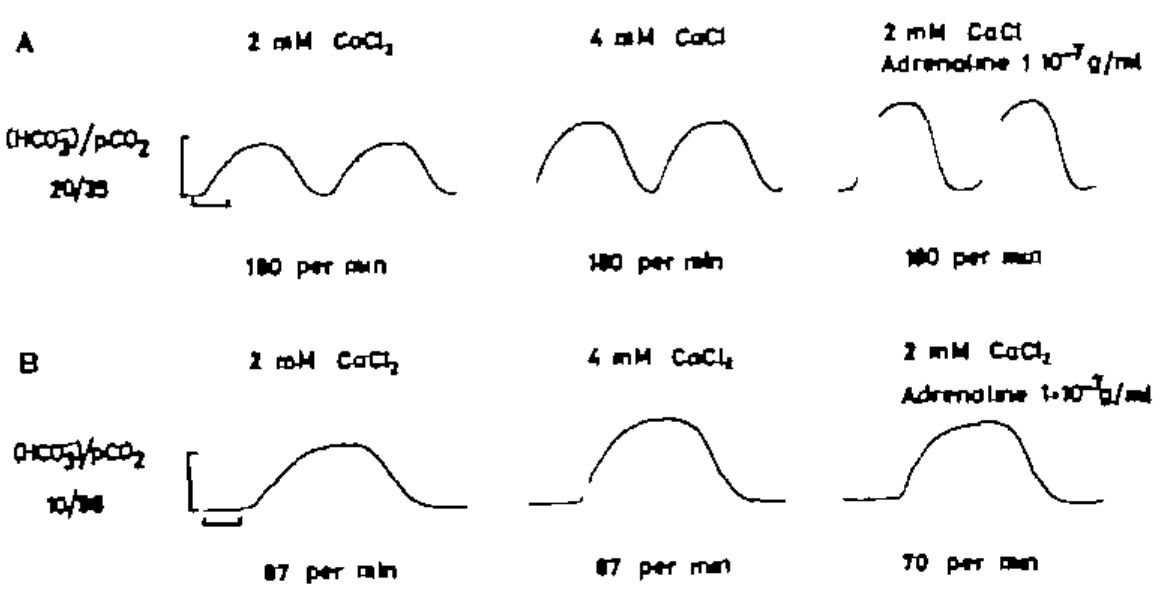


Fig. 7 Intraventricular pressure curves showing effects of adrenaline and changes in [$CaCl_2$] at pH 7.33 (A) and pH 6.62 (B). Sinus node of the preparation was excised. Pressure recorded by means of a catheter in the left ventricle. Numbers indicate [$NaHCO_3$] and pCO_2 as in Fig. 3. Stimulation frequencies indicated. Calibration: Vertical bars 20 mm Hg, horizontal bars 100 msec.

external calcium concentration is also illustrated. In contrast with an increased calcium concentration adrenaline reduced the time to maximal intraventricular pressure and the duration of the contractile cycle. Fig. 7 B shows the inotropic effects of adrenaline and calcium at pH 6.62 (10 mM $NaHCO_3$, pCO_2 96 mm Hg). No quantitative analysis was performed concerning the influence of pH changes upon these inotropic effects. It should be noted, however, that also at this low pH both calcium and adrenaline exerted a positive inotropic effect.

Electrogram changes induced by acid-base alterations

At any given pH in the range 7.04—7.75 atrio-ventricular conduction time and the duration of the ventricular complex increased with increasing stimulation frequency (cf. preceding paper (Andersson, Gemzer and Nilsson 1970). This may be ascribed to a decrease in conduction velocity of the myocardial action potential at high contraction rates (Gemzer and Nilsson 1970). At a given contraction frequency a decrease in pH increased the atrio-ventricular conduction time and the duration of the ventricular complex, an increase in pH inducing the reverse changes. A parallel increase in [$NaHCO_3$] and pCO_2 shortened the atrio-ventricular conduction time and decreased the duration of the ventricular complex. These electrogram alterations indicate that a lowering of pH reduces conduction velocity in the foetal myocardium, whereas an increase of both [$NaHCO_3$] and pCO_2 at constant external

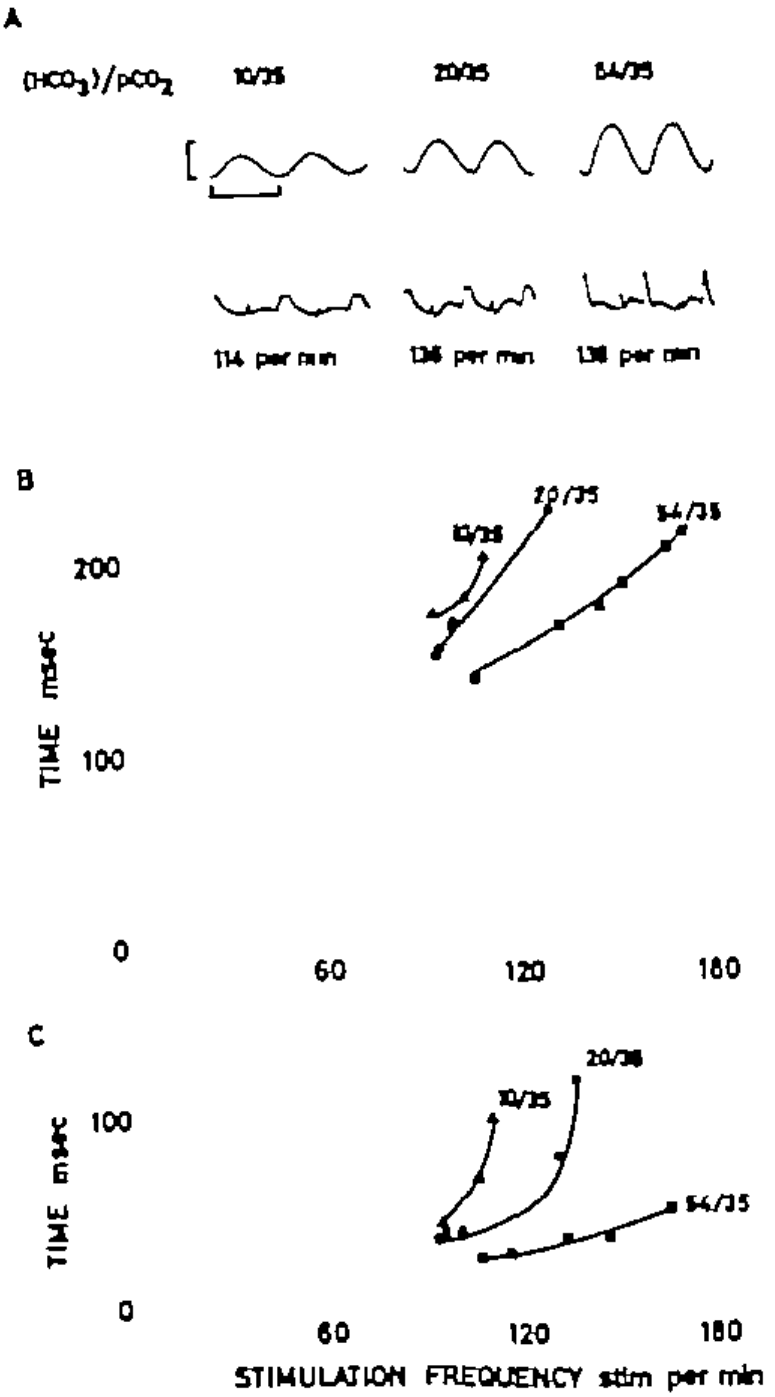


Fig. 8. Influence of pH-changes induced by variation of [$NaHCO_3$] on electrogram of one foetal heart preparation. Numbers indicate [$NaHCO_3$] and pCO_2 as in Fig. 3.

A. Recordings of intraventricular pressure (upper curves) and electrogram (lower curves) during perfusion with solutions of pH 7.75, 7.53 and 7.04 (right to left). Solution 10/35, pH 7.04; atrioventricular conduction block occurred at stimulation frequencies above 114 per min. Calibration: Vertical bar 20 mm Hg, horizontal bar 0.5 sec. Pressure recorded by means of an intraventricular balloon. Sinus node of the preparation was excised.

B. Atrio-ventricular conduction time measured from stimulation artefact to beginning of ventricular complex, as function of stimulation frequency.

C. Duration of ventricular complex, measured at the 50% level, as function of stimulation frequency.

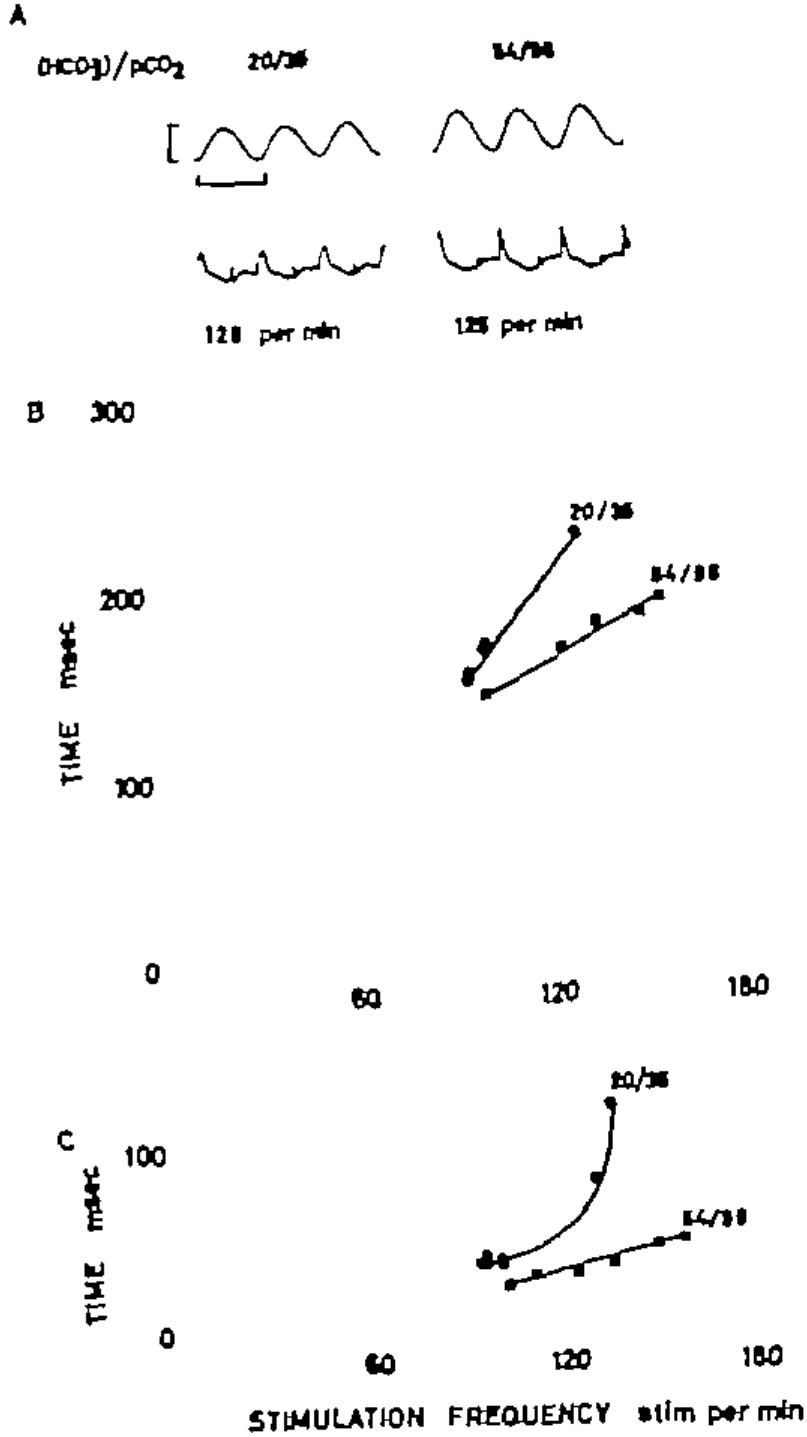


Fig. 9 Influence of parallel changes in [$NaHCO_3$] and pCO_2 on electrogram. Same preparation as in Fig. 8. Numbers indicat [$NaHCO_3$] and pCO_2 as in Fig. 3 pH 7.33 in both solutions.

A. Recordings of intraventricular pressure (upper curves) and electrogram (lower curves) during perfusion with solutions of pH 7.33, before (20/35) and after (54/96) parallel 2.7-fold increase in [$NaHCO_3$] and pCO_2. Calibration: Vertical bar 20 mm Hg, horizontal bar 0.5 sec.

B. Atrio-ventricular conduction time measured from stimulation artefact to beginning of ventricular complex, as a function of stimulation frequency

C. Duration of ventricular complex, measured at the 50 % level, as a function of stimulation frequency

pH raises the conduction velocity Tracings and diagrams obtained in one typical experiment are shown in Fig 8 and 9 Similar results were obtained in two other experiments.

Discussion

The present report deals with the effect of changes in acid-base variables upon spontaneous contraction rate, electrogram and intraventricular pressure in isolated, isovolumetrically contracting human foetal heart preparations. The pH and pCO_2 range studied exceeds that found in foetal blood at delivery of both normal foetuses and depressed foetuses (Jacobson 1970) In the latter investigation a pH of *7 11±0 01 (mean±SD) and a pCO_2 of 66±6 mm Hg was found in* umbilical artery blood of depressed foetuses (with Apgar score < 7)

Chronotropic and inotropic effects induced by changes in external pH

In the present study a decrease in pH within the range studied (6.62—7 75) was found to reduce the spontaneous contraction rate and the maximum frequency at which the preparations could be stimulated without the appearance of A—V-conduction block This is in agreement with earlier investigations on isolated hearts or heart lung preparations (McElroy Gerdes and Brown 1958 Nejad and Ogden 1967) A lowering of pH also reduced the conduction velocity within the ventricles, as judged from widening of the ventricular electrogram complex. The latter finding agrees with direct measurements of influence of pH-changes on conduction velocity in isolated rabbit atrial preparations (Vaughan Williams and Whyte 1967) A *decrease in external pH at constant pCO_2 diminished the contractility of the isolated* human foetal hearts in accordance with earlier observations on excised myocardial preparations (Vaughan Williams 1955 Vaughan Williams and Whyte 1967 Pannier and Leuven 1968) isolated hearts and heart lung preparations (McElroy Gerdes and Brown 1958 Opie 1965 Nejad and Ogden 1967 Ng, Lewy and Zieske 1967 Kolliardt Wirth and Dudeck 1967) The present study was not aimed at elucidating the mechanism( ) behind the negative inotropic effect of acidosis. However it should be pointed out that a *reduction in conduction velocity reduced* by e g. acidosis can in itself have a negative inotropic effect on the whole heart and to a less extent on excised myocardial preparations by causing an asynchronous contraction (cf Andersson, Gennser and Nilsson 1970) It is reasonable to assume that inotropic changes observed in the present study following alterations of external pH may be explained in part by diff rences in intra entricular conduction velocity

Factors influ enci g the negat v inot opic ff ct f acido is

In the intact organism a negative inotropic effect of reduction in pH can, at least temporarily be compensated by an activation of the sympathetic nervous system

induced by acidosis (Downing, Talner and Gardner 1965 Downing Talner and Gardner 1966 a, b Wildenthal *et al* 1968) It has been shown that during beta receptor blockade with propranolol a decrease in pH from 7.34 to 7.08 significantly reduced cardiac contractility in cats, whereas cardiac function was unaffected by a similar pH-change in cats with intact adrenergic function (Rocamora and Downing 1969) Divergent results have been obtained, however concerning the time course of the activation of the sympatho-adrenal system induced by acidosis (Talner Gardner and Downing 1966 Wildenthal *et al.* 1968) The present report indicates that at midgestation adrenaline can cause a positive inotropic response of the human foetal heart also at low extracellular pH. The negative inotropic effect induced by a decrease in pH may therefore to some extent be offset by adrenergic influences also in the human foetus.

In the present study an increase in external calcium concentration was found to exert a positive inotropic effect. It has been shown (Moore 1970) that a reduction in pH from 7 4 to 6.8 causes approximately a 15 % increase in $[Ca^{2+}]$ of serum and heparinized whole blood. Such an increase in $[Ca^{2+}]$ might be of importance *in vivo* in reducing the negative inotropic effect of acidosis.

As described in a preceding paper (Andersson, Gennser and Nilsson 1970) a lowering of the contraction rate of the isolated human foetal heart below the spontaneous rate enhanced the amplitude of the isovolumetric contraction. *In vivo* such a positive inotropic effect of bradycardia might be of significance for maintenance of the efficiency of the individual heart beat during acidosis.

Inotropic effects induced by changes in pCO_2

The influence of pCO_2 upon myocardial contractility is still a matter of controversy Hypercapnia has been found to increase cardiac stroke volume in man (Richardson, Wasserman and Patterson 1961 Robseth 1966 Cullen, Eger and Gregory 1969) adult dog (Carson *et al.* 1965) and foetal lamb (Koivikko 1969) The interpretation of the observed effects of pCO_2 upon myocardial contractility is, however complicated by simultaneous variations in pH and by activation of the sympathetic nervous system. Early investigations showing negative inotropic effects of carbon dioxide on isolated hearts or heart lung preparations did not distinguish pH-effects from those due to pCO_2-alterations (Jerusalem and Starling 1910 Ketcham, King and Hooker 1912, Boniface and Brown 1953 Nahas and Cavert 1957) In experiments with isolated whole heart preparations for elucidating the selective influence of pH and pCO_2, it has been concluded that pCO_2 changes do not alter the mechanical performance of the heart (McElroy Gerdes and Brown 1958, Nejad and Ogden 1967 Ng, Lewy and Zieske 1967) In rabbit atrial preparations a parallel increase in $[NaHCO_3]$ and pCO_2 at constant pH in the external medium was found not to alter the contractile force of the preparations (Vaughan Williams and Whyte 1967) whereas in cat papillary muscles a transient (Pannier and Leusen 1968) or a slight permanent reduction of the contractile strength (Cingolani *et al.* 1970) was noted

after such an alteration of the perfusing medium. The divergency between these conclusions is not surprising, as the investigations differ not only in animal species and type of preparation used but also in experimental conditions which can influence the results such as temperature, mode of stimulation and stimulation frequency. Furthermore, the fact that an increase in $[HCO_3^-]$ decreases the ionized calcium concentration seems to have received no attention in previous studies.

The present study shows that in human foetal hearts an increase in $[NaHCO_3]$ and pCO_2 at constant external pH has a moderately positive inotropic effect despite a decrease in $[Ca^{2+}]$ concomitant with this change. (A shift in $[NaHCO_3]$ from 20 to 54 mM reduced $[Ca^{2+}]$ by 21 % see Methods). This positive inotropic effect may be of clinical relevance in the discussion of the pathophysiology of acid-base changes in the foetus. It should be pointed out, however that the present results were obtained during a relatively short period of exposure to high pCO_2 (< 20 minutes). Electrogram recordings indicate a shortened intraventricular conduction time following a parallel increase in pCO_2 and $[NaHCO_3]$. This suggests that a more synchronous activation of the myocardium may be responsible for the observed increase in contractility. In this connection it is of interest that a raised conduction velocity has been demonstrated in atria after a parallel increase in $[NaHCO_3]$ and pCO_2 (Vaughan Williams and Whyte 1967).

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Influence of Mepivacaine on Isolated Human Foetal Hearts at Normal and Low pH

By

K. E. Andersson G Gennser and E. Nilsson

Abstract

Andersson K. E., G Gennser and E. Nilsson *Influence of mepivacaine on isolated human foetal hearts at normal and low pH* Acta physiol. scand. 1970 *80* Suppl. 353 31—47

In human foetal hearts from midgestation, perfused *in vitro* in Langendorff the influence of mepivacaine (concentrations 2.5—20 μg/ml) on spontaneous contraction rate, active systolic pressure, and electrogram was studied at pH 7.35 and 7.00. In each individual heart the effects of the local anaesthetic were dependent on its concentration in the perfusion fluid. Active systolic pressure and $(dp/dt)_{max}$ was reduced by 23 and 30 per cent, respectively at a concentration of 2.5 μg/ml. Only a moderate reduction (12 per cent) of the spontaneous contraction rate was seen even at the highest concentration used (20 μg/ml). In the electrogram, changes indicating a prolongation of the atrio-ventricular and intra-ventricular conduction time were seen at mepivacaine concentrations in the perfusion fluid exceeding 2.5 μg/ml. A lowering of pH from 7.35 to 7.00 induced changes in the parameters studied similar to those evoked by mepivacaine. It was found that the effects of a lowering of pH were added to those produced by the local anaesthetic. Possible mechanisms behind the mepivacaine induced changes are discussed.

Introduction

After the administration of local anaesthetics for obstetrical analgesia as paracervical block (PCB) changes in the heart rate of the foetus have been observed. Both bradycardia and tachycardia have been noted, bradycardia being the more common finding (for references, see Teramo and Widholm 1967). The significance of changes in the foetal heart rate during labour is difficult to evaluate (Assali, Bekey and Morrison 1968 Sureau 1969) and the nature of foetal bradycardia during PCB is not clear. It has been suggested that rapid absorption of the local anaesthetic into the foetal circulation would give concentrations in foetal blood high enough to have a direct depressive action on the myocardium (Page Kamm and Chappel 1961 Teramo and Widholm 1967 Gordon 1968 Asling *et al* 1970). High concentrations of local anaesthetics are known to diminish excitability conduction velocity and contractility of mammalian myocardium. These effects are seen in intact mammals including man they can also be demonstrated in isolated heart preparations (Harrison Sprouse and Morrow 1963, Austen and Moran 1965 Lieberman *et al* 1968, Nayler *et al* 1969 Nahas, Lachapelle and Tremblay 1969)

The aim of the present study was to analyse the influence of mepivacaine on the electrical and mechanical performance of isolated, human foetal hearts. The parameters investigated were spontaneous contraction rate, intraventricular pressure, and electrogram. A fall in pH of foetal scalp-blood samples has been observed in association with bradycardia during PCB (Teramo and Widholm 1967, Hickl and Kirchner 1968, Asling *et al.* 1970). Combined effects of local anaesthetics and acidosis on foetal myocardium could be of clinical importance. It was therefore considered of interest to study the effects of mepivacaine both at normal and at low pH.

Methods

Material

Foetal hearts were obtained at *legal interruptions of pregnancy performed by* hysterotomy. Fourteen hearts with no signs of malformation were used. The estimated gestational age of the foetuses ranged from 14 to 24 weeks and their crown-heel length from 14 to 30 cm. The wet weight of the hearts was 0.5—3 g and their spontaneous contraction rates ranged from 83 to 135 beats per min at the beginning of the experiments.

Preparation and mounting

As soon as possible after the evacuation of the uterus, the hearts were dissected, perfused *via* the coronary arteries *ad modum* Langendorff and mounted in a thermostated bath under the same experimental conditions as described previously (Andersson, Gennser and Nilsson 1970 a).

Stimulation and recording technique

Electrical stimulation and recording of left intraventricular pressure and electrogram were performed as described in an earlier report (Andersson, Gennser and Nilsson 1970 a). In five hearts, the pressure was registered by means of a thin, non-elastic balloon, and in five preparations, an open catheter was inserted into the ventricle through the *mitral ostium*. *In two other hearts, pressures within the right* and left ventricles were recorded simultaneously. For the pressure recording within the right ventricle, a catheter was introduced through the valve of the pulmonary artery. This catheter was fixed to the pulmonary artery by a ligature just above the valvular plane. In these two hearts, left ventricular pressure was recorded by means of an open catheter through the mitral ostium.

Simultaneous registrations of intraventricular pressure and electrogram were performed in eight preparations. Either the electrogram or the intraventricular pressure alone was studied in two and four hearts, respectively.

Contraction rate, active systolic pressure, maximum rate of pressure development ($(dp/dt)_{max}$) and electrogram measurements were performed as described (Andersson, Gennser and Nilsson 1970 a).

Solutions

A Ringer's solution of the following composition was used as a standard solution (mM) NaCl 117 $NaHCO_3$ 20 $MgSO_4$ 1.5 NaH_2PO_4 1.5 KCl 5 $CaCl_2$ 2, glucose 3.3 *Rheomacrodex® to a final concentration of 2.5 %* was added. The standard solution was equilibrated with a gas mixture containing 80 vol.% O_2, 15 vol.% N_2 and 5 vol. % CO_2, and in this way pH was kept at 7.35 (range 7.30—7.40) In order to obtain a pH of 7.00 (range 6.95—7.05) during equilibration with the described gas mixture, a Ringer's solution containing $NaHCO_3$ 10 mM and NaCl 1.97 mM was used, the other components being as indicated for the standard solution. To these Ringer's solutions mepivacaine (mepivacaine hydrochloride, Carbocain®) was added to final concentrations of 2.5 5 10 and 20 μg/ml. In two cases, a Ringer's solution (pH 7.35) containing lidocaine (lidocaine chloride, Xylocain®) 20 μg/ml was used.

The temperature of the perfusion solutions was kept constant at 37±0.5 C.

Chemical analysis

Chemical determination of mepivacaine was performed by gas-liquid chromatography

Results

Only heart preparations were used in which before the experiment began, a steady state condition could be obtained, as judged from the electrical and mechanical recordings. All preparations were perfused with the different solutions in periods of 1—15 min. During these perfusion periods, a new steady state was obtained in all spontaneously beating hearts (except in two preparations in high mepivacaine concentrations, see below) The active systolic pressure and the spontaneous contraction rate of the preparations in the standard solution were slightly reduced during the experiments (2—4 hours) The mean change of the active systolic pressure was 3.5% (n 12, range −19 — +14 %) and that of the contraction rate 2.5% (n 13 range −13 — +8 %) To reduce this source of error the values obtained during steady state in the test solution were expressed in per cent of the mean of the corresponding values obtained in the preceding and following standard Ringer's solution

Influence of mepivacaine on spontaneously beating hearts

The effects of mepivacaine on spontaneous contraction rate, active systolic pressure and/or electrogram were investigated in 1 hearts. The results of these experiments are summarized in Table 1 The changes in the spontaneous contraction rate in the A—V conduction time and in the duration of the ventricular complex in the electrogram were directly related to the concentration of mepivacaine in the perfusion

TABLE I. Influence of mepivacaine on spontaneously beating hearts. Values indicate deviation, in per cent, from measurements in standard Ringer' solution without mepivacaine, pH 7.35

Preparation No.	Mepivacaine conc. µg/ml	Spontaneous contraction rate	$(dp/dt)_{max}$	Activ systolic pressure	A V conduction time	Duration of ventricular complex	Type of pressure registration
4	2.5	± 0	—13	—13	± 0	+10	open catheter
5	2.5	— 6	—39	—39	—	—	
6	2.5	— 4	—25	—14	—	—	"
7	2.5	— 4	—44	—24	+10	± 0	"
Mean		— 3.5	—30.3	—22.5	(+5)	(+5)	
1	5	— 6	—22	—11	+15	+21	open catheter
9	5	—10	—49	—26	—	—	balloon
10	5	— 6	—29	—17	+10	+100	
11	5	— 5	—25	— 6	+16	+16	"
Mean		— 6.8	—31.3	—15.0	+13.7	+45.7	
1	10	—10	—31	—16	+30	+58	open catheter
2	10	— 6	—58	—27	+17	+63	
6	10	— 7	—48	—34	—	—	
7	10	— 4	—87	—79	+25	+85	
8	10	— 23	—	—	—	—	
Mean		—10.0	—55.8	—39.0	+28.0	+68.7	
1	20	—17	—58	—31	+46	+105	open athete
13	20	— 11	—48	—29	+140	+86	balloon
14	20	— 9	—36	—14	+73	+48	
Mean		— 1.3	—44.0	—21.3	+86.3	+79.7	

fluid. In every heart in which more than one concentration of mepivacaine was tested, the changes in all parameters studied increased with increasing concentration of the local anaesthetic (as in preparation No. 1 Fig. 1 A—F) although not evident in the mean values of Table I. As each heart was not perfused with all concentrations of mepivacaine used, the lack of correlation in Table I between the mepivacaine concentration and the mean changes in active systolic pressure and $(dp/dt)_{max}$ can be explained by differences in sensitivity of the hearts towards the local anaesthetic.

In this connection it should be recalled, that a reduction in contraction rate has a positive inotropic effect in this type of heart preparation (Andersson, Gennser and Nilsson 1970 a). The slowing of the contraction rate, which was more pronounced at high mepivacaine concentrations than at low, therefore tends to counteract the negative inotropic action of the local anaesthetic.

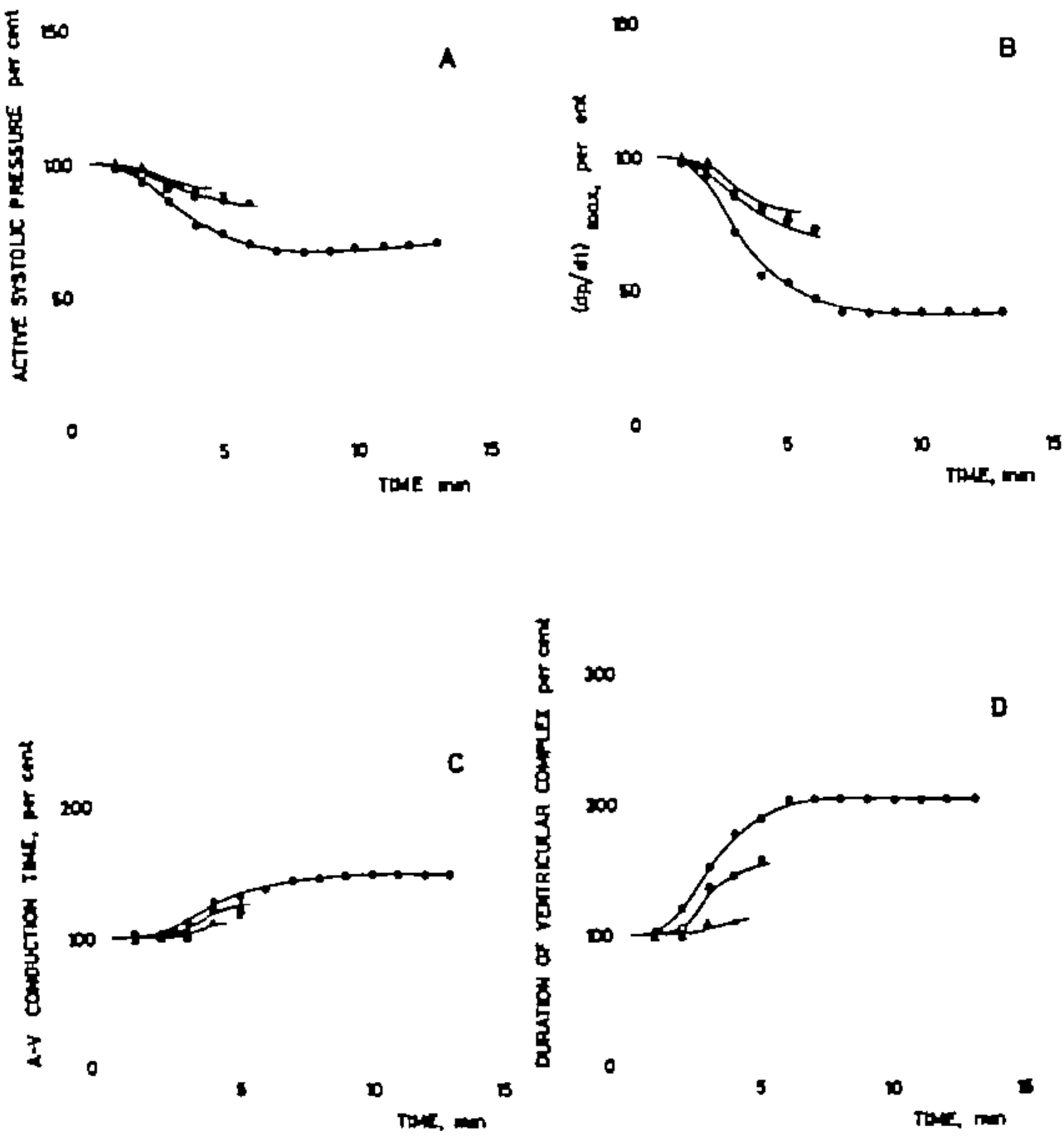
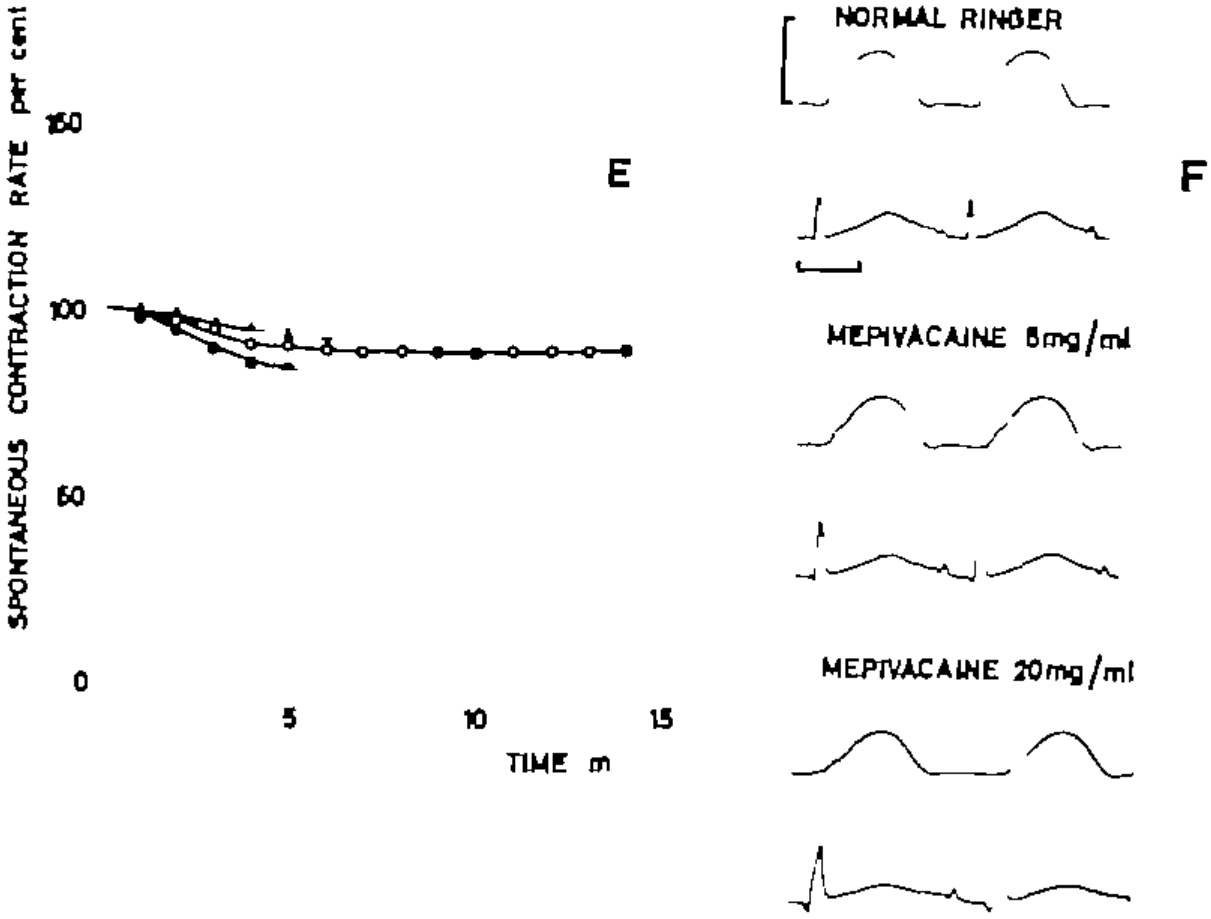


Fig. 1 A—F Effect of three different concentrations of mepivacaine on active systolic pressure (A) $(dp/dt)_{max}$ (B) A—V conduction time (C) duration of ventricular complexes (D) and spontaneous contraction rate (E) in one heart preparation. Panel (F) shows typical tracings of the mechanical and electrical registrations at two different concentrations of mepivacaine 5 and 20 μg/ml respectively. Calibration horizontal bar 200 msec vertical bar 20 mm Hg. Symbols mepivacaine 5 μg/ml Δ mepivacaine 10 μg/ml ( ) mepivacaine 20 μg/ml ( )

Fig. 1 E and F on opposite page



The effects of mepivacaine were evident as soon as the mepivacaine solutions reached the hearts and were maximum after 3—5 min. They disappeared with the same time course after changing to perfusion with the standard Ringer's solution.

a) Spontaneous contraction rate

The contraction rate of the spontaneously beating hearts was reduced only to a moderate extent even at high mepivacaine concentrations (a 12 per cent reduction was seen at a mepivacaine concentration of 20 μg/ml). In two hearts (results not included in Table I) an arrhythmia due to atrio-ventricular conduction block occurred at concentrations of 10 and 20 μg/ml, respectively. This arrhythmia ended in slow regular idio-ventricular rhythm.

b) Intraventricular pressure

Even at the lowest concentration used 2.5 μg/ml, a reduction of the intraventricular pressure could be recorded (Table I). Maximum rate of pressure development, $(dp/dt)_{max}$ was often more reduced than active systolic pressure. No significant change in the duration of the contraction was found. In the two hearts where the intraventricular pressure in both right and left ventricle was recorded simultane

ously the reduction of the developed pressure was more pronounced in the right ventricle.

c) Electrogram

Changes in electrogram were inconspicuous at a mepivacaine concentration of 2.5 μg/ml, but were pronounced at higher concentrations. The atrio-ventricular conduction time and the duration of the ventricular complexes increased with increasing mepivacaine concentration (Table I and Fig. 1 C, D and F) In the two hearts where an arrhythmia was registered, a reversible atrio-ventricular conduction block occurred. In both hearts, the pressure was recorded by means of a balloon in the left ventricle. It should be noted that electrogram changes were constantly more pronounced in this type of preparation than in those where an open catheter was used for pressure registration (Andersson, Genmer and Nilsson 1970 a)

Lidocaine

Changes in spontaneous contraction rate, intraventricular pressure, and electrogram similar to those produced by mepivacaine, were seen in the two hearts where the perfusion fluid contained lidocaine 20 μg/ml. No analysis of possible quantitative differences between the effects of lidocaine and mepivacaine was performed.

Influence of mepivacaine on electrically stimulated hearts

In order to avoid inotropic influences due to changes in spontaneous contraction rate five heart preparations were electrically stimulated. Two hearts were paced at a constant frequency throughout the experiment and three preparations at various selected frequencies during each of the different perfusion periods.

a) Intraventricular pressure

Fig 2 A and B illustrate data from one experiment in which a constant stimulation frequency was used. The changes in $(dp/dt)_{max}$ and active systolic pressure were dependent on the concentration of mepivacaine and were more pronounced at high stimulation frequencies than at low (compare Fig 1 A, B and E with Fig. 2 A and B, which show results obtained with the same preparation at an initial spontaneous contraction rate of 118 beats per min and during electrical stimulation 128 stimulations per min, respectively)

b) Electrogram

Similar mepivacaine effects on the electrogram as were seen in spontaneously beating hearts could be demonstrated in electrically stimulated preparations. The atrio-ventricular conduction time and the duration of the ventricular complexes were prolonged in proportion to the mepivacaine concentration used. In all five preparations a reversible atrio-ventricular conduction block could be produced at high concentra-

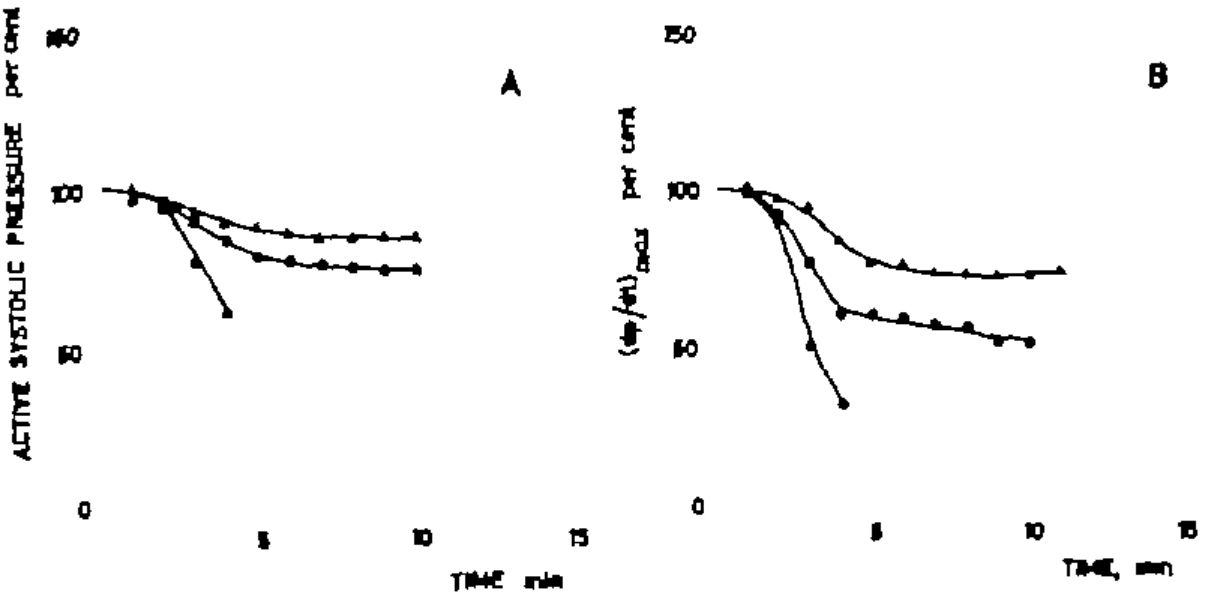


Fig. 2 A and B. Effects of three different concentrations of mepivacaine on activ systolic pressure (A) and $(dp/dt)_{max}$ (B) Constant stimulation frequency 128 stim./min. Same preparation and same symbols as in F g. 1 A—E. At the mepivacaine concentration 20 μg/ml A—V-conduction block occurred after four min perf sion.

tions of mepivacaine (10—20 μg/ml) Even at a concentration of 5 μg/ml, atrio-ventricular conduction block occurred at stimulation frequencies just above the spontaneous contraction rate in standard Ringer's solution.

I fluence f pH on the effects of mepivacaine

The effects of mepivacaine at pH 7.00 and 7.35 were compared in five spontaneously beating hearts the results are summarized in Table II The combination of mepivacaine and low pH caused a reduction of spontaneous contraction rate, active systolic pressure (Fig 3) and $(dp/dt)_{max}$ it also caused an increase in atrio-ventricular and intraventricular conduction time all these responses being more pronounced than to either intervention separately The negative inotropic effect of mepivacaine on electrically stimulated heart preparations was augmented by acidosis at all frequencies studied The ability of the preparations to respond to rapid frequencies of stimulation was reduced by mepivacaine and further diminished by a simultaneous lowering of extracellular pH (Fig. 4)

Concentrations f mepivacaine in heart tissue

Three heart preparations were perfused for 10 min with a Ringer s solution of pH 7 35 containing mepivacaine 10 μg/ml. After perfusion the hearts were immediately frozen and stored at −20 C. Their content of mepivacaine was analysed by gas-liquid chromatography The values obtained were 9 10, 7.36 and 8.91 μg/g wet heart tissue (mean 8.29 μg/g) Foetal hearts at midgestation contain approximately 850 g w ter per kg wet tissue (Widdowson and Dickerson 1960 Gemmer Johanson and

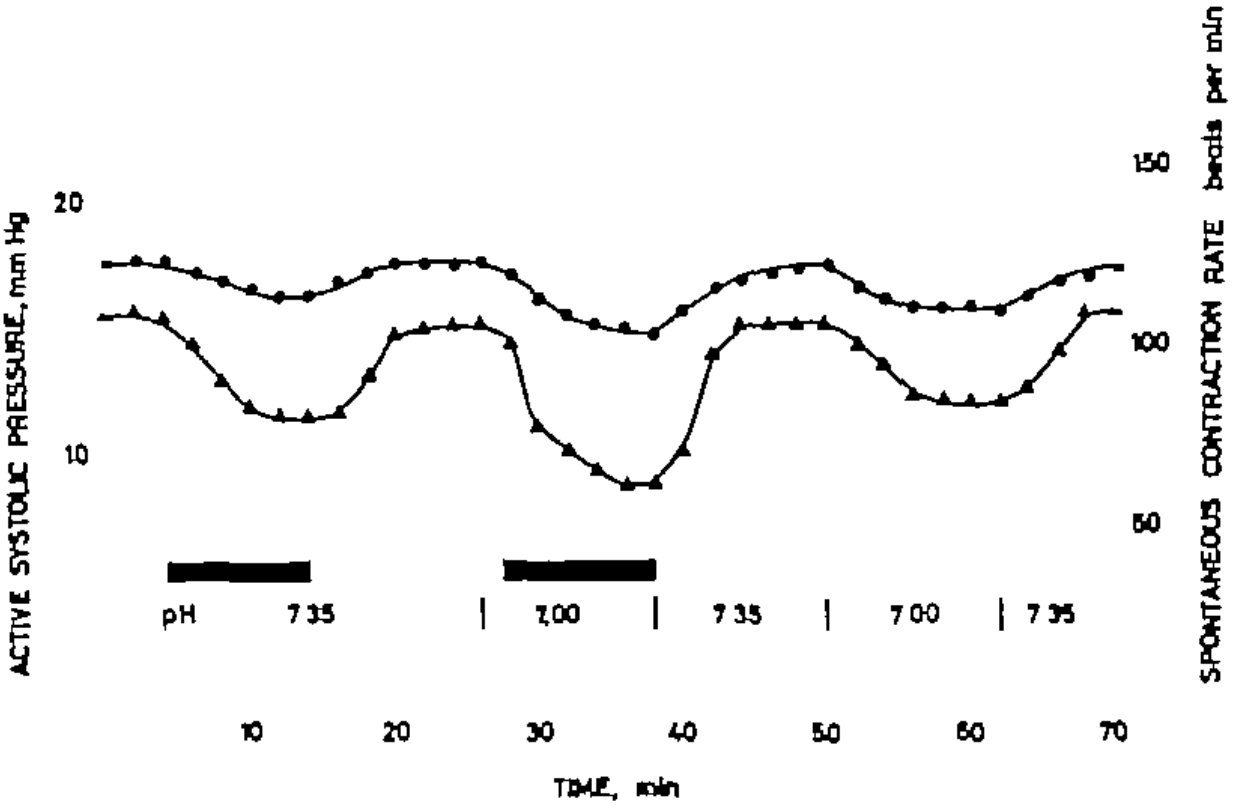


Fig. 3 Effects of mepivacaine at pH 7.35 and 7.00 on spontaneous contraction rate ( ) and active systolic pressure (▲) Black bars indicate perfusion with Ringer's solution containing mepivacaine 10 μg/ml. Heart preparation No. 2, see Table II

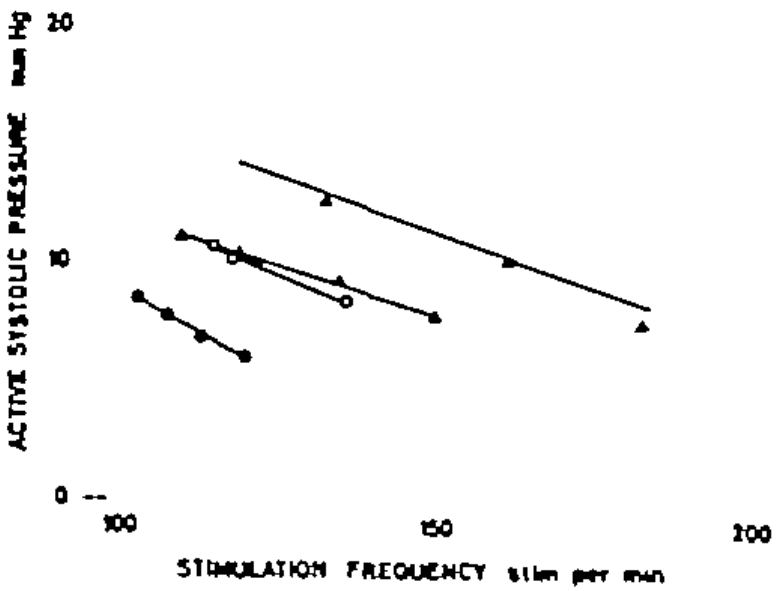


Fig. 4 Relation between active systolic pressure and stimulation frequency at pH 7.35 and 7.00 with and without mepivacaine in the perfusion fluid. Symbols: pH 7.35 (▲) pH 7.00 (△) pH 7.35 + mepivacaine 10 μg/ml ( ) pH 7.00 + mepivacaine 10 μg/ml ( ) Right ends of lines denote maximum contraction rate in response to stimulation. Same preparation as in Fig. 3

Kullander 1968). Assuming an even distribution of mepivacaine in total tissue water the intracellular concentration of mepivacaine would be very close to the concentration in the perfusion fluid. (A ratio of 0.98 for concentration in tissue water/concentration in perfusion fluid was obtained.)

TABLE II Influence of pH on the effects of mepivacaine. Values indicate deviation, in per cent from measurements in standard Ringer's solution without mepivacaine.

Preparation No.	Mepivacaine conc. μg/ml	pH	Spontaneous contraction rate	$(dp/dt)_{max}$	Active systolic pressure	A-V conduction time	Duration of ventricular complex	Type of pressure registration
2	10	7.35	— 6	—58	—27	+17	+ 63	open catheter
	0	7.00	— 9	—38	—20	+10	+ 5	
	10	7.00	—16	—70	—41	+26	+164	
3	5	7.40	—10	—49	—26	—	—	balloon
	0	7.05	—15	—48	—31	—	—	
	5	7.05	—25	—60	—41	—	—	
10	5	7.36	— 6	—29	—17	+10	+200	balloon
	0	6.99	— 7	—21	—13	+ 3	+105	
	5	6.99	—14	—44	—23	+21	+495	
8	10	7.31	— 3	—	—	—	—	—
	10	7.02	—33	—	—	—	—	
9	5	7.31	—	—	—	+14	+ 50	
	5	6.96	—	—	—	+29	+100	

Electrical stimulation, 110 stim./min.

Discussion

In the present study heart preparations from human foetuses at midgestation were used. There are reasons to assume that the results obtained are valid also for the human foetal heart at term. Organogenesis of the heart is considered to be complete before midgestation (Patten 1953) and it is possible that the human foetal heart at this stage of the gestation has developed an adrenergic innervation (Gennser and Owman, unpublished observation). It has also been shown (Gennser and Nilsson 1970) that human foetal heart cells from this period have resting membrane potentials of the same magnitude as and action potential characteristics similar to cells from adult mammalian hearts. Furthermore isometric tension developed by papillary muscle preparations from human foetal hearts at midgestation show a frequency dependence similar to that demonstrated in ventricular preparations from adult hearts of other mammalian species (Gennser and Nilsson 1972).

The mepivacaine concentrations used in the present investigation (5–10 μg/ml) are of the same magnitude as foetal blood levels of mepivacaine reported to have foetal bradycardia and/or foetal depression after paracervical block or epidural anaesthesia (Morishima et al. 1969, Gordon 1968, Hon 1970, Teramo and Rajamäki 1970).

Pacemaker activity

The results obtained have shown that mepivacaine produces a moderate slowing of the contraction rate by reducing the pacemaker activity of the sinus node. It is of interest to note that lidocaine which is chemically and pharmacologically similar to mepivacaine, has been demonstrated to depress the automaticity of the dog heart, primarily the ventricular but also the atrial (Lieberman *et al.* 1968 Sugimoto *et al.* 1969) However in several studies on adult man no consistent changes in heart rate have been observed at therapeutical concentration of lidocaine (plasma levels 1—4 μg/ml) (Harrison, Sprouse and Morrow 1963 Schumacher *et al.* 1968, Cullhed 1969) Initial tachycardia followed by bradycardia is seen as the typical pattern of *local anaesthetic toxicity (Steinhaus 1957) and a progressive bradycardia has been* noted during lidocaine infusion in dogs (maximum plasma levels 11.3±3.30 μg/ml mean and S.E.) (Binnion 1968, Binnion *et al.* 1969) In view of the moderate reduction in contraction rate obtained in the present study it seems unlikely that a direct effect of mepivacaine on the sinus node is the only cause of foetal bradycardia during PCB.

Electrogram changes

Large doses of lidocaine are known to prolong atrio-ventricular conduction time (Lieberman *et al.* 1968) This finding is in accordance with the present results for mepivacaine in which a concentration-dependent increase of the atrio-ventricular conduction time was noted. Also a broadening of the ventricular complexes could be demonstrated, which probably reflects an impaired intraventricular conduction. Both effects were more pronounced at high stimulation frequencies than at low The ability of the preparations to respond to rapid frequencies of stimulation was reduced by mepivacaine This is in accordance with results obtained in a recent electrophysiological study in which the lidocaine effects on Purkinje fibres and papillary muscles from dogs were analysed (Davis and Temte 1969)

Sinclair *et al* (1965) reported four cases of accidental injection of mepivacaine in the foetus in connection with PCB. In two of these cases (blood level determined in one 52 μg/ml) the electrogram showed an atrio-ventricular rhythm with ventricular conduction delay It is of interest that the same changes could be seen in two preparations of the present study (at concentrations 10 and 20 μg/ml, respectively)

Contractility

It is well known that high concentrations of local anaesthetics reduce the contractility of the heart, and the present results show that mepivacaine in all concentrations used reduced both $(dp/dt)_{max}$ and active systolic pressure Furthermore the results indicate that mepivacaine induces a direct negative inotropic effect independent of inotropic changes due to alterations in contraction rate

Mechanisms of mepivacaine effects

Mepivacaine at a concentration of 2.5 μg/ml or more caused a moderate slowing of the pacemaker activity of the sinus node, produced changes in the electrogram indicating a reduced conduction velocity and reduced the contractility of the hearts. The mechanisms behind these actions are not obvious. Local anaesthetics are known to "stabilize" excitable membranes raising the threshold for electrical excitation, suppressing the spontaneous pacemaker activity and slowing the propagation of action potentials. The rate of rise and the overshoot of the action potential are reduced but resting membrane potential is not consistently affected (Weidmann 1955 Ritchie and Greengard 1966). These actions are considered to be produced by a reduction in the conductance of sodium and possibly other ions in the cell membrane.

The negative inotropic action of mepivacaine may to some extent be ascribed to a reduction of intraventricular conduction velocity causing an asynchronous activation of the ventricles (cf. Andersson, Gennser and Nilsson 1970 a). It is also possible that mepivacaine directly interferes with the excitation-contraction coupling in the myocardial cell. Local anaesthetics have been shown to antagonize calcium movements across the cell membrane (Feinstein 1963) as well as across the internal membrane system of muscle cells, the sarcoplasmic reticulum (Feinstein 1963 Bianchi and Bolton 1967 Lüttgau and Oetliker 1968). These calcium movements are necessary for activation and relaxation of the contractile system (for references, see Langer 1968, Katz 1970). Furthermore, local anaesthetics have been shown to reduce the accumulation of calcium by the sarcoplasmic reticulum (Johnson and Inesi 1969). This ability might possibly be correlated with their negative inotropic action on the myocardium, as suggested by Gertz *et al.* (1967).

Mepivacaine and pH

The present results show a depressive effect on the spontaneous contraction rate and the myocardial contractility of both mepivacaine and a lowering of pH (cf. Andersson, Gennser and Nilsson 1970 b). The combined effect of these interventions may reflect a simple addition of the effects of mepivacaine and the increased proton activity in the external medium, but the present study was not aimed at a quantitative analysis of this point. It is not clear whether the cationic or the uncharged form of a local anaesthetic is responsible for its cardiac effects (see Ritchie Cohen and Dripps 1970). The pk_a of mepivacaine is 7.8 (Aberg, Friberger and Adler personal communication). Hence the relative amount of total anaesthetic in cationic form increases from 74 % at pH 7.35 to 86 % at pH 7.00. It seems unlikely that such a small change in the relation between the ionized and the non-ionized form of mepivacaine would have markedly affected the results of the present study.

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ACTA PHYSIOLOGICA SCANDINAVICA

SUPPLEMENTUM 344

STUDIES ON THE SYMPATHETIC REGULATION OF CIRCULATION AND METABOLISM IN ISOLATED CANINE SUBCUTANEOUS ADIPOSE TISSUE.

BY

BERTIL B FREDHOLM

STOCKHOLM 1970

Kuhlström & Sunes Bokutrychers AB Stockholm 1970

CONTENTS

I	Introduction	5
II.	Methodology	9
	a Experimental procedure	9
	b Comments	11
	c Analytical methods	12
III	Results and Discussion	14
	a Composition of adipose tissue	14
	b Metabolism during basal conditions	14
	c Blood circulation	16
	d Adrenergic transmission	19
	e Metabolic effects of nerve stimulation	22
	f Modification by receptor antagonists	24
	g Role of cyclic AMP	25
	h Role of prostaglandins	28
	i Changes in re-esterification	30
	j Modification of the metabolic responses by vasoconstriction	34
IV	Summary	36
	Acknowledgements	38
	References	39

presence in white adipose tissue of measurable amounts of noradrenaline metabolizing enzymes i.e. monoamineoxidase (Stock and Westermann 1963) and catechol-O-methyl transferase (Traiger and Calvert 1969 Horst *et al* 1969) has also been reported Inhibition of tyrosine hydroxylation, the rate limiting step in noradrenaline biosynthesis, in adipose tissue leads to a progressive decline in the transmitter content (Gessa *et al.* 1968) This indicates the presence in adipose tissue of a functioning noradrenaline biosynthesis. The release of noradrenaline from adipose tissue upon nerve stimulation, which would provide the final evidence for a transmitter function of noradrenaline in this tissue, has not been reported, however The fate of noradrenaline in intact adipose tissue is similarly unknown.

Electrical stimulation of nerves to rat and rabbit adipose tissue *in vitro* for prolonged periods of time and with high impulse frequencies (16—50 cps) has been reported to release FFA into the medium (Nash *et al.* 1961 Correll 1963) Recently an experimental technique permitting the study of vascular and metabolic reactions in dog subcutaneous adipose tissue was described (Orö Rosell and Wallenberg 1965 Rosell 1966) With this technique Rosell demonstrated the frequency-response characteristics of the FFA releasing effect of nerve stimulation. Maximal release rates during the stimulation were seen with 3 impulses/sec. At higher rates of stimulation the pronounced vasoconstriction apparently counteracted lipolysis during nerve stimulation. It has also been shown that electrical stimulation of certain diencephalic and mesencephalic areas connected to the sympathetic nervous system cause an elevation of plasma levels of FFA (Orö, Wallenberg and Bolme 1965)

Attempts to demonstrate adrenergic nerves by means of the fluorescence histochemical method of Falck and Hillarp (Falck 1962, Corrodi and Jonsson 1967) around individual fat cells in rat (Winsén 1965 Ballantyne and Raftery 1969) and dog (Fredholm and Hamberger unpublished) adipose tissue have proved unsuccessful. On the other hand, a rich supply of adrenergic nerves to the blood vessels has been shown. From this observation it has been suggested that the adrenergic nerves in adipose tissue regulate lipolysis by varying the blood flow through the tissue (Winsén 1965 Ballantyne 1968) However Rosell (1966) has shown that sympathetic nerve stimulation does cause mobilization of FFA even when the blood flow is kept constant.

The administration of the sympathetic neurotransmitter noradrenaline to intact animals and man increases the FFA and glycerol levels in plasma (cf Hagen 1967) This effect is probably due to a direct action on adipose tissue and not primarily to other factors, such as inhibition of insulin release (Porte 1967) This opinion is further strengthened by the massive collection of data showing that sympathomimetic amines are lipolytic when added to isolated

adipose tissue *in vitro* (see Steinberg and Vaughan 1965 Hagen 1967) and even to isolated fat cells from the rat (Rodbell 1964) Adrenaline and nor adrenaline cause an increase in the activity of the enzyme catalyzing the first step in triglyceride breakdown, which is probably rate-limiting in many situations (Gorin and Shafrir 1964 Strand, Vaughan and Steinberg 1964) The enzyme, hormone-sensitive triglyceride lipase exists in two forms (Vaughan *et al* 1965) and it appears that catecholamines are able to convert the inactive form into an active one. Moreover there is evidence that this conversion is brought about by adenosine 3',5'-monophosphate, cyclic AMP (Rizack 1964 Rizack 1965) possibly as a consequence of phosphorylation induced by a cyclic AMP-stimulated protein kinase (Corbin and Kreba 1969) The synthesis of cyclic AMP in adipose tissue is increased by catecholamines (Butcher *et al*, 1965 Butcher Baird and Sutherland 1968) The activity of the cyclic AMP synthesizing enzyme, adenyl cyclase also increased in adipose tissue following exposure to adrenaline (Vaughan and Murad 1969)

In accordance with the hypothesis of Ahlquist (1948) it is customary to divide the effects of catecholamines into two categories, α- and β- This classification is based on relative agonist potencies and the use of presumably specific receptor antagonists. Attempts to place the lipolytic response to catecholamines into this scheme have not been entirely successful (cf. Hagen 1967) This is particularly true with regard to the FFA and glycerol increases in plasma following the administration of sympathomimetic amines to intact animals and man. The usefulness of the classification in terms of "receptors" of the metabolic effects of adrenaline and noradrenaline in intact animals and man has been seriously questioned (Hagen 1967) On the other hand, a careful analysis of dose-response relationships of adrenomimetic amines with or without receptor blocking agents in epididymal fat pads *in vitro* led Wenke (1966) to conclude that the structure function relations agree extremely well with those for muscular β-effects. This conclusion was also reached by Stock and Westermann (1966a, 1966b)

Sympathetic nerve activity and catecholamines affect metabolic functions other than lipolysis both in intact animals (see Hagen 1967) and in isolated adipose tissue *in vitro* (see Ball and Jungas 1965 Flatt and Ball 1965 Lynn, Macleod and Brown 1960 Shafrir and Wertheimer 1965) The metabolic effect of sympathetic nerve activity in adipose tissue perfused with blood is not known, however Similarly the effect on adipose tissue of metabolic changes induced by sympathetic nerve activity elsewhere in the body such as increased blood lactate, is essentially unknown.

Adipose tissue is well vascularized (Gersh and Still 1945 Sheldon 1965) and clearcut vascular changes, such as arterial and venous vasoconstriction

and an increased hydrodynamic conductivity are seen when the nerves to adipose tissue of the dog are activated (Ngai, Rosell and Wallenberg 1966 Öberg and Rosell 1967) There are also indications that these vascular changes have some influence on the metabolic action of sympathetic nerve activity (Rosell 1966) this problem has not been studied in detail, however

The present experiments were undertaken to clarify the role played by sympathetic nerves in the regulation of circulation and metabolism in adipose tissue. The above mentioned experimental technique (Rosell 1966) permitting the study of the effects of nerve stimulation in isolated bloodperfused canine subcutaneous adipose tissue has been used. The release and fate of the adrenergic transmitter has been studied indirectly by following labelled nor adrenaline. The metabolic and vascular changes induced in adipose tissue by nerve stimulation have been studied in some detail. By pharmacological means attempts have been made to clarify the relative importance of different factors responsible for determining the overall response in adipose tissue to nerve stimulation. Particular emphasis has been put on the relationship between vascular changes and metabolic responses.

II. METHODOLOGY

a. Experimental procedure

Materials The studies were performed on female mongrel dogs weighing between 6 and 25 kg The dogs were fasted overnight (self-imposed) but no further caloric restrictions were imposed. Anaesthesia was induced by sodium pentobarbital (25—30 mg/kg) i.v with supplement during the experiment when necessary

Surgical procedure Subcutaneous adipose tissue (10—104 g) was isolated from all surrounding tissues, including skin, and scrupulous hemostasis was maintained (see Rosell 1966) In the majority of the experiments the right subcutaneous adipose tissue was used, but the left one appeared similar in every respect. An analysis of variance with regard to vascular resistance before and during nerve stimulation, glycerol and FFA release, and glucose uptake revealed no significant differences in ten dogs. Subcutaneous adipose tissue can be isolated and perfused in male dogs also (Ballard, Fredholm and Rosell, unpublished) Moreover from a qualitative point of view the responses are similar to those obtained in the female, but no quantitative comparisons have been carried out. The surgical procedure is considerably easier in the female.

Methods of perfusion The inguinal adipose tissue is supplied by one artery and drained by one vein (Rosell 1966) After canulation the tissue is therefore easily perfused. Three different types of perfusion have been used in these studies a) autoperfusion, b) constant flow perfusion using defibrinated blood and c) constant flow perfusion using heparinized blood. The different methods have been compared (a with b in papers V and VI b with c in paper IV) No significant differences with regard to the uptake or release of metabolites were found.

Constant flow perfusion was used in the majority of the experiments for three major reasons. First of all there have been proposals (Wirsén 1965 Ballantyne 1968) that the metabolic effects of nerve stimulation are mediated via changes in gross blood flow in adipose tissue. Secondly it was necessary to study the responses of the adipose tissue in complete isolation from the rest of the dog since it has been suggested that humoral factors (Wirsén 1965) play a major role in the response of white adipose tissue to an increased sympatho-adrenal tone. Finally it was hoped that by the use of an arterial

blood reservoir there would be no or at least more predictable, changes in the blood composition. In papers IV and V changes in the reservoir blood were reported (glucose concentration decreased and the lactate concentration increased) but it was found that these changes could be described by a linear change with time. It was thus possible to obtain a valid estimate of the blood composition at any given time during the course of the experiment.

In order to evaluate whether the constant flow perfusion caused any alterations in the activity of adipose tissue free flow experiments were also carried out. The basal metabolism was similar in autoperfused and constant flow perfused adipose tissue (papers V and VI) the similarity in the lipolytic response to nerve stimulation is documented below (section III e)

The vascular effects of drugs and of nerve stimulations were always studied with free flow perfusion. In some experiments the tissue was placed in a plethysmograph to measure volume changes and the capillary filtration coefficient CFC, as described in detail elsewhere (Folkow Lundgren and Wallentin 1963 Cobbold *et al* 1963 Öberg and Rosell 1967)

Nerve stimulation. The nerve to adipose tissue which runs together with the main artery and vein, was cut at the level of the external hiatus of the inguinal canal and placed on a bipolar electrode. The nerve was protected from moisture and air by Plastibase (Squibb) and stimulated electrically with approximately rectangular pulses delivered from a Grass stimulator

The pulses (2 msec duration, 8—15 V) were delivered at a rate of 3 to 10 cps. In most experiments 4 cps was used a frequency which is within the range considered by Folkow (1957) to be the physiological rate of firing in skeletal muscle adrenergic fibres. This frequency was known to give clearcut, although not maximal, vasoconstriction (Ngai, Rosell and Wallenberg 1966) and almost maximal rates of FFA release during a 30 min stimulation Rosell 1966)

Sampling procedure Samples of arterial and venous blood were collected. The sampling procedure varied between the series of experiments. The arterio-venous concentration difference was determined. If the concentration difference is negative there is considered to be a release of the metabolite in question, similarly a positive arterio-venous difference is in this report called uptake The uptake and "release of the metabolite is expressed as μmoles/min/100g by multiplying the concentration difference with the blood or plasma flow in ml/min/100g tissue.

b. Comments

Hahn (1943) found that heparin cleared lipemic plasma and Korn (1958) ascribed this phenomenon to the release of a lipase activity clearing factor lipase (CFL) by heparin. For this reason the use of heparin as an anticoagulant in a study of lipid metabolism deserves comment. Although CFL was at one time thought to be responsible for the mobilization of FFA from white adipose tissue (Gordon and Cherkes 1956 Olson and Vester 1960) this is not the current opinion (e.g. Ho Ho and Meng 1967) The administration of heparin causes release of lipase activity from canine subcutaneous adipose tissue (Ballard Fredholm Meng and Rosell 1970) but does not affect the release of FFA and glycerol therefrom induced by sympathetic nerve stimulation (Fredholm Meng, Öberg and Rosell 1968) Nerve stimulation did not release lipase activity into venous plasma (unpublished exp.) Therefore the two lipase activities, the one causing hydrolysis of intracellular triglycerides and that released by heparin, are in all probability dissociated There was no difference in the basal metabolism of adipose tissue perfused with defibrinated or heparinized blood (paper IV) Moreover heparin did not change the vascular resistance (Ballard *et al* 1970) For these reasons the use of heparin in the present studies appears justifiable.

The nerve to the adipose tissue is mixed in character as is evident from the fact that some, but not all, of the nerve fibres exhibit the specific catecholamine fluorescence after formaldehyde treatment according to Falck and Hillarp (Fredholm and Hamberger unpublished experiments) There is, however good reason to believe that the responses seen upon electrical activation of the nerves are secondary to the release of noradrenaline from nerve endings within the tissue. The responses to nerve stimulation could be mimicked by noradrenaline (paper II Ballard, Cobb and Rosell 1970) The vasoconstriction occurring upon nerve stimultaion can be converted to a vasodilatation by the use of an adrenergic α-receptor antagonist. Papers I III IV Ngai Rosell and Wallenberg 1966) This vasodilation is similar to that produced by isoprenaline (paper II) a relatively pure β-receptor agonist. There is also a close similarity between the lipolytic response to isoprenaline and to nerve stimulation after adrenergic α-blockade. The lipolysis, as well as the vasodilatation, can be inhibited by adrenergic β-receptor antagonists, including pronethalol, propranolol and INPEA (paper I) After dual adrenergic receptor blockade, nerve stimulation had neither vascular nor metabolic effects (paper I) Guanethidine (1.5 mg i.a.) blocked the vascular and metabolic effects of nerve stimulation (Fredholm, unpublished) Further evidence concerning the presence, uptake, release and metabolism of noradrenaline in

canine subcutaneous adipose tissue is summarized elsewhere (section III d)

Although histamine is present in appreciable amounts in canine subcutaneous adipose tissue and the release of endogenous histamine is accompanied by vasodilatation and lipolysis (Fredholm, Meng and Rosell 1968 Fredholm and Frisk Holmberg 1969) histamine release does not seem to be a factor in the responses to nerve stimulation (II) The possibility that prostaglandins play an important role in determining the responses to nerve stimulation in canine subcutaneous adipose tissue is discussed elsewhere (section III h)

c. Analytical methods

FFA were determined titrimetrically in plasma according to Dole (1956) as modified by Trout *et al.* (1960)

Glycerol was determined enzymatically as described by Laurell and Tibbling (1966)

Glucose was measured enzymatically using a commercial reagent (Glox, Kebo Stockholm Sweden) It was found that the sampling error was considerably lower (less than 2 per cent) when plasma was used instead of blood (about 4 per cent) Consequently glucose was measured in plasma in the majority of the experiments. As discussed in paper V plasma and blood glucose values covaried and the determination of glucose uptake with either of the two methods gave statistically indistinguishable results.

Lactate and *pyruvate* were measured according to Bücher *et al.* (1967) and Hohorst (1962) respectively using test kits obtainable from Boehringer and Sohne Mannheim, Germany

H no adrenaline and its metabolites The methods used for separation and determination have been described in detail in paper III

Noradrenaline was measured in 0 4 M perchloric acid extracts of adipose tissue according to the procedure of Bertler Carlsson and Rosengren (1958)

Glucos lactat and glycogen were determinated fluorimetrically (Karlsson, Diamant and Saltin 1970) in biopsy specimens (20—50 mg) excised before, during and after nerve stimulation and frozen in liquid nitrogen within 4 sec. The biopsies were cleansed from gross visible contaminants such as blood and connective tissue. The remainder was weighed, pulverized and mixed with frozen 3M perchloric acid (about 150 µl) After thawing the extract was titrated to pH 7.8—8 5 The supernatant after centrifugation was used for the determination of glucose and lactate The former was measured

as increase in NADPH after incubation with hexokinase and glucose-6-phosphate dehydrogenase in 0 1 M Tris buffer (pH 8 0) Lactate was measured as increase in NADH after incubation with heart lactic dehydrogenase in 0.2 M carbonate buffer (pH 9 7)

Triglycerides were determined according to Martinsson (1967)

Fatty acids in biopsy specimens (0.5—1 0 g) were determined titrimetrically (Trout *et al.* 1960) after homogenization in Dole s medium (25 ml)

Tissue water was determined in biopsy specimens (50—100 μg) by the weight decrease following freeze-drying for 14 days.

Na^+ *and* K^+*-ions* were determined in digests of adipose tissue with twice the weight of conc. HNO_3. After dilution and centrifugation, the ion concentration in samples were measured flame spectrophotometrically

III RESULTS AND DISCUSSION

a. Composition of adipose tissue

The composition of canine subcutaneous adipose tissue, as revealed by the present studies, is presented in Table 1

The extravascular ion contents could be calculated to be about 30 μEq Na^+/g tissue and 2.3 μEq K^+/g, since the plasma concentrations averaged 143 and 3 8 mM respectively The sodium space is calculated to average 21 per cent Using this estimate and assuming that the adipocyte potassium concentration is 110 mM (Denton, Yorke and Randle 1966) the intracellular water space is found to be 1 4 per cent. The values obtained are similar to those determined by Denton, Yorke and Randle (1966) in rat epididymal fat pads. Due to the variability in the ion determinations and the many approximations involved in the calculations the present estimate is very rough. Similarly the estimate of the extracellular water space from the determination of tissue glucose content (Ballard and Hanson 1969) is subject to considerable error One might conclude that the adipose tissue contains between 10 and 21 ml extracellular water per 100 g and that the intracellular water is one order of magnitude less.

b. The uptake and release of metabolites during basal conditions (I IV V IV)

During basal conditions (i.e. in unstimulated adipose tissue) the glycerol release rate averaged 0.24 μmoles/min/100 g The range was wide (0.0⁵— 0 71) This variation could not be explained on the basis of differences in the arterial concentrations. There was also no significant association between the rate of glycerol release and the fat weight, or the ratio between fat and dog weight, which might give a rough indication of the adiposity of the dog

Throughout this study the release of glycerol has been taken as an indicator of the rate of lipolysis The arguments in favour of this assumption were summarized by Steinberg and Vaughan (1965) Glycerol is utilized in adipose tissue (Lynn, MacLeod and Brown 1960 Antony White and Laudau 1969) but the rate is low This is probably due to the fact that the enzyme that phosphorylates glycerol glycerokinase is scarce in white adipose tissue.

TABLE 1 Composition of canine subcutaneous adipose tissue.
Numbers within parantheses represents number of determinations.

Glycogen (μmoles/100 g)	310 ± 120 (13)	P per IV
Triglycerides (g/100 g)	73 ± 3 (6)	
FFA (μmoles/100 g)	540 ± 100 (14)	
Glucose (μmoles/100 g)	50 ± 10 (14)	Paper IV
N (μEq/100 g)	3450 ± 450 (10)	
K (μEq/100 g)	450 ± 12 (10)	
Water (ml/100 g)	17 ± 4.5 (10)	Paper IV
Intravascular volume (ml/100 g	5	Öberg and Rosell (1967)
Extracellular water (ml/100 g)	21	(From N determination)
Extracellular water (ml/100 g)	11 ±	(From glucose determination)
Intracellular water (ml/100 g)	14	(From Na and K determination)

It was originally thought to be absent (Margolis and Vaughan 1962) but its presence was recently demonstrated (Robinson and Newsholme 1967) It is of importance that glycerol cannot by itself support triglyceride synthesis in otherwise suitably fortified homogenates of rat epididymal adipose tissue (Steinberg, Vaughan and Margolis 1961 Margolis and Vaughan 1962)

Basal FFA release was lower (0 12 μmoles/min/100 g) and the range was wider (—0.96 to 1.25) than the basal glycerol release. There was no correlation between the arterial FFA concentration and the net release rate. Similarly the fat weight and the adiposity did not correlate with the release rate.

The basal glucose uptake was extremely variable between dogs, but this variation can be ascribed, at least partly to an increased uptake with increasing arterial glucose concentration (V VI) The glucose uptake was weakly correlated ($p<0.05$) to an indicator of the re-esterification (3 x glycerol release — FFA release)

The uptake of lactate and pyruvate by adipose tissue was strongly dependent upon the arterial concentration (V) The dependence could be approximated by a straight line regression, the steepness of which was greater for pyruvate than for lactate. The lactate regression line intercepted with the arterial concentration between 1 and 2 mM the pyruvate regression line between 0.05 and 0.1 mM. These arterial concentration values are close to those found normally in the dogs. It might be concluded that the adipose tissue does not normally contribute significantly to the net uptake or release of these anions.

The oxygen extraction was found to be 2.4 ±0.6 μmoles/min/100 g and the carbon dioxide production 2.7 ± 1 7 μmoles/min/100 g. (IV)

c. Blood circulation in canine subcutaneous adipose tissue

1 *Resting conditions*

Blood circulation in canine subcutaneous adipose tissue has been studied extensively in this laboratory (see review by Rosell 1969). The resting blood flow was 8.4 ml/min/100 g (range 3.2—19) (Ngai, Rosell and Wallenberg 1966; Öberg and Rosell 1967 paper II). This value is of the same order of magnitude as that reported for resting skeletal muscle (Mellander and Johansson 1968). These authors also calculated that adipose tissue would take about 15 per cent of resting cardiac output in a 70 kg man, which is a larger proportion than any other organ system.

It was noted (II) that the highest blood flow values were generally seen in adipose tissues with the lowest weights. This finding indicates that increases in fat weight are not necessarily accompanied by corresponding increases in the magnitude of the vascular resistance section. Hausberger and Widelity (1963) in a study of the distribution of Cr tagged erythrocytes concluded that the size of the vascular bed decreased with ageing, when fat accumulation increased.

Blood flow measured by Xe^{133} clearance, in abdominal subcutaneous adipose tissue in man (Häggendal, Steen and Svanborg 1970) is similar to that obtained in the abdominal subcutaneous adipose tissue in dogs. On the other hand blood flow values in other depots of subcutaneous adipose tissue in man, similarly determined by Xe^{133} or Kr^{85} clearance, are generally somewhat lower (2.6 ml/min/100 g, Larsen, Lassen Quaade 1966; 2.3 ml/min/100 g Nielsen *et al* 1968; 2.1 ml/min/100 g Lesser and Deutsch 1967). The flow in canine subcutaneous adipose tissue is similar to that in rat as measured directly (5.3 ml/min/100 g) and by Rb^{86} clearance (5.7 ml/min/100 g) (Mayerle and Havel 1969 a) or by clearance of H DDT (10—25 ml/min/100 g, Herd Goodman and Grose 1968) and also similar to rabbit epigastric adipose tissue (Lewis and Matthews 1968 8.1 ml/min/100 g).

There is considerable evidence that blood flow may in some conditions, such as fasting diabetes and glukagon administration, increase with lipolysis in man (Nielsen *et al* 1968; Häggendal *et al* 1970) rat (Mayerle and Havel 1969 b) and rabbit (Lewis and Matthews 1968).

The results of Scow *et al* (1965) studying the release of FFA from perfused rat parametrial fat, do not suggest such a good correlation, however. Thus ACTH which strongly stimulated lipolysis and FFA release had no effect on blood flow and adrenaline decreased blood flow in lipolytic concentrations. Our own results (see below) also indicate that there is not always a correlation between lipolysis and blood flow.

The maximal blood flow rate in canine subcutaneous adipose tissue, which might give an indication of the dimension of the resistance section, ranged between 25 and 45 ml/min/100 g tissue (Ngai *et al.* 1966 Öberg and Rosell 1967 paper II) This suggests that the resistance section is less well developed than in skeletal muscle for example.

The regional blood content in adipose tissue was estimated by Öberg and Rosell (1967) to be 5 ml/100 g tissue, which is 1.5—2 times higher than the corresponding value in skeletal muscle. Hausberger and Widelitz (1963) reported a regional blood content of one to eight ml/100 g tissue in white adipose tissue from various locations in the rat. This value was similar to that of skeletal muscle in the same study

The capillary filtration coefficient, CFC, which gives a measure of the total surface area available for filtration of water indicated a richer capillary supply than that present in skeletal muscle (0.024 ml/mm/100 g/mmHg as compared to 0.010—0.015 ml/min/100 g/mmHg in skeletal muscle) On the other hand Gerah and Still (1954) in a morphological study concluded that adipose tissue was less well capillarized than skeletal muscle, but yet, for purposes of metabolism, the capillary bed of adipose tissue was far richer than that of muscle.

2. *The effect of drugs.* A brief summary is given in Table 2.

Table 2. Vascular effects of drugs in canine subcutaneous adipose tissue.

Drug	Dose	% change in flow	% change in CFC	% change in reg. blood vol.
Acetylcholine	10^{-6}—10^{-5}	107	20	4
Bradykinin	10^{-6}	46	114	15
Histamine	10^{-7}—10^{-6}	113	82	6
5-hydroxytryptamine	10^{-6}	23	9	— 3
Isoprenaline	10^{-6}	151	15	3
Noradrenaline	10^{-5} —10^{-4}	—68	63	—14
PGE_1	10^{-5}	127	27	8
Theophylline	10^{-5}	165	122	9
I. a.				
K^+	5×10^{-5}	30		
Ca^{++}	5×10^{-4}	—20	12	— 2
Mg^{++}	3×10^{-4}	10		
Ba^{++}	5×10^{-5}	—30		
Na-EDTA	3×10^{-5}	190	25	4
L(+)-lactate	-20×10^{-4}	— 3		
D(—)-lactate	10^{-5}	0		
Pyruvate	5×10^{-5}	18		
Vag. *stimulation*	3—8 cps	—66	73	—14

Acetylcholine, isoprenaline 5-hydroxytryptamine and prostaglandin E all increased blood flow in adipose tissue (paper II) The most potent of these drugs on a molar basis was PGE_1 PGE_2 was 2 to 10 times less effective (Fredholm, Öberg and Rosell, unpublished) These drugs also caused a small (5—10 per cent) increase in CFC. Histamine and bradykinin also increased blood flow but produced larger increases in CFC (paper II) per unit increase in blood flow Noradrenaline (II) and adrenaline (Ballard, Cobb and Rosell 1970) are predominantly vasoconstrictor in subcutaneous adipose tissue. The former drug gives vascular changes, including CFC increase, identical to those given by nerve stimulation (Table 2)

Of the anions studied in paper V only pyruvate produced a significant vasodilatation. This is in agreement with earlier observations (see Haddy and Scott 1968)

3 *The effect of nerve stimulation. Earlier studies*

The nervous control of adipose tissue has been studied in some detail in this laboratory (Ordö Rosell and Wallenberg 1965 Ngai, Rosell and Wallenberg 1966, Öberg and Rosell 1967 paper II Fredholm, Linde, Öberg and Rosell 1969 Rosell 1969) The blood flow through canine subcutaneous adipose tissue decreases during electrical activation of the nerve to the tissue or the lumbar sympathetic chain. The rate of the resistance increase as well as the "steady state" level of the constrictor response is frequency dependent. Maximal vasoconstriction is obtained with frequencies between 10 and 20 cps. Stimulation with 4 cps, the frequency most commonly used in the present experiments, gave 70 per cent of maximal vasoconstriction (Ngai Rosell and Wallenberg 1966)

Comparative studies on the sympathetic regulation of circulation in adipose tissue from other species are lacking. On the other hand, studies of circulation in canine adipose tissue from other locations, have revealed differences from subcutaneous adipose tissue (Ballard and Rosell 1969 1970)

During nerve stimulation about one third of the blood contained in adipose tissue is expelled (Öberg and Rosell 1967) This is similar to skeletal muscle (Mellander and Johansson 1968)

Nerve stimulation, moreover increased CFC markedly This response appears to be unique. The CFC has been regarded as an indication of the capillary surface area and/or the capillary porosity (Cobbold *et al.* 1963) The morphological basis for the increase in adipose tissue is as yet unclear *In skeletal muscle CFC increases somewhat, although much less than in* adipose tissue, during prolonged nerve stimulation. This phenomenon has been ascribed to a sphincter relaxing effect of metabolites accumulating in

the tissue. It does not seem possible to explain the CFC increase in adipose tissue similarly. Thus metabolism in adipose tissue is stimulated by a mechanism that can be inhibited by adrenergic β-receptor antagonists (I) yet the administration of propranolol will not abolish the CFC increase during nerve stimulation in adipose tissue. Moreover Linde and Rosell (unpublished) have found that the clearance of locally injected radioisotopes is decreased in adipose tissue during nerve stimulation, in situations where CFC increases, even if the blood flow is kept constant by artificial means.

4. The effect of nerve stimulation. Own studies (II)

In order to obtain further evidence on the background to these apparently conflicting findings the studies reported in paper II were performed. It was noted that the effect of nerve stimulation could be mimicked by noradrenaline and after α-receptor blockade it could be imitated by isoprenaline. The CFC increases seen during nerve stimulation were larger than those produced by acetylcholine, isoprenaline and PGE_1 in concentrations giving apparently maximal relaxation of the resistance section in adipose tissue (Table 2). Since procedures which maximally increase blood flow are generally capable of opening all capillaries, mere relaxation of precapillary sphincters was apparently not capable of allowing CFC rises comparable to those seen with nerve stimulation. On the other hand, histamine and bradykinin, which are generally supposed to increase capillary permeability (Elliott, Horton and Lewis 1960, Spector 1958) did give CFC values comparable to those seen with nerve stimulation (Table 2). When nerve stimulation was superimposed upon infusion of acetylcholine, isoprenaline or PGE_1 a further increase in CFC was obtained even if blood flow was kept constant. Nerve stimulation during infusion of histamine or bradykinin, however rather decreased CFC.

These results, those obtained by Linde and Rosell (unpublished) and others can possibly be unified, as discussed earlier (Fredholm *et al.* 1969, Rosell 1969) by assuming that nerve stimulation induces a closure of pre capillary sphincters and an increase in the permeability of the capillaries left open. Such a mechanism would give increased diffusion distances, leading to a decreased clearance of radioisotopes, and simultaneously an increased CFC.

d. Adrenergic transmission in canine subcutaneous adipose tissue (III)

Canine subcutaneous adipose tissue contained 37 $\pm$10 ng noradrenaline/g (n=10). This value is in the lower range reported for white adipose tissue (see Introduction).

During prolonged sympathetic nerve stimulation there was a net production of lactate seen as a net outflow of lactate and an accumulation of lactate in the tissue (IV) The tissue lactate level during stimulation was approximately 2.5 μmoles/g. Assuming distribution solely in tissue water this would correspond to a concentration above 10 mM. On the other hand, shorter nerve stimulation had no significant effect on the net lactate release by adipose tissue (V)

f. Modification of metabolic effects of nerve stimulation by adrenergic receptor antagonists (I IV)

There is general agreement that catecholamines stimulate lipolysis mainly by activating receptors which are similar to muscle β receptors (Havel 1965 Stock and Westermann 1966 a, Wenke 1966) On the other hand, in the two published reports concerning the effect of nerve stimulation on lipolysis dibenamine an adrenergic α-receptor antagonist, inhibited lipolysis induced by nerve stimulation (Nash *et al.* 1961 Correll 1963)

In contrast in our experiments (paper I) the lipolytic effect of nerve stimulation was potentiated by adrenergic α-receptor blockade induced by dihydroergotamine or phentolamine. Administration of β receptor antagonists such as pronethalol, propranolol or INPEA (D( ) N isopropyl-p-nitrophenyl-ethanolamine) inhibited the lipolytic effect as well as the vasodilatation occurring during nerve stimulation after dihydroergotamine or phentolamine. The fact that Correll (1963) was able to produce inhibition of lipolysis might be due to the high concentration of dibenamine (0.5 mg/ml) he used, since it has been shown that α-blocking agents in concentrations of this magnitude cause unspecific inhibition of lipolysis (Stock and Westermann 1966 b)

Oxygen uptake increased during nerve stimulation after dihydroergotamine pretreatment, but decreased after propranolol as it did when no receptor antagonist was given. The oxygen uptake appeared to vary inversely with vascular resistance (IV)

The glycogenolysis during nerve stimulation was unaffected by dihydroergotamine, but was inhibited by propranolol It appears that glycogenolysis as well as lipolysis is activated by stimulating fat cell receptors which are similar to muscular β-receptors.

Lactate production during nerve stimulation was inhibited by both propranolol and dihydroergotamine (IV) It seems reasonable to assume that the lactate production is a consequence of both a stimulation of metabolism effected by β-receptor activation and a decreased transport of metabolites, including oxygen, secondary to vasoconstriction caused by stimulation of α-receptors.

g. Evidence for the participation of cyclic AMP in the regulation of lipolysis in canine subcutaneous adipose tissue

The evidence for a role of cyclic adenosine 3',5'-monophosphate (cyclic AMP) in the activation of lipolysis by different lipolytic hormones, including sympathomimetic monoamines, was briefly summarized in the Introduction. Methyl xanthines, including caffeine and theophylline, can be used as tools to evaluate the role of cyclic AMP in lipolysis as discussed by Brodie and coworkers (Hynie, Krishna and Brodie 1966, Weiss, Davies and Brodie 1966). These substances are known to inhibit the enzymatic degradation of cyclic nucleotides (Butcher and Sutherland 1962).

In a series of experiments theophylline-1-aminopropanol (a kind gift from AB Astra, Södertälje, Sweden) was infused in graded amounts (0.05—10 mM in blood). Theophylline above 0.1 mM was found to increase the rate

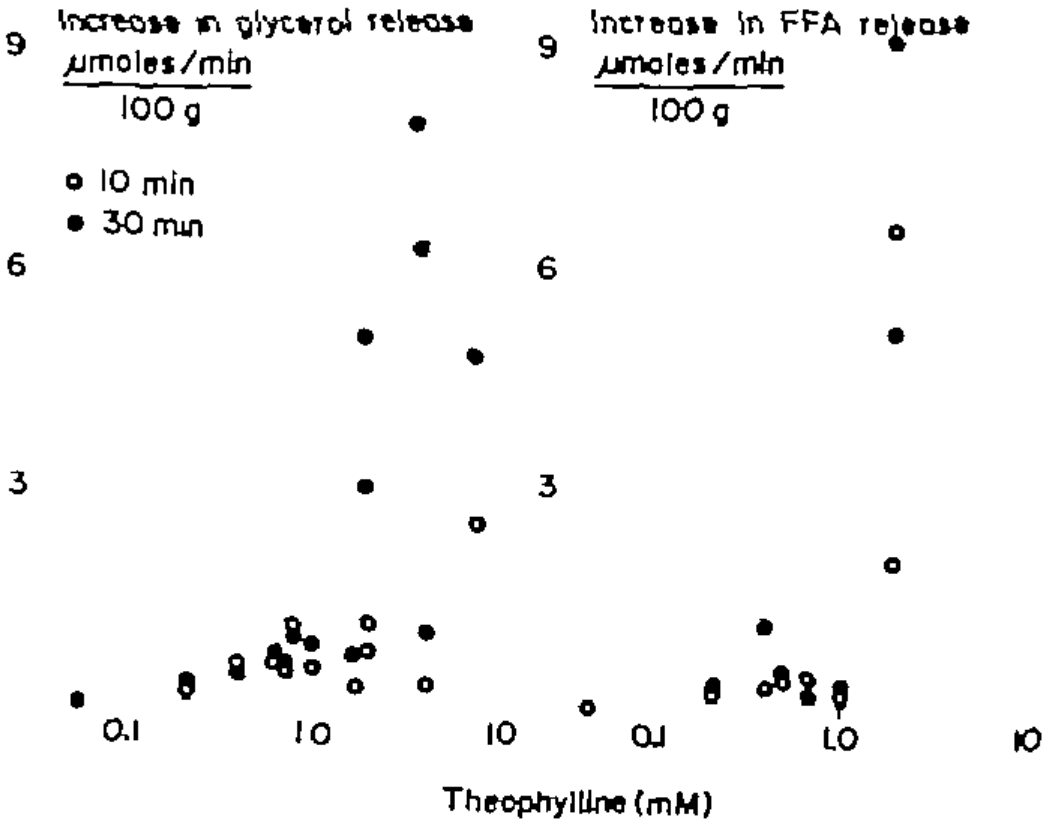


Fig. 3. Increase in glycerol and FFA 10 (○) and 30 min (●) after the start of an infusion of theophylline-1-aminopropanol in graded amounts. Each point represents results from one experiment.

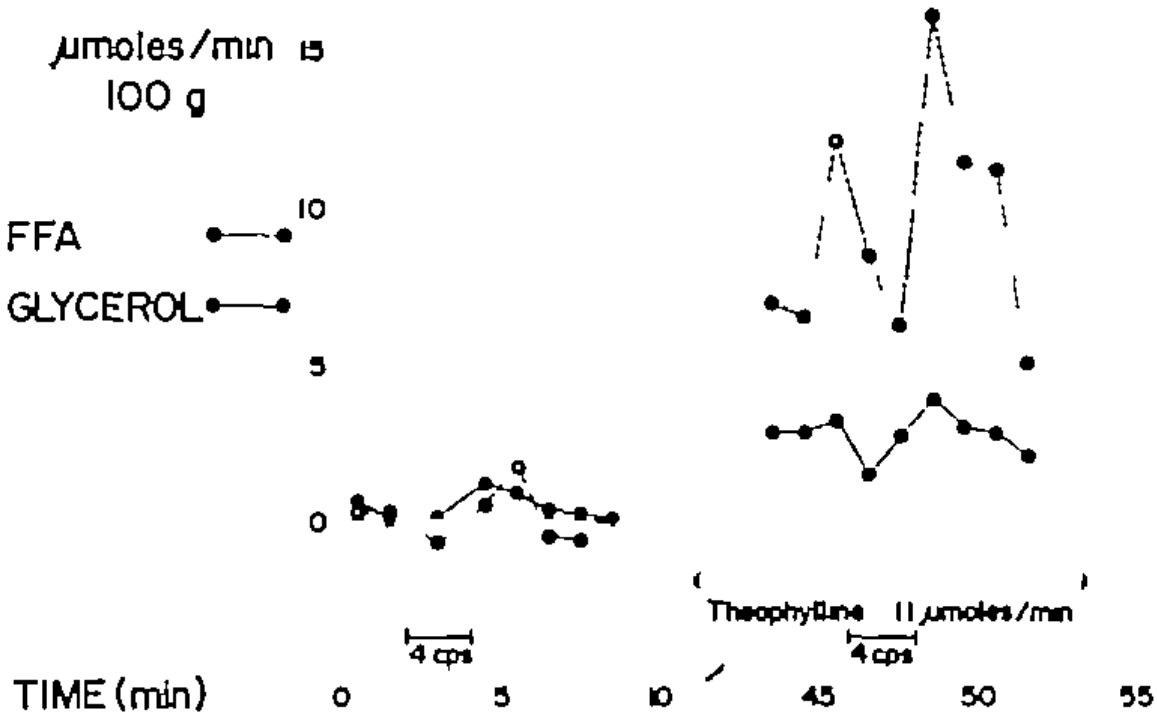


Fig. 4 The effect of a min sympathetic nerv stimulation on the release of FFA and glycerol before and during an infusion of theophylline-1-aminopropanol (11 µmoles/min) Adipose tissue weight 24 g Mean systemic blood pressure 110—100 mmHg PCV 40.0.

of glycerol and FFA efflux from adipose tissue (Fig 3) 0 1 mM was threshold for producing lipolysis, increasing cyclic AMP levels and significantly inhibiting phosphodiesterase in rat epididymal adipose tissue and isolated fat cells (Weiss, Davies and Brodie 1966 Hynie, Krishna and Brodie 1966) Theophylline above 2 mM produced marked increases in FFA and glycerol release (Fig 3) These concentrations produced apparently maximal stimulation of lipolysis in the rat and human adipose tissue *in vitro* (Hynie, Krishna and Brodie 1966, Weiss, Davies and Brodie 1966, Carlson, Hallberg and Michell 1969) Nerve stimulation during infusion of these high concentrations of theophylline had little if any further stimulatory effect on lipolysis (Fig 4) This indicates that these concentrations of theophylline had indeed caused a maximal stimulation of lipolysis in canine subcutaneous adipose tissue *in situ* The fact that theophylline by itself can markedly increase lipolysis can be taken as evidence that cyclic AMP is rapidly synthesized and broken down even in unstimulated adipose tissue (Weiss, Davies and Brodie 1966) at least if it is assumed that theophylline only inhibits phosphodiesterase

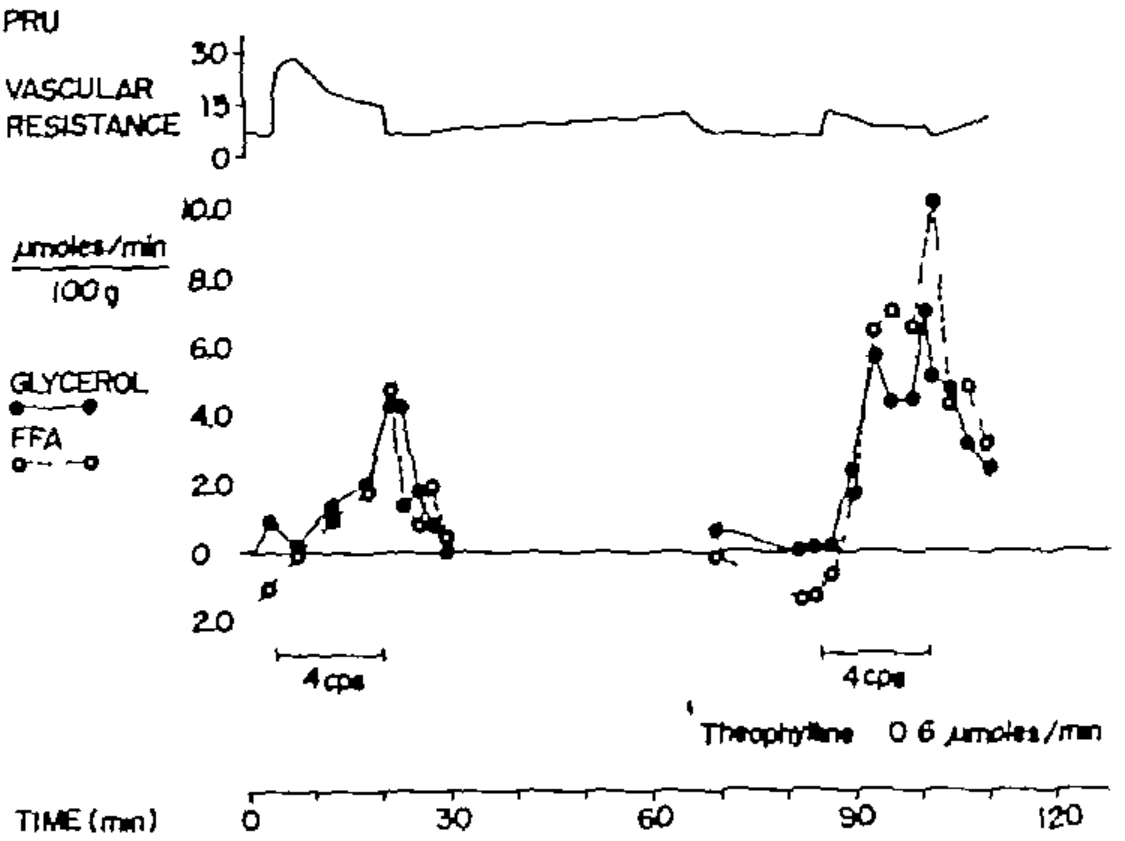


Fig. 5. Potentiation of the release of glycerol and FFA evoked by nerve stimulation by theophylline-1-aminopropanol (0.6 µmoles/min). Adipose tissue 16 g. PCV 34 0. Mean systemic blood pressure 140—120 mm Hg.

The lipolytic effect of nerve stimulation was potentiated by theophylline in low (0 1—1 mM) concentrations (Fig 5) Noradrenaline induced lipolysis is potentiated by low amounts of theophylline in rat epididymal fat pads in *vit o* (Vaughan and Steinberg 1963 Weiss, Da ies and Brodie 1966) The increase in cyclic AMP after adrenaline is considerably enhanced by methyl xanthines in rat epididymal fat pads (Butcher Ho Meng and Sutherland 1965) and isolated fat cells therefrom (Butcher Baird and Sutherland 1968) Therefore the present data might indicate that nerve stimulation in canine subcutaneous adipose tissue *in situ* increases the rate of cyclic AMP formation.

It can be seen in Fig 5 that the vasoconstriction induced by nerve stimulation is reduced by theophylline. (0.1—1 mM theophylline inhibited the vasoconstriction by 34 per cent 1—10 mM by 66 per cent) The lipolytic response to nerve stimulation is affected by the vasoconstriction (section *III j*) Therefore the interaction between theophylline and α-receptor stimulation on blood circulation might contribute to the potentiation.

h. *Effects of prostaglandins on lipolysis during nerve stimulation (VI)*

The prostaglandins are a group of acidic lipids found in many tissues and body fluids. Prostaglandins, especially E-compounds, decrease lipolysis in adipose tissue *in vitro* (see Bergström, Carlson and Weeks 1968) presumably by inhibiting the formation of cyclic AMP (Stock, Aulich and Westermann 1968 Butcher and Baird 1968) Stimulation of the nerve to rat epididymal fat pads *in vitro* was recently found to cause an increased prostaglandin formation and release of prostaglandin-like material into the medium (Shaw and Ramwell 1968) The possibility that prostaglandins act as feed-back inhibitors of stimulated lipolysis has therefore been proposed (Shaw and Ramwell 1968 Bergström, Carlson and Weeks 1968 Horton 1969)

Studies on the isolated dog spleen revealed that prostaglandin-like material could be released during nerve stimulation and that the release was inhibited by adrenergic α-receptor antagonists (Davies, Horton and Withrington 1968) Since it was found in the course of the present work (paper I) that α-receptor blockade potentiated the release of glycerol and FFA we were interested to see whether this was mainly due to removal of inhibition of lipolysis by prostaglandins, released by α-receptor stimulation. Efendic (1970) concluded from a series of studies on human adipose tissue *in vitro* that stimulation of adrenergic α-receptors inhibits lipolysis.

In a recent study (Fredholm Rosell and Strandberg 1970) we were able to confirm the results of Shaw and Ramwell (1968) by showing that sympathetic nerve stimulation at "physiological" frequencies caused an increased outflow of acidic lipids, resembling prostaglandins chromatographically from blood perfused canine subcutaneous adipose tissue. In general less than 1 ng prostaglandin like activity (in PGE units) was found in the venous effluent per ml. There was no detectable increase in extractable prostaglandin-like material in stimulated adipose tissue relative to control. We were unable to find inhibition of prostaglandin release by dihydroergotamine. Recently it was reported that prostaglandin release from the diaphragm is actually increased by α-receptor blockade (Laity 1969) which indicates that the findings of Davies, Horton and Withrington (1968) may not be representative of all tissues from all species. Therefore the possibility that the potentiation of lipolysis by dihydroergotamine or phentolamine was due to removal of inhibition secondary to prostaglandin release, as also suggested by Bergström, Carlson and Weeks (1968) appeared less attractive.

In paper VI prostaglandin E — the most potent inhibitor of lipolysis in rat epididymal fat pads *in vitro* (Bergström, Carlson and Weeks 1968) was infused in graded amounts into adipose tissue and its effect on the glycerol and

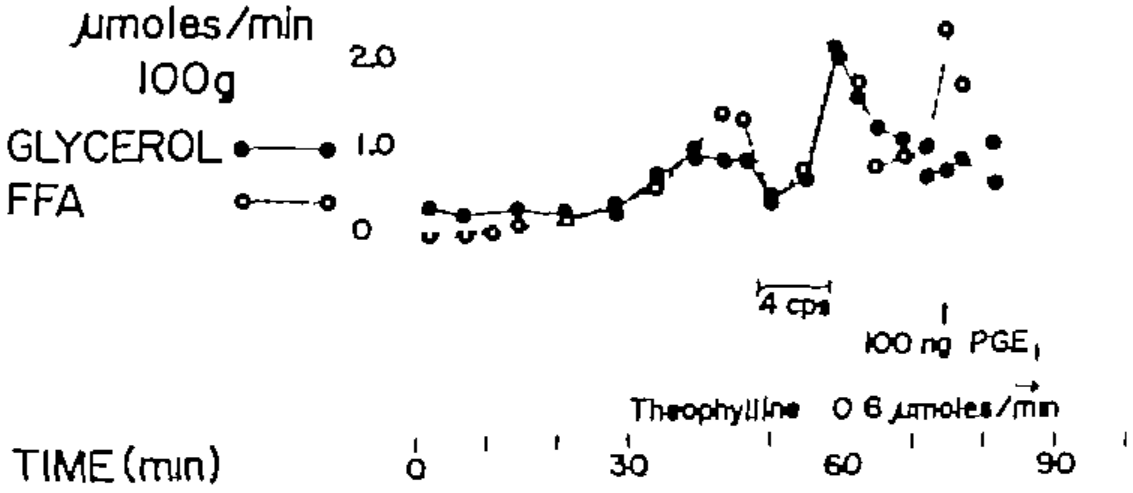


Fig. 6. The effect of nerve stimulation (10 min, 4 cps, 12 V 2 msec) and PGE_1 (100 ng i.a.) during infusion of theophylline (0.6 μmoles/min) after pretreatment of the tissue with propranolol (400 μg i.a.) on FFA and glycerol release. Adipose tissue 35 g. PCV 40.0. Mean systemic blood pressure 105—100 mm Hg.

FFA release induced by nerve stimulation determined. It was found that lipolysis induced by nerve stimulation was inhibited dose-dependently by PGE_1 (ID_{50} appr 1.2×10^{-7} M). The concentrations needed to produce significant inhibition of lipolysis were considerably larger than those found in the venous effluent after nerve stimulation (10—100 times). Thus it would appear that the amounts of prostaglandin released are insufficient to significantly affect lipolysis during nerve stimulation. It was, however found (Fredholm, Rosell and Strandberg 1970) that the rate of inactivation of infused PGE was considerable. It is consequently possible that a small pool of prostaglandin, with a very rapid turnover might regulate lipolysis, although it can not be shown by merely analyzing for total tissue prostaglandin content.

PGE_1 was also found to produce a very marked increase in the rate of glucose uptake by adipose tissue, as described earlier *in vitro* by Böhle *et al.* (1966). On the other hand, nerve stimulation did not cause an increased uptake of glucose (papers IV, V, VI).

PGE was found to be a powerful vasodilator (II). It did not produce the very marked increase in CFC as seen after nerve stimulation, however. It is difficult to ascribe any of the vascular reactions during nerve stimulation to the release of prostaglandins in adipose tissue (II).

Polyphloretinphosphate has recently been shown to antagonize the action of prostaglandin on many smooth muscle preparations (Eakins, Karim and Miller 1970). Polyphloretinphosphate (10—20 mg/ml) did not, however

affect the basal or nervously stimulated lipolysis in canine subcutaneous adipose tissue (unpublished)

Finally in a series of six experiments no evidence for the presence in canine subcutaneous adipose tissue of an inhibitory α-receptor mechanism was obtained. Stimulation after propranolol administration, leaving only the α-receptors intact caused a decrease in the release rate during the stimulation period, but afterwards there was a compensatory increase (Fig 6) In the same experiment and in three others PGE actually increased the release of glycerol as was seen occasionally when lipolysis had not been previously stimulated by theophylline (VI) The results did not support the opinion that prostaglandins, released by adrenergic α-receptor stimulation, are important inhibitors of lipolysis activated by nerve stimulation.

To summarize We have been able to confirm the *in vitro* observation that PGE_1 inhibits lipolysis stimulated by nerve activity (Berti and Usardi 1964) and extended it by demonstrating dose response characteristics in a blood perfused organ stimulated with frequencies more similar to those occurring physiologically We have also confirmed the results of Shaw and Ramwell (1968) that prostaglandin-like material is released from adipose tissue upon nerve stimulation. It is, however difficult to define the part played by prostaglandins in the lipolytic response to nerve stimulation in canine subcutaneous adipose tissue and none of the vascular responses could be directly attributable to prostaglandin release. Whether prostaglandins play a role in the long-term regulation of adipose tissue metabolism in the dog cannot be determined from these experiments.

5. Changes in re-esterification (V)

When triglycerides in adipose tissue are hydrolysed three moles of fatty acid are formed per mole of glycerol. It has been found repeatedly in *in vitro* experiments (see Steinberg and Vaughan 1965) that less than three moles of fatty acids are released per mole of glycerol even if corrections are made for the contents of free fatty acids and of glycerol in the incubated tissue Steinberg and Vaughan (1965) discussed this phenomenon at length and concluded that the most reasonable explanation is that fatty acids are re esterified.

In the present experiments there was often a net uptake of FFA in spite of a definite net release of glycerol during basal (i.e. unstimulated) conditions. Since no estimate of the fluxes of fatty acids in adipose tissue were made conclusions about re-esterification and esterification could not be

drawn in the basal state. For this reason studies of the changes of the re esterification were made on the basis of differences in the amounts of FFA and glycerol released upon nerve stimulation. The increase in FFA and glycerol release over the basal rate were considered to be due to increased release and not to decreased uptake.

Increasing the arterial L(+) lactate concentration decreased the mobilization of FFA upon nerve stimulation, while the release of glycerol was essentially unchanged. On the other hand, infusion of Na-pyruvate in very high amounts had the opposite effect more FFA were mobilized but the glycerol release was significantly lowered. This implies that lactate increased re-esterification and pyruvate decreased it. Before discussing possible mechanisms behind this effect some of what is known about adipose tissue intermediary metabolism will be briefly reviewed (Fig 7)

The rate of triglyceride synthesis in defatted homogenates of rat epididymal adipose tissue was found to be strongly dependent upon the concentration of α-glycerol phosphate and fatty acid (Steinberg, Vaughan and Margolis 1961 Angel and Rondel 1967) Many authors have proposed a control for α-glycerol phosphate in the regulation of triglyceride biosynthesis *in vivo* (e.g. Leveille 1967) On the other hand attempts to correlate the contents of α-glycerol phosphate or long-chain fatty acyl-CoA with *observed rates of glyceride biosynthesis* have not been successful (Denton and Randle 1967 Denton and Halperin 1968)

Although less effective than glucose, pyruvate can be a substrate for synthesis of glyceride-glycerol (Ballard, Hanson and Leveille 1967 Leveille 1967 Reshef Hanson and Ballard 1969 Wieegrad and Renold 1958) This is presumably true also for lactate. *Adipose tissue contains pyruvate carboxylase (Ballard and Hanson 1967)* and phosphoenolpyruvate carboxykinase (Ballard, Hanson and Leveille 1967) These findings indicate that pyruvate carbon can be incorporated into triglycerides via a pathway essentially similar to gluconeogenesis in liver Pyruvate carboxylase and citrate synthetase activities are higher in mitochondria from white adipose tissue than from liver (Patel and Hanson 1970) which supports the view that this route might be of physiological significance. Adipose tissue apparently lacks the enzymes fructose 1,6-phosphatase and glucose 6-phosphatase (Weber *et al.* 1965) and glucose and glycogen cannot be synthesized from e.g. pyruvate. Instead, fructose 1,6-diphosphate and the triosephosphates, including dihydroxyacetonephosphate, the immediate precursor to α-glycerol phosphate, would accumulate. The physiological significance of the pathway might be the production of glycerol necessary to keep up with triglyceride turnover in conditions such as fasting, when the supply of glucose is limited (Steinberg, Vaughan and Margolis 1961) Evidence supporting this view was recently provided (Reshef Ballard and Hanson 1969) Pyruvate kinase in adipose tissue is converted *to* a less active form upon fasting (Pogson 1968) and the conversion of oxaloacetate to malate decreases during fasting (Gorin, Tal-Or and Shafrir 1969) Both these changes would enable more pyruvate to form glyceride-glycerol in fasting.

When the animal is fed the conversion of oxaloacetate to malate is presumably of great importance, since *this step is crucial in a cyclic process in adipose tissue with the*

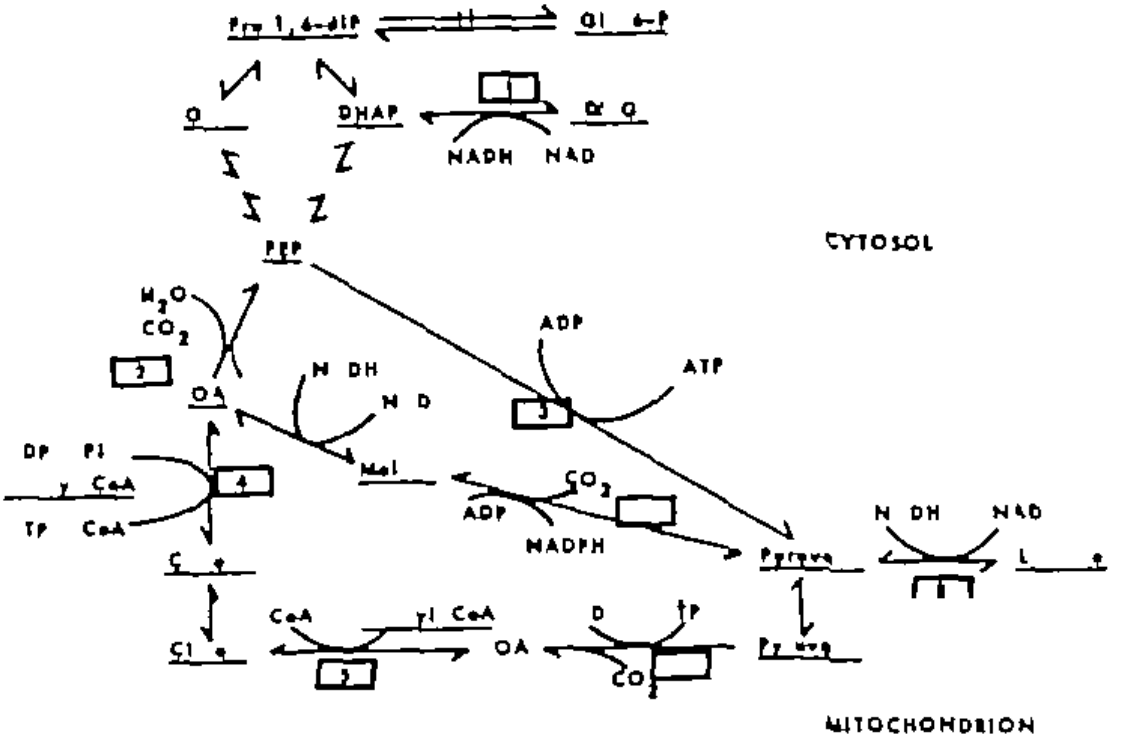


Fig. 7 Schematic representation of pathways involved in glucose and pyruvate metabolism relevant to a discussion of the regulation of re-esterification in adipose tissue. Glu-6-P = glucose 6-phosphate Fru 1,6-di P = fructose 1,6-diphosphate GADP = glyceraldehyde 3-phosphate DHAP = dihydroxyacetone phosphate α-GP = glycerol 3-phosphate PEP = phosphoenolpyruvate OA = oxaloacetate.

Reaction 1) is catalyzed by glycerolphosphate dehydrogenase (1.1.1.8) 2) by phosphoenolpyruvate carboxykinase (4.1.1.3) 3) by pyruvate kinase (2.7.1.40) 4) by ATP citrate lyase (citrate cleavage enzyme) (4.1.3.8) 5) citrate synthetase (4.1.3.7) 6) Pyruvate carboxylase (6.4.1.1) 7) Malate dehydrogenase-decarboxylating enzyme (1.1.1.40) and 8) by lactate dehydrogenase (1.1.1.8)

twofold purpose of transferring acetyl-CoA from the mitochondria to the cytosol and transhydrogenation of NADH to NADPH both essential processes in lipogenesis (Young, Shrago and Lardy 1961 Wise and Ball 1964 Rognstad and Katz 1968 Leveille and Hanson 1966) This cycle, which found experimental support by Rognstad (1969) involves the transfer of pyruvate into the mitochondria, carboxylation to oxaloacetate, oxaloacetate and acetyl-CoA forming citrate which flows out of the mitochondrion and is split by citrate cleavage enzyme into oxaloacetate and acetyl CoA. The oxaloacetate is reduced to malate with NADH and the malate converted back to pyruvate generating NADPH. Since the first steps in the pyruvate cycle and the pathway leading from pyruvate to glyceride-glycerol are identical there should be competition between glyceride and fatty acid formation from pyruvate as discussed for liver (Ballard, Hanson and Kronfeld 1969) and found experimentally in rat epididymal fat pad (Gorin, Tal-Or and Shafrir 1969) In fed animals, which have been used throughout this study the cyclic process might be the quantitatively more important one.

If prevalon of glycerol equivalents for triglyceride biosynthesis was the only explanation for the observed increase in re-esterification during lactate infusion it is surprising that pyruvate does not have the same effect. Indeed one would expect that pyruvate would be a more efficient substrate for α-glycerolphosphate biosynthesis since lactate would tend to shift the oxaloacetate-malate reaction towards malate, thus providing less phosphoenolpyruvate.

The final step in the conversion of glucose — or for that matter pyruvate — into glycerol phosphate involves the reduction of dihydroxyacetone-phosphate. This reaction it is generally accepted, is strongly dependent upon the ratio between reduced and oxidized NAD (Bücher and Klingenberg 1958, Hohorst *et al* 1965) Another tentative explanation for the observed actions of lactate and pyruvate on the re-esterification can be stated. It is possible that lactate and pyruvate change the concentration of α-glycerolphosphate less by themselves being the substrate than by changing the utilization of glucose for glycerol synthesis. When lactate is infused a certain proportion of the anion is oxidized to pyruvate, furnishing reduced nucleotides in the process. The reduced nucleotides shift the dihydroxyacetonephosphate—α-glycerolphosphate equilibrium to the right. The converse could be true during pyruvate infusion. Cahill Leboeuf and Flinn (1960) found that 5 mM pyruvate significantly decreased the incorporation of ^{14}C-label from glucose into glyceride glycerol. Incubation of rat epididymal fat with adrenaline increased both lactate production and triglyceride synthesis (Flatt and Ball 1965 Landau *et al* 1965) Similarly lactate production and re-esterification seemed to increase in parallel during prolonged sympathetic stimulation (IV) and it was shown by Peterson and co-workers (1964) that pyruvate and dihydroxyacetonephosphate compete for NADH in skeletal muscle.

Evidence for an inhibition of FFA release by lactate has appeared earlier Thus Mueller (1961) found that lactate inhibited the FFA increase in plasma of dogs after systemic 2-deoxy-D-glucose administration. Issekutz and Miller (1962) found that infusion of Na-lactate in amounts similar to those produced in exercise and giving the same blood concentrations decreased plasma FFA levels. This finding was confirmed by Miller *et al.* (1964) who also found that the effect was stereospecific for L(+)-lactate. These authors concluded that the effect was presumably due to an increased re-esterification secondary to an increased availability of α-glycerolphosphate, but could not prove this, since glycerol turnover rates in the dog were not determined. Björntorp (1965 1966) investigated the effect of Li-lactate on glycerol production in rat epididymal fat pads *in vitro* He found that already at a concentration of 3 mM lactate caused a significant inhibition of glycerol production and lipo-

lytic activity and that this effect was not clearly dose-dependent since 30 mM lactate had the same effect.

The results (V) with infusion of lactate and pyruvate clearly demonstrate that the mobilization of FFA can be varied independently of the mobilization of glycerol, i.e. of lipolysis. Moreover it appears that such modification could occur under physiological conditions. Decreased mobilization of metabolizable fat by increasing re-esterification would seem to be a rapid and efficient means of adjusting the substrate availability in conditions such as heavy exercise, when the need for FFA is small, and yet a high sympathoadrenal tone is present

j Modification of the metabolic responses by vasoconstriction.

It was essential to clarify the metabolic consequences of vasoconstriction and the other vascular effects of nerve stimulation (see section III c) In paper IV the metabolic consequences of nerve stimulation were studied after administration of propranolol in amounts sufficient to inhibit adrenergic activation of lipolysis. The vasoconstrictor response was essentially the same as without propranolol. During such pure α receptor stimulation the glycerol release rate decreased, but immediately after the cessation of the stimulation the release rate increased. This pattern of response resembled the disappearance of radioactive xenon or iodide from canine subcutaneous adipose tissue during and after α-receptor stimulation (Linde and Rosell unpublished) Similarly as illustrated in Fig 6, a decreased rate of glycerol release was seen *during α-receptor stimulation, and a compensatory increase afterwards*, even when the level of glycerol release was increased by theophylline. The findings are best explained by assuming that vasoconstriction caused trapping of glycerol and FFA in the tissue and that the excess is free to diffuse out when the stimulation is terminated.

In paper III it was suggested that vascular responses might actually modify the adrenergic transmission between nerve endings and fat cells. It is possible that the vascular responses might delay the onset of lipolysis stimulation owing to hindrance of transmitter transport from nerve endings to fat cells. The possibility that the lipolysis is delayed in its onset when nerve stimulation produces vasoconstriction received circumstantial support (see above section III d)

Vasoconstriction might therefore affect the lipolytic response in two ways The products of lipolysis formed upon stimulation of triglyceride breakdown are to some extent trapped in the tissue and the onset of lipolytic stimulation apparently also delayed, possibly due to inhibition of noradrenaline transport

The vascular effects of nerve stimulation seem to have other metabolic consequences as well. Some of these might be secondary to the accumulation of products of lipolysis, especially fatty acids in adipose tissue. The oxygen uptake decreases during stimulation. This is most likely due to a diminished capillary surface area available for diffusion of oxygen. Accumulated fatty acids might play a role since it has been shown that they decrease oxygen utilization in adipose tissue *in vitro* (Hagen and Ball 1961). On the other hand, the failure of nerve stimulation to increase glucose uptake, which was expected from available *in vitro* data (Hagen 1967), can probably not be explained similarly since nerve stimulation did not increase glucose uptake after dihydroergotamine pretreatment either. The accumulation of lactate in adipose tissue upon prolonged nerve stimulation appears to depend upon the vasoconstrictor response, since it was blocked by α-receptor antagonists.

Finally, we must consider the possibility that the observed potentiation of the lipolytic response to nerve stimulation after adrenergic α receptor blockade could depend upon the abolishment of vasoconstrictor response. Two principally different mechanism are conceivable. Firstly it is known (Bally *et al.* 1965, Rodbell 1965) that fatty acids function as feed-back inhibitors of lipolysis. Accumulation of excessive amounts of fatty acids might thus inhibit lipolysis. Secondly it is possible that abolishment of vasoconstriction in adipose tissue makes more of the released transmitter substance available for stimulation of lipolysis. α-Receptor antagonists abolish vasoconstrictor responses facilitating the diffusion of noradrenaline thereby producing smaller concentrations of transmitter near the nerve ending. This could lead to a smaller re-uptake of the transmitter into the nerve ending (Iversen 1968). It is also known that different α-receptor blocking agents have a direct inhibitory effect on the neuronal uptake process as well as effects on the granular storage mechanism.

IV SUMMARY

The present results have elucidated several aspects of the sympathetic regulation of circulation and metabolism in canine subcutaneous adipose tissue.

1 Noradrenaline is present in canine subcutaneous adipose tissue in measurable amounts. H-noradrenaline is taken up into cell structures in the tissue and released by nerve stimulation. The released transmitter is metabolized the same way as in organs known to have a functioning adrenergic innervation.

2. Nerve stimulation causes an arteriolar vasoconstriction, an increased hydrodynamic conductivity (CFC increase) and an expulsion of blood. These effects are mimicked by noradrenaline and are abolished by adrenergic α-receptor antagonists. The ensuing arteriolar vasodilatation, limited increase in CFC and venodilatation are all mimicked by the β-receptor agonist isoprenaline. The possibility is discussed that the CFC increase during nerve stimulation is due to an increased capillary permeability simultaneously with a decrease in the number of open capillaries.

3 The rate of free fatty acid (FFA) and glycerol release increased during and after nerve stimulation. This lipolytic effect was inhibited by β-receptor blocking agents. The maximal release rates were generally seen after the cessation of the nerve stimulation. After adrenergic α-blockade totally more glycerol and FFA were released and the maximal release rates were generally observed already during the stimulation period. α Receptor blockade apparently brought about a similar change in the magnitude and time course of the evoked transmitter overflow to judge from the efflux of radioactivity from H-noradrenaline

It appears that vasoconstriction influences the mobilization of FFA and glycerol in two major ways it delays the stimulation of the fat cells by released noradrenaline and it causes a trapping in the tissue of lipolytic products already formed.

4 Glycogenolysis is activated by nerve stimulation, presumably via a β receptor mechanism On the other hand, net glucose uptake in adipose tissue in unaffected by nervous activity Oxygen extraction in adipose tissue decreased during nerve stimulation provided vasoconstriction occurred. If vasoconstriction was prevented by dihydroergotamine oxygen uptake increased. Pro-

longed sympathetic nerve stimulation caused a net production of lactate which also accumulated in the tissue. This effect was prevented by adrenergic α- or β-blockade.

5 Lactate, in concentrations present in blood during severe muscular exercise or in shock or calculated to occur in adipose tissue during prolonged sympathetic nerve stimulation, inhibited the mobilization of FFA. Since the glycerol release was not significantly affected, this implies an increased re esterification during lactate administration. Pyruvate had the opposite effect. It appears that alterations in the degree of re-esterification could be an efficient means of regulating the mobilization of fat from adipose tissue, and also that the cytoplasmatic red-ox state might be a factor in this regulation.

6. Prostaglandins are released from canine subcutaneous adipose tissue by nerve stimulation and administered PGE inhibits its lipolytic effect. The amounts recovered in venous blood following nerve stimulation are lower than those necessary to produce inhibition, however There is no effect of nerve stimulation that can be directly attributed to prostaglandin release and formation.

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ACTA PHYSIOLOGICA SCANDINAVICA

Supplementum 355

FROM THE WIHURI RESEARCH INSTITUTE
HELSINKI, FINLAND

MOVEMENTS OF SODIUM AND POTASSIUM IONS AND THEIR TRACERS IN PROPRANOLOL TREATED RED CELLS AND DIAPHRAGM MUSCLE

BY

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CONTENTS

	Page
ACKNOWLEDGEMENTS	3
INTRODUCTION	7
General features of red cells in studies of transport phenomena	7
Some earlier observations on the transfer of material across the red cell membrane	8
Theories of the distribution of ions across the red cell membrane	9
Various chemical modifiers of ion permeability of the red cell membrane	13
Review of the biological effects of propranolol	14
Isolated rat diaphragm as an object for transport studies	15
OUTLINES OF THE PRESENT INVESTIGATION	16
MATERIALS AND METHODS	17
Experiments with red cells	17
General arrangements	17
Bulk ion and tracer ion concentrations	18
Experiments with ^{14}C-sucrose	18
Experiments with ^{14}C-propranolol	18
Adenosine triphosphate (ATP) concentration	19
Adenosine triphosphatase (ATPase) activity	20
Experiments with isolated rat diaphragm	20
Chemicals	21
Calculation of the tracer rate constants	21
General case	22
Stationary state	24
Practical arrangements	24
RESULTS	26
Inflow and outflow of ^{14}C-propranolol	26
Accumulation by whole red cells	26
Outflow and binding by whole red cells	28
Uptake by red cell membrane stroma	30
Movements of bulk potassium and sodium ions and their tracers in red cells during the propranolol-induced non-stationary state	30
Different optical isomers of propranolol	30
Effect of the pH of the medium	34
Effect of potassium and sodium ions	35
Effect of calcium and magnesium ions	37
Ouabain and propranolol	41

Effect of protamine 44
Reversibility of normal cation permeability after propranolol treatment 46
Sucrose permeability 47
Adenosine triphosphate concentration 47
Adenosine triphosphatase activity 47
Experiments with isolated rat diaphragm 49
Cation and water contents 49
Extracellular space 50
Intracellular cation and water concentrations 51
Inflow of ^{22}Na and ^{42}K tracer ions 51
Outflow of ^{22}Na, ^{42}K, and ^{86}Rb tracer ions 52
Effect of lidocaine and dibucaine on ^{86}Rb tracer outflow 54

DISCUSSION 55
Accumulation and exchange of propranolol 55
Effect of calcium, magnesium, potassium and sodium bulk ions on propranolol induced cation shifts 56
Different optical isomers of propranolol 57
Propranolol and ouabain 58
ATP and ATPase activity 60
Isolated rat diaphragm 61
Possible effect of propranolol on the membrane structure 62
Mechanism of propranolol induced accelerated cation exchange 64

SUMMARY 66

REFERENCES 68

INTRODUCTION

General features of red cells in studies of transport phenomena

At present the literature concerning erythrocyte membrane transport phenomena is so voluminous, and not without conflicts of opinion, that attempts to try to understand even a bit of it are difficult. The reviews of Ponder (1948) Harris (1960), Prankerd (1961) Passow (1964) Whittam (1964) and Nims (1967 a) are helpful and give comprehensive data.

The mature mammalian red cell is structurally one of the simplest cells, oversimplified it is a parcel of haemoglobin enveloped by a thin membrane. The cell weight is made up of about 33 % haemoglobin, 1 % lipids and lipoproteins, 1 % alkali salts, and the rest is water The lipids and lipoproteins are almost totally confined to the cell membrane. Mature mammalian red cells have few of the properties of other cells with specialized functions, such as secretion, absorption, and protein synthesis. Red cells, however have the common fundamental characteristics of all living cells they have a finite life span, and as a result of metabolic reactions they are capable of maintaining concentration gradients of permeable compounds across the cell membrane.

Isolated red cells maintain their stationary state even in artificial medium, probably because of their simple metabolic requirements. The main metabolic pathway of the mature mammalian red cells is the anaerobic glycolysis of glucose to lactic acid. Human red cells convert about 2 mmoles of glucose to lactic acid per litre of cells per hour (Bartlett and Marlow 1953, De Loecker and Prankerd 1961 Murphy 1963) About 6 % of the glucose is consumed via the hexose monophosphate pathway (De Loecker and Prankerd 1961 Szeinberg and Marks 1961). Red cells cannot metabolize fatty acids and the tricarboxylic acid cycle is not functioning in mature mammalian red cells (Rubinstein and Denstedt 1953, Dejani and Orten 1958, Bishop 1964).

Due to their availability as single cells and ease with which they can be maintained in a stationary state, even in laboratory conditions, mature mammalian red cells have become widely used as tools in transport studies. The amount of medium trapped between packed red cells is about 2—3 % (Beilin et al. 1966, Funder and Wieth 1967), while the interspace volume in skeletal muscle is about 10—20 % and in cardiac muscle 20—25 % (Harris 1960) The rate of movement of material across the red

cell membrane is often slow enough to allow the cells to be washed with cold saline, which in many cases makes consideration of the effect of interspace volume unnecessary

The surface area of the human red cell is about 150 μm^2 and the cell volume is 90 μm^3 (Whittam 1964). One litre of human red cells consists of 1.1×10^{13} cells. From these values it follows that the total membrane area per litre of cells is as high as 1650 m^2. The thickness of the red cell membrane is 8—10 nm (Robertson 1967). Due to the biconcave disc shape of the human red cell its surface/volume ratio is nine times that of a sphere of equal volume. One consequence is that the membrane can be considered to be the main limiting factor in the transfer of material between the cell and the medium. Thus a red cell suspension, when slowly stirred, can with reasonable accuracy be considered to be composed of two compartments the cell or cell interior and the medium. This allows general two-compartment analysis, solvable in an exact form, to be used in the kinetic treatment of the flow of material across the membrane. In the case of ion transport with radioactive tracers the most widely used method of analysis is that developed by Sheppard and Martin (1950). It is based on changes on each side of the membrane in the specific activities of the ions studied. A still more convenient method for the calculation of the tracer rate constants both in the stationary and the non-stationary state was presented by Ekman, Manninen and Salminen (1969). This treatment makes use of the changes of the concentration ratios in the two compartments.

Red cells contain about 5 mmole of haemoglobin per litre of cells. Haemoglobin is retained inside the cell, and, depending on the pH, may provide an anion concentration as high as 50 mEq/litre cells (Adair 1925, Harris 1960). If a system like red cells that contains small anions and cations capable of passing through the membrane were in Donnan equilibrium, the intracellular osmolality would be expected to exceed that of the medium by 25—30 mOsm/kg H_2O and the water pressure excess to be about 6000 mm H_2O. These values are far greater than the experimental estimates. We can conclude that red cells have the ability, with the aid of metabolism, to cancel out the effect of colloid *osmotic accumulation of water (Tosteson and Hoffman 1960, Tosteson 1964)*. In fact, when determined by freezing point depression, the intracellular and extracellular water activities are the same (Appelboom et al. 1958, Maffly and Leaf 1958) and the internal water pressure excess over that outside is 2 mm H_2O (Rand and Burton 1964).

Some earlier observations on the transfer of material across the red cell membrane

After the introduction of the concept of haematocrit (Hedin 1890), changes in red cell volumes in various circumstances were measured in the first

systematic studies of red cell permeability at the turn of the century Gryns (1896), Hedin (1897 1898) and Hamburger (1902) incubated red cells in isotonic solutions of solutes such as urea, ethylene glycol and glycerol, and found that the red cell volumes and water contents increased. In hypertonic solutions of sucrose, mannitol, and sodium or potassium chloride the red cells shrank, while in hypotonic solutions the cell volume increased. It was concluded that the red cell membrane was permeable to water and certain other substances, but impermeable to some others, such as sucrose, mannitol, and alkali salts.

The very rapid movement of water across the red cell membrane was demonstrated later in several studies (Parpart 1935 Paganelli and Solomon 1957 Villegas, Barton and Solomon 1958). The half-exchange time of tritiated water is about 4 msec.

Soon it became evident that the red cell membrane was easily permeable to small anions, such as chloride and bicarbonate ions (Hamburger 1902, van Slyke, Wu and McLean 1923) The half-exchange time of radioactive chloride ions in red cells has been found to be 0.2 sec (Tosteson 1959). This shows that chloride ions can rapidly pass through the membrane, but that the friction in their movement is greater than in that of water

Until the 1940 s the intact red cell membrane was believed to be impermeable to sodium and potassium ions. Then it was found that red cells that had lost potassium and gained sodium during cold storage at 4 C, could reverse these ion shifts after *in vivo* transfusion or after rewarming the cell suspension *in vitro* (Maizels and Paterson 1940 Danowski 1941 Harris 1941). Simultaneously it was shown with radioactive isotopes that the red cell membrane was permable to sodium and potassium ions (Hahn, Hevesy and Rebbe 1939 Dean *et al.* 1941 Hahn and Hevesy 1941 Mullins *et al.* 1941) Detailed studies with tracers of the red cell permeability to sodium and potassium ions were first carried out by Solomon (1952) He found that the half-exchange times for sodium and potassium tracers were about 20 hr and 30 hr respectively Thus the small anions pass through the red cell membrane at a rate 10^4 times higher than that of the small cations, while the water permeability is still 10^4 times higher than that of the small anions.

Theories of the distribution of ions across the red cell membrane

The high permeability of the red cell membrane to chloride and bicarbonate ions allows their rapid equilibration between the cells and the external environment. Their distribution is related to the difference in pH across the membrane and does not significantly differ from that predicted by the Donnan equilibrium theory (van Slyke *et al.* 1923 Passow 1965 Funder and Wieth 1966)

In the stationary state the cell to medium concentration ratios of potassium and sodium ions are 20 and 0 05 respectively Clearly some force is needed to sustain such a high deviation from equilibrium. Two different approaches to this problem are reviewed here. One connects the metabolic reactions and cation movements with a special »pump At the expense of ATP which is split to ADP and inorganic phosphate, potassium and sodium ions are believed to be transported to different sides of the membrane. The other predicts that the non-equilibrium distribution of potassium and sodium ions follows from their different friction with the transmembrane flows of metabolites.

Danowski (1941) and Harris (1941) in their pioneering experiments found that the uptake of potassium and outflow of sodium in cold-stored red cells after rewarming depended on glycolysis. The connection between glycolysis and cation transport was later studied in detail by several authors. Since metabolism was needed for the uphill transport of cations from a lower to a higher concentration, and the downhill movements from a higher to a lower concentration occurred in the absence of metabolism, the cation transport process was divided into two parts an actively pumped uphill transport and a non metabolically driven passive downhill leak (Maizels 1949 Solomon 1952, Shaw 1955 Glynn 1956, Solomon, Gill and Gold 1956) The leak is thought to be parallel to the pump, but independent of it and not representing a backwash through the pump (Post, Albright and Dayani 1967 Post 1968).

Schatzmann (1953) contributed to progress towards the active cation pump concept by first using cardiac glycosides, of which ouabain has been the most popular to study the mechanism of the selectivity between sodium and potassium ions by the red cell membrane. Cardiac glycosides were found to reduce the rate of uptake of potassium ions in cells rewarmed after cold-storage Glynn (1957) used alkali ion tracers. He found that inward unidirec tional flux of potassium and outward unidirectional flux of sodium were greatly diminished by cardiac glycosides, whereas outward unidirectional potassium flux was only slightly reduced, and inward unidirectional sodium flux was almost unaffected. Thus the main effect of cardiac glycosides was ascribed to inhibition of the active transport of potassium and sodium ions. As a consequence, cardiac glycosides have been used to separate the ion flows into the actively pumped flows and the passive diffusing or leaking ones.

Cardiac glycosides were originally found not to affect the rate of glucose conversion to lactic acid (Schatzmann 1953 Glynn 1964) Later it became evident that ouabain decreases the lactic acid production rate by 15—50 % (Murphy 1963 Minakami, Kakinuma and Yoshikawa 1964 Whittam and Ager 1965 Minakami and Yoshikawa 1966).

Cardiac glycosides in relatively high concentrations reduce the rate of ATP breakdown to ADP and inorganic phosphate in whole red cells (Whittam 1958

1964) and in isolated red cell membrane stroma or red cell ghosts (Post *et al.* 1960 Dunham and Glynn 1961) This was believed to be the only metabolic alteration in red cells caused by administration of cardiac glycosides (Glynn 1964). The enzyme especially inhibited by cardiac glycosides is a special $(Na^+ + K^+)$-activated, Mg^{++}-dependent, ATPase (see e.g Skou 1965 a or Katz and Epstein 1968 for a detailed review). Since cardiac glycosides were found to inhibit both the active uphill movements of sodium and potassium ions and the activity of the $(Na^+ + K^+)$-activated ATPase without affecting overall cell metabolism, the $(Na^+ + K^+)$-ATPase enzyme system is commonly considered somehow to be connected with the cation pump. This enzyme system has been detected in a large variety of cells.

The other approach reviewed here to the problem of transfer and distribution of material across biological membranes is based on the principles of thermodynamics of irreversible processes. The fundamental characteristics are that all reactions and processes occurring in the system are coupled, and the flow of material is a linearly dependent function of all forces, such as chemical potential and temperature gradients, in the system, provided that the system is observed from one point inside the membrane. If the phenomenological coefficients expressing the linear dependence are known as functions of concentrations, it is possible to solve the flows of components as functions of concentrations on each side of the membrane. It then becomes evident that the flow of a substance does not only depend on its own concentration, but also depends on the concentrations of all the other substances. It is possible that a given substance can flow across the membrane against its concentration gradient. According to the Curie principle the chemical reactions, being scalar processes, cannot be directly coupled to the flow of material, but affect the flows across the membrane because of the stationary state coupling (Prigogine 1955 Nims 1961 1967 a and b).

This approach is independent of the molecular mechanism of the transport process, and its principles can be applied generally In a living cell nutrients and metabolic end products are continually flowing in and out. These flows may lead to non-equilibrium stationary state distribution of compounds for which the system is closed, such as alkali ions. Indeed, Thurber and Thompson (1967) and Coster George and Rogers (1968) could demonstrate that the logarithm of the deviation of potassium and sodium ions from the equilibrium distribution is in a predictable way linearly dependent on the rate of flow of reactants. Thus potassium and sodium ions, without participating in the chemical reaction, were supposed to be separated and brought to a non equilibrium distribution across the membrane by their different friction with the flows of reactants. Coupling of certain flows has been found to lead to movement of some other substances against their concentration gradients in nerves (Baker *et al.* 1969 Blaustein and Hodgkin 1969) and ascites-tumour cells

(Eddy 1968 a and b). In these cases uphill movement of calcium ions and glycine was achieved by a downhill movement of potassium and/or sodium ions.

In living cells there are far too many components to allow an exact mathematical analysis of the transport processes. However simple models may be illustrative. Salminen (1963) and Ekman, Rastas and Salminen (1963) separated potassium and sodium ions by their different friction against the flow of water and acid, the end products of oxidative metabolism. Across a homogeneous porous membrane it was possible to keep potassium predominantly in one compartment and sodium in the other and to solve the system mathemati cally A still more sophisticated model has been constructed by Nims (1968). In his model the energy source needed for the non-equilibrium distribution of cations was an enzyme-induced chemical reaction, the hydrolysis of urea to ammonia and carbon dioxide.

If the membrane is non-homogeneous some component may circulate across the membrane even though there is no net flow Then it could act as a »carrier» for substances whose flows are coupled to it. This differs from the traditional carriers in that the formation of a chemical compound from the carrier and the substance to be transported would not be necessary The selectivity is achieved by different frictional coefficients in the membrane. Such a system has been studied in a model where circulation of water induced by electric current was found to lead to non-equilibrium distribution and accelerated exchange of various test substances across a non-homogeneous membrane (Salminen and Ekman 1970) The stationary state distribution ratios and the isotopically-determined exchange rates differed greatly from those obtained with the same current across a homogeneous membrane.

The water permeability of red cells is high and if the red cell membrane properties are different at different sites, circulation of water inwards and outwards across the cell membrane would be possible as a result of flows of metabolites. Salminen, Ekman and Manninen (1968) proposed that coupling the potassium and sodium ions to the water circulation could lead to their non-equilibrium distribution in red cells.

It is evident that, on the basis of the thermodynamics of irreversible processes, it is difficult to separate transport processes into active and passive systems. If active transport means movement of a substance against its chemical potential gradient, it can be brought about by coupling simultaneous flows of several components. Such coupling may arise from chemical reac tions of the components, or only from friction between various molecules. The situation in the membrane at a given time determines the direction of movement of an individual component. There is no fundamental difference between flows along or against the chemical potential gradient. If active transport means a flow of material connected to some chemical reaction the following points have to be taken into account maintaining a stationary

non-equilibrium state in living cells needs continuous inflow of some compounds, and simultaneous outflow of others. This means that the transport process predicts a chemical reaction to occur in the system. On the other hand there cannot be direct coupling of the chemical reaction and the flow of material according to the Curie principle: the coupling is the indirect stationary state coupling. In principle it is not possible to resolve a flow coupled to a given reaction: the whole system has to be considered as a single entity.

Various chemical modifiers of ion permeability of the red cell membrane

Many chemically unrelated substances have been found to cause the human red cell membrane to leak alkali ions. Since the cells usually begin to lyse at higher concentrations, the effect is called pro-lytic (Davson and Danielli 1938, Ponder 1948). It is characteristic that certain agents can induce a selective leakage of potassium ions, while some other agents affect both sodium and potassium ions. Propranolol and pronethalol (Ekman *et al.* 1969), lead salts (Ørskov 1935, Joyce, Moore and Weatherall 1954, Grigarzik and Passow 1958, Vincent and Blackburn 1958, Hasan *et al.* 1967), fluoride in certain concentrations (Wilbrandt 1940, Eckel 1958, Gardos 1959, Lepke and Passow 1960), valinomycin and certain other cyclic polypeptides and polyethers (Tosteson *et al.* 1967, Lardy 1968, Tosteson 1968, Henderson, McGivan and Chappell 1969) induce a greatly increased net flow and exchange of potassium or rubidium ions. However they have little effect on the movements of sodium ions. Butydrine induces a flow of potassium twice as great as that of sodium (Manninen 1969). On the other hand primaquine (Weed, Eber and Rothstein 1961) and alprenolol (Manninen 1969) increase the flows of potassium and sodium ions to similar extents, while chlorpromazine increases the flow of sodium ions somewhat more than that of potassium ions (Kwant and Stevenínck 1968).

Valinomycin and a number of its natural and synthetic analogues also selectively increase potassium and rubidium permeabilities in mitochrondria and artificial bimolecular lipid leaflets (Pressman 1965, 1968, Andreoli, Tieffenberg and Tosteson 1967, Lev and Buzhinsky 1967, Henderson et al. 1969).

In certain cases the changes in ion behaviour induced by chemical agents are not restricted to cations. For example dinitrofluorobenzene is known to decrease permeability to anions and increase that to cations (Berg, Diamond and Marfey 1965, Passow 1969). Others like benzote and dinitrophenol, have little if any effect on cation movements but effectively decrease movements of anions (Passow and Schnell 1969).

Review of the biological effects of propranolol

In the last few years propranolol (Fig. 1) has been widely used in clinical research because of its favourable effects in certain cardiac dysfunctions. Pharmacologically propranolol is classified as a potent beta-adrenergic blocking agent, which selectively suppresses the so-called beta receptor effects (as described by Ahlquist 1948) without having any sympathomimetic intrinsic activity (Shanks 1966) Propranolol effectively suppresses adrenaline-induced cardiac arrhythmias in animals (Shanks 1966). In man propranolol has also proved useful in controlling those cardiac arrhythmias exacerbated or induced by sympathetic activity (Besterman and Friedlander 1965, Irons, Ginn and Orgain 1966). Propranolol effectively blocks many catecholamine-induced metabolic effects (Zoter et al. 1966, Hunninghake, Azarnoff and Waxman 1967 Sailer et al. 1967) It has also been used to lower the cardiac work and to supress cardiac pain in patients with ischaemic heart disease (Epstein and Braunwald 1966, Glanelly et al. 1967 Mason and Braunwald 1967)

Propranolol has many other effects. It is a potent local anaesthetic, its potency being twice that of procaine (Morales-Aquiléra and Vaughan Williams 1965). Propranolol has an effect similar to quinidine on cardiac muscle action potentials (Vaughan Williams 1966). Propranolol can protect animals and humans from cardiac arrhythmias induced by cardiac glycosides (Howe and Shanks 1966 Irons et al. 1966 Turner 1966, Dohadwalla, Freedberg and Vaughan Williams 1969). These effects have been called the non-specific actions of propranolol. Alterations in membrane properties might be involved in some of them, such as those connected with cardiac glycosides, whose effects on electrical phenomena in cardiac muscle are believed to be based on altered transport of potassium ions in cardiac muscle membranes (Lyon and DeGraff 1966, Weatherall 1966).

In an organ like cardiac muscle, which is capable of carrying out complicated functions, it may be difficult to decide between pure membrane effects and effects mediated by a changed metabolic state in other parts of the cells (Bülbring 1960). If experiments are carried out in situ connections between different organs also have to be considered. For example adrenaline is known to elevate the plasma potassium concentration, the source of potassium being the liver (Ellis and Beckett 1963) while in cardiac and skeletal muscle potassium uptake may be enhanced (Powell and Skinner 1966, Stafford 1969) Increased plasma potassium concentration may per se change cardiac function. Similarly propranolol by inhibiting catecholamine-induced lipolysis (Zoter et al. 1966 Hunninghake et al. 1967 Sailer et al. 1967) might secondarily lead to changes in the behaviour of potassium and sodium ions in remote organs in the body As Jacob (1967 1968) and Cooper and Jandl (1968) showed lipids are in some unknown way linked to cation tranport even in red cells.

When studying the direct membrane effects of propranolol it may be advantageous that mature human red cells do not contain the adenylcyclase and 3'5' AMP system (Sutherland and Robison 1966). Many probably all, of the beta-receptor effects are believed somehow to be mediated through this system (Robison, Butcher and Sutherland 1967). As stated earlier adrenaline leads to cation shifts in certain organs, but does not influence the movements of sodium and potassium ions in red cells (Kahn and Acheson 1955).

Isolated rat diaphragm as an object for transport studies

Rat diaphragm is one of the most suitable objects for studying transport in the complicated cell systems. It is mammalian skeletal muscle naturally thin enough provided that small animals are used (Creese 1960) Potassium and sodium ions in rat diaphragm have been extensively studied by several authors (Creese 1954, Flückiger and Verzár 1954 Lüllman 1958 Rixon and Stevenson 1958, Creese and Northover 1961, Fozzard and Kipnis 1967). The diaphragm is capable of gaining potassium and expelling sodium in a way comparable to other cell systems.

OUTLINES OF THE PRESENT INVESTIGATION

Propranolol induces a massive outflow of bulk potassium ions from human red cells. Against the outflow of bulk potassium ions, tracer potassium ions are rapidly taken up into the cells. Their intracellular concentration transiently reaches much higher levels than that in the final propranolol induced stationary state. The rate of exchange of sodium tracer ions is also somewhat increased, but the concomitant net uptake of bulk sodium ions is small. The net loss of potassium salts is associated with a loss of water and the cell volume decreases. These phenomena were first investigated by Ekman *et al.* (1969). Now an attempt has been made to investigate these effects in more detail, for instance by changing to composition of the incubation medium, and simultaneously adding other membrane active compounds. The kinetics of propranolol uptake and outflow especially in relation to the cation flows, was studied using ^{14}C-labelled propranolol. The effectiveness of different optical isomers of propranolol was compared. Since cation transport and ATP breakdown to ADP and inorganic phosphate are generally believed to be causally connected, the effect of propranolol on ATP content and ATPase activities in red cells and isolated red cell membrane stroma was measured. Finally an attempt was made to compare the phenomena observed in red cells with those in other more complicated systems using isolated rat diaphragm.

MATERIALS AND METHODS

Experiments with red cells

General arrangements

Freshly drawn blood from apparently healthy human donors of both sexes, aged 20—53 years, was used. About 100—150 ml of blood was drawn at a time. There was at least a month between successive bleedings of one donor. Heparin (0.5 mg/ml blood) was used as anticoagulant. The blood was centrifuged at 3000 g for 15 min, then plasma, buffy coat, and the topmost cell layer were discarded. The cells were then washed 2—3 times with a twofold volume of buffered saline of the composition described below. About 90 % of the original white cells were removed by the washing. The cells were subsequently resuspended in the buffer to give, unless otherwise stated, a packed red cell volume of about 55 %.

The cells were incubated in a water bath at 38°C, mixed gently and usually equilibrated with humidified gas containing 95 % of O_2 and 5 % of CO_2.

The medium used in most experiments contained (mmole per litre) NaCl 115, $MgCl_2$ 1.0, $CaCl_2$ 0.5, $NaHCO_3$ 30, Na_2HPO_4 1.7 KH_2PO_4 0.4, KCl 4.3, and glucose 14. The pH of the medium during the experiments was 7.4.

To study the effect of calcium or magnesium ions their concentrations in the medium were either increased to 20 mM, or alternatively both were first omitted and later added in appropriate amounts to the cell suspension with the drugs and tracers. In some experiments enough disodium ethylenediaminetetra-acetate (EDTA Na_2) was added to chelate calcium and magnesium in the medium.

To study the effect of increased sodium ion concentration on the propranolol induced non-stationary state the cells were first suspended in the usual medium to give a packed cell volume of 50 %. Then the suspension was divided into three portions, to one 5 M NaCl was added to bring the sodium concentration to 310 mM, to another 0.35 M of sucrose was added, the third was a control. The packed red cell volumes were reduced by the NaCl and sucrose additions to 29 %. In the sucrose-treated cell suspension the pH was reduced to 7.15. Enough 0.2 N NaOH was added to bring the pH to 7.4.

When the potassium concentration was increased a corresponding amount of sodium was withdrawn, so that the osmolality remained unchanged.

For experiments of pH 7.0 and 8.0 the pH of the suspension was adjusted by adding 0.2 N HCl or NaOH in small amounts and thoroughly mixing the suspension between successive additions. With this treatment the cells were not lysed.

The pH was measured with a glass capillary electrode system (Radiometer Copenhagen).

When the reversibility of normal cation permeability in the propranolol treated cells was studied the cells were suspended in a fractional cell volume of 0.5 in a medium containing 100 mM potassium and 45 mM sodium. Propranolol (0.5 mM) was added and the suspension was incubated at 38°C for 1 hr Then the cells were first washed 3 times in a threefold volume of the KCl medium, resuspended in a tenfold volume of this medium and stored overnight. After that the cells were washed twice in the KCl and at last twice in the usual medium, to which they were subsequently resuspended. Control cells without propranolol were treated as above.

Bulk ion and tracer ion concentrations

Drugs and tracers were usually added simultaneously in saline to intact red cell suspensions. In most experiments the initial packed red cell volume after addition of the drugs and tracers was about 50 /i. From time to time an aliquot of 2.0 ml of the cell suspension was pipetted into an isotope counting tube and centrifuged at 3000 g for 5 min A 0.5 ml sample of the supernatant was carefully removed. After discarding the remaining supernatant without disturbing the cell column, the cells were washed once with a fiftoenfold volume of ice-cold buffer To determine the cell volume in the sample the relative packed red cell volume of the suspension was measured separately using microcapillary haemotocrit centrifugation at 12,000 g for 5 min with Hawskley & Sons (London) centrifuge.

The radioactive tracers were determined with an automatic dual channel well type crystal scintillation spectrometer (Nuclear Chicago, 874., or Wallac Decem). In most experiments ^{42}K and ^{22}Na isotopes were simultaneously present, and they were separated by their different half-lives and radiation energies. In some experiments ^{86}Rb was used and then sodium tracer was not present. The ^{22}Na and ^{86}Rb tracers were supplied by the Radiochemical Centre (Amersham) and ^{42}K by the Reactor Laboratory Helsinki University of Technology The specific activity of ^{86}Rb was high, so that the system contained very low concentrations of non-radioactive rubidium ions.

Non-radioactive potassium and sodium ions were determined with an internal standard flame photometer (Beckman 105).

Experiments with ^{14}C sucrose

Universally labelled sucrose was obtained from the Radiochemical Centre (Amersham) It was dissol ed in saline before use. The incubation and sample preparation procedures were as in experiments with alkali ion tracers. After separating the cells, they were lysed in a fivefold volume of distilled water

Round pieces of lens paper were placed so as to cover the bottom of the counting planchets. Test samples were pipetted onto the lens paper and dried at 22°C. The counts were measured with a gaiger gas flow counting apparatus (Beckman, Lowbeta II) The counts were corrected for dead times and self-absorption.

Experiments with ^{14}C p opranolol

The ^{14}C propranolol was was dissolved in saline before use. The basic principles were as described a lier for experiments with alkali ion tracers, except that the

packed red cell volume in the final incubation mixture was usually 10 /. Aliquots of 0.5 ml of the whole suspension and the medium, and the sedimented cells from samples of 2.0 ml of the suspension were used to determine the radioactivities.

A great deal of radioactivity was lost from the cells even in one wash in ice-cold buffer. Therefore the cellular ^{14}C-propranolol concentrations in the inflow experiments had to be calculated from those of the whole suspension and the medium with the aid of the packed red cell volume.

When the outflow of propranolol was studied, the cells were preincubated for 30 min in a medium containing either 1 / M or 0.4 mM propranolol including the ^{14}C-propranolol tracer. Then the cells were either washed 1—6 times with a fiftyfold volume of cold propranolol free buffer or the tracer outflow was followed against time after suspending the cells in the usual medium in a fractional cell volume of 0.1 with various concentrations of propranolol. The initial cellular radioactivities were calculated as before, the remaining activity after each wash was counted directly from the cell samples.

Red cell membrane stroma were prepared as described later in the ATPase experiments. The final incubation medium contained (mmole per litre) NaCl 100, KCl 10, $MgCl_2$ 5, $CaCl_2$ 5, imidazole Cl 5, tris Cl 6, adjusted to pH 7.5. The fractional stroma volume was 0.1. The suspension was incubated with ^{14}C-propranolol for an hour at 38 C, pH 7.5. Aliquots (0.5 ml) of the suspension and the medium, and 0.2 ml of the sedimented stroma after centrifugation at 20,000 g for 10 min were used to determine radioactivities.

In alkaline solutions propranolol can be quantitatively extracted as a free amine in heptane or in other non-polar solvents. The procedure used for extracting propranolol was a modification of those described by Black, Duncan and Shanks (1965) and Potter (1967). Aliquots of 4 ml of 1 N NaOH were added to the samples, and they were allowed to stand at least for an hour. Then 4.0 ml of n-heptane was added, and the tubes were gently shaken for 15 min. The phases were separated by centrifugation at 2000 g for 10 min. Aliquots of 2.0 ml of the heptane phase were added to 10 ml of scintillator containing 4 g of 2,5-diphenyloxazole (PPO) and 100 mg of 1,4,-di(2,5-phenyloxazolyl)-benzene (POPOP) dissolved in a mixture of equal volumes (500 ml) of toluene and ethyleneglycolmonoethylether.

The radioactivities in the samples were counted with a dual channel automatic liquid scintillation apparatus (Wallac Decem 314). The recoveries of various concentrations of ^{14}C-propranolol added together with different amounts of non-radioactive propranolol to the cell, medium and whole suspension samples were found to vary from 96 / to 100.5 / with the heptane extracting procedure. For correction of quenching the external standard channels ratio method was used.

Adenosine triphosphate (ATP) concentration

The cells were handled as above except that after discarding the buffy coat and the topmost cell layer the remaining cells were resuspended in a medium containing 50 / of their own plasma and 50 /o of the usual buffered saline. Aliquots of 2.0 ml were taken immediately and after 60 min. They were then deproteinized in equal volume of cold 0.6 N $HClO_4$. After centrifugation at 3000 g for 10 min, the ATP concentration was determined enzymatically from the clear supernatant using reagent kits of Boehringer & Soehne (Mannheim).

Adenosine triphosphatase (ATPase) activity

Red cells were washed three times in the usual medium, from which calcium had been omitted. They were then resuspended in the same medium at 38 C to give a packed red cell volume of 20 /o. The drugs, ATP (4 mM) and in some cases $CaCl_2$ (5mM), were added simultaneously. Aliquots of the suspension were withdrawn immediately and after 60 min, and were deproteinized by adding 0.2 vol. of cold 60 (w/w) $HClO_4$. After centrifugation at 3000 g for 10 min inorganic phosphate (Pi) was determined immediately from the clear supernatant according to Fiske and Subbarow (1925). ATPase activity was calculated from the increase in Pi content in the suspension during the first hour. The method used to determine Pi did not cause any significant hydrolysis of ATP as revealed by adding ATP and 0.2 vol 60 % $HClO_4$ to the buffer, determining the Pi and comparing the result to that obtained after heating the same ATP-containing solution with an equal volume of 1 N H_2SO_4 at 160°C for 3 hr.

The method used to prepare red cell membrane stroma was a modification of that used by Wiley (1969). Red cells were washed three times with 0.145 M NaCl. Centrifugation time was 10 min at 3000 g. The buffy coat and topmost cell layer were discarded. The cells were then lysed at room temperature in a tenfold volume of 6 mM tris Cl, adjusted to pH 7.5. The lysate was allowed to stand about 10 min, then the stroma were separated by centrifugation at 2°C at 20,000 g for 15 min. All visible traces of haemoglobin were removed by four further washes in this medium. The white stroma were suspended in 5 mM tris Cl, pH 7.5, at a concentration of 1.5—2 mg protein/ml solution. The stroma were preincubated at 38 C for 10 min with the tested drugs before the reaction was initiated by adding ATP and co-factors in 0.2 vol. to give final concentrations (mmole per litre) of NaCl 100, KCl 10, $MgCl_2$ 5, imidazole Cl 5 and ATP 1.5. In some cases 5 mM $CaCl_2$ was added. Aliquots were withdrawn at 0 min and 60 min. They were deproteinized in 0.5 vol of cold 1 N $HClO_4$, and the Pi was determined from the clear supernatant after centrifugation at 3000 g for 10 min. Protein in the incubation mixture was determined by the Biuret method (Gornall, Bardawill and David 1949). A commercial protein standard (Lab-Trol, Dade, Evanstown), diluted to the protein concentration of the incubation mixture, was used as a reference.

Experiments with isolated rat diaphragm

Diaphragms of Sprague-Dawley rats of both sexes weighing 150—200 grams were used. For the tracer outflow experiments the diaphragms were loaded with ^{22}Na, ^{42}K, and ^{86}Rb tracer ions in vivo. Amounts of the tracers that would give enough counts in the hemidiaphragms for reliable measurement were given intraperitoneally in 2—3 ml of 0.145 N NaCl in one or two injections 12—36 hr before the experiment. Only one isotope was given to each rat. The rats were anaesthetized with ether and exsanguinated by cutting the abdominal aorta. The diaphragms were rapidly rinsed in 0.145 M NaCl, and the tracer outflow was followed by incubating isolated hemidiaphragms at 38 C in a succession of about 10 tubes containing 3 ml non-radioactive buffered saline as used in red cell experiments except that the $CaCl_2$ concentration was 1 mM. The diaphragms were soaked 5—10 min in one tube, the contents of which were mixed with a vigorous bubbling of gas containing 95 / O_2 and 5 /o CO_2. The radioactivities released in the tubes, and the remaining activity in the diaphragm after soaking, were counted with a well-type automatic gamma spectrometer as described in experiments with red cells.

For the ion inflow experiments pieces of diaphragm weighing about 100 mg were removed rapidly and dissected radially to minimize fibre damage. They were incubated at 38°C in a two hundredfold volume of the buffered saline. The volume was large enough to maintain a practically constant composition. Propranolol and ^{42}K and ^{22}Na tracers were added to the medium beforehand.

From time to times pieces of muscle were removed, rapidly rinsed with 0.145 M NaCl, dried with blotting-paper and weighed. The radioactivities were counted as previously. To determine bulk potassium and sodium ion concentrations, the muscle pieces were digested by heating in 2 ml of 30 % HNO_3 at 80°C for 6 hrs. The whole muscle samples except for a little fat piece were digested. Potassium and sodium ion concentrations were then determined as in experiments with red cells.

The water content of the muscles was measured by calculating the loss of weight after drying to constant weight (24 hr at 100°C).

The extracellular space was determined by measuring the distribution of ^{14}C-sucrose between the muscle and the medium. Experimental procedure was as above. Activity of ^{14}C was counted in the medium and digested muscle samples with a gas flow geiger tube (Beckman Lowbeta II).

Chemicals

Propranolol (1-isopropylamino-3-(1-naphthyloxy)-propan-2-ol hydrochloride (Fig. 1) both different optical isomers and the ^{14}C labelled propranolol, were kindly supplied

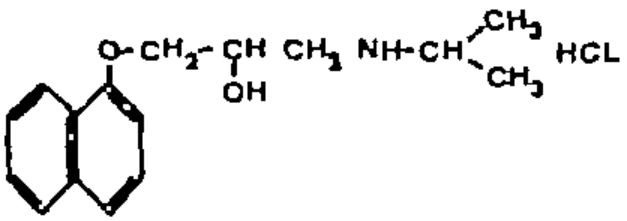


Fig. 1. Structure of propranolol.

by Drs J. D. Fitzgerald and A. Hayes (I.C.I., Macclesfield). Propranolol was dissolved in distilled water to which NaCl was added to bring the solution to 300 mOsm/kg H_2O. Unless otherwise stated, racemic propranolol was used. Ouabain was obtained from Fluka (Buchs). Protamine sulphate was obtained from Orion (Helsinki) as a 1 % solution in hydrochloric acid, and was neutralized before use. ATP was obtained from Boehringer Soehne (Mannheim).

Concentrations of the drugs are expressed, if not otherwise stated, as moles per litre of the whole suspension.

Calculation of the tracer rate constants

The tracer rat constants, k_{in} and k_{out}, give the fraction of the tracer that moves in one direction per unit time. The tracer rate constants do depend on the concentrations of all bulk substances, but are independent of the low tracer concentrations.

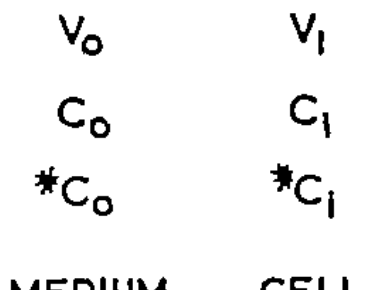


Fig. — Two compartment model system. V and V_i denote the volumes of the compartments and c and *c the concentrations of two isotopes of the same species.

The following equations follow from this general principle of the tracer experiments. Calculation of the tracer rate constants in a two-compartment system composed of the »cell or inside« and the medium« or »outside« separated by a thin membrane (Fig. ") are described by the following equations. A general case, during which all properties of the system may change with time, and a stationary state case are presented. This approach conforms to the analyzing method suggested by Ekman et al. (1969) and Ekman (personal communication 1970).

General case

The inside and outside volumes of the two compartments are denoted by V_i and V_o. The system contains two isotopes of the same species, so that their behaviour is similar but they can be separated analytically One is a tracer and the other may be another tracer or the corresponding bulk substance. The concentrations inside are denoted by c_i and $^*c_i$ and outside by c_o and $^*c_o$.

The flow of the first isotope through the membrane from one compartment to be other is given by

$$J = k_{in} C_o - k_{out} C_i \tag{1}$$

J is the flow into the cell in moles/litre cells/min.

The flow of the other isotope at the same time is

$$J = k_{in} {}^*C_o - k_{out} {}^*C_i \tag{2}$$

with the same rate constants.

The flow of the first isotope into the cell is equal to the rate of change of the concentration in the cell

$$J = \frac{dC_i}{dt} \tag{3}$$

The flow of the other isotope is

$$J = \frac{d^*C_i}{dt} = -\frac{V}{V_i}\frac{d^*C_o}{dt} \tag{4}$$

The ratios of concentrations are denoted by

$$r = \frac{C_i}{C_o} \qquad \text{and} \qquad {}^*r = \frac{{}^*C_o}{{}^*C_i} \tag{5}$$

and the ratios of the total amounts of the isotopes in the compartments are denoted by

$$R = \frac{V_i}{V}\,\frac{C_i}{C_e} \qquad \text{and} \qquad {}^*R = \frac{V}{V_i}\,\frac{{}^*C_i}{{}^*C_i} \tag{6}$$

By taking the derivatives with respect to time we get

$$\frac{dR}{dt} = \frac{V_i}{V}\,\frac{1}{C_e}\left(\frac{dC_i}{dt} - \frac{C_i}{C_e}\,\frac{dC_e}{dt}\right) \tag{7}$$

and

$$\frac{d^*R}{dt} = \frac{V_e}{V_i}\,\frac{1}{{}^*C_i}\left(\frac{d^*C_e}{dt} - \frac{C_e}{{}^*C_i}\,\frac{d^*C_i}{dt}\right) \tag{8}$$

Because the total amount of the isotopes in the system is constant, we have

$$V_e\frac{dC_e}{dt} + V_i\frac{dC_i}{dt} = 0 \quad \text{and} \quad V\,\frac{d^*C_e}{dt} + V\,\frac{d^*C_i}{dt} = 0 \tag{9}$$

Thus equations (7) and (8) may be written

$$\frac{dR}{dt} = \frac{V_i}{V}\,\frac{1}{C_e}(1+R)\frac{dC_i}{dt} \tag{10}$$

$$\frac{d^*R}{dt} = \frac{V}{V_i}\,\frac{1}{{}^*C_i}(1+{}^*R)\frac{d^*C_e}{dt} \tag{11}$$

From equations (1—4), (10) and (11) follow that

$$\frac{C_e V}{V_i(1+R)}\,\frac{dR}{dt} = k_{in}C_e - k_{out}C_i \tag{12}$$

and

$$-\frac{C_i V_i}{V\ (1+\ R)}\,\frac{d\ R}{dt} = k_{in}{}^*C_e - k_{out}{}^*C_i \tag{13}$$

By dividing equation (12) by (13) we get

$$\frac{k_{in} - k_{out}\,r}{k_{out} - k_{in}\,{}^*r} = \frac{V}{V_i}\,\frac{\dfrac{1}{1+R}\,\dfrac{dR}{dt}}{\dfrac{1}{1+\ R}\,\dfrac{d^*R}{dt}} = A \tag{14}$$

where definitions from equation (5) have been used.

From equation (14) the ratio k_{in}/k_{out} can be solved

$$\frac{k_{in}}{k_{out}} = \frac{A + r}{1 + A^*r} \tag{15}$$

k_{in}/k_{out} is the cell/medium concentration ratio that the tracer tends to attain at time t.

The rate constant k_{out} can be calculated from

$$k_{out} = \frac{V}{V_i}\,\frac{1}{1+R}\,\frac{\dfrac{dR}{dt}}{\dfrac{k_{in}}{k_{out}} - } \tag{16}$$

which is obtained from equations (1), (3) and (10).

The k_{in} can be calculated from the values of k_{out} and k_{in}/k_{out}. The values of k_{in} and k_{out} are the same for all isotopes, so that the flow of any isotope or the total flow of the species can be calculated from equation (1) by substituting the concentrations c_e and c_i by the inside and outside concentrations in question.

Stationary state

When the system is in a stationary state with respect to the bulk concentrations, the tracer rate constants are independent of time. The time derivative of r is, according to equations (5) and (9)

$$\frac{d^*r}{dt} = \frac{1}{C_o}\left(1 + r\,\frac{V_1}{V}\right)\frac{dC_1}{dt} \tag{17}$$

Equations (1), (3) and (17) yield

$$\frac{d^*r}{dt} = (k_{in} - k_{out}\,{}^*r)\left(1 + {}^*r\,\frac{V_1}{V}\right) \tag{18}$$

The variables can be separated by integrating from $^*r = 0$ to $^*r = {}^*r$ and $t = 0$ to $t = t$

$$k_{in}\,t = \frac{1}{\frac{V_1}{V} + \frac{k_{out}}{k_{in}}}\ln\frac{1 + {}^*r\frac{V_1}{V_o}}{1 - \frac{k_{out}}{k_{in}}\,{}^*r} \tag{19}$$

By infinite time the tracer flow stops and we may write

$$\frac{{}^*C_1^{\infty}}{C^{\infty}} = {}^*r^{\infty} = \frac{k_{in}}{k_{out}} \tag{20}$$

Thus equation (19) takes the form

$$k_{in} = k_{out}\,{}^*r^{\infty} = \frac{B/t}{1/{}^*r^{\infty} + V_1/V} \tag{21}$$

where

$$B = \ln\frac{1 + {}^*r\,V_1/V}{1 - {}^*r/{}^*r^{\infty}} \tag{22}$$

When the system is in the stationary state with respect to the bulk component, the tracer tends to attain the same cell/medium concentration ratio as the corresponding bulk substance, so that $^*r^{\infty} = r^{\infty}$. When the corresponding bulk substance is not present, or its distribution is not known, the ratio $^*r^{\infty}$ can be obtained by choosing by trial and error a value of $^*r^{\infty}$ that gives a straight line in the plot of the term B against time. When the tracer movement is slow so that its distribution is still far from the stationary state at the end of the experiment, only large errors in the value of $^*r^{\infty}$ will lead to a visible deviation from the straight line in the plot. In such cases the most accurate method of obtaining the right value for $^*r^{\infty}$ is to examine the rates of tracer movements both inwards and outwards.

Practical arrangements

The calculations were performed with a small digital computer (Olivetti, Programma 101). In equations (15 and 16) the tangents dR/dt and d*R/dt can be determined, if the time-interval $t_2 - t_1$ is short enough, as follows

$$\frac{dR}{dt} = \frac{\frac{V_{11}}{V_1}r_1 - \frac{V_{12}}{V_2}r_2}{t_2 - t_1} \quad \text{and} \quad \frac{d\,{}^*R}{dt} = \frac{\frac{V_1}{V_{11}}\,{}^*r_1 - \frac{V_2}{V_{12}}\,{}^*r_2}{t_2 - t_1} \tag{23}$$

in which 1 and 2 denote the measured parameters at times t_1 and t_2. So the rate constants could be calculated with the available computer

In the calculations of the stationary state rate constants the term B in equation (22) was calculated with different values of "r" When plotted against time, the one that showed the least deviation from the straight line was chosen by the least square method.

Calculations of the diaphragm inward tracer rate constants were based on the above treatment. The diaphragms were incubated in a large volume of medium so that the composition of this other compartment remained constant. This made it possible to leave the volume parameter out of the equations.

In outflow experiments the standard technique for one compartment analysis was used. The radioactivities in the diaphragm at a given time, after replacing the the outer compartment often enough with non-radioactive medium, were plotted semi-logarithmically against time. The half-exchange time ($T_{½}$) was then calculated graphically The tracer outward rate constant, $k_{out} = \ln 2/T_{½}$, was then calculated.

RESULTS

Inflow and outflow of ^{14}C propranolol

Accumulation by whole red cells

Fig 3 shows that propranolol added initially to the medium was rapidly taken up by the cells. The cell/medium concentration ratios of the ^{14}C-propranolol varied from 7 to 10 in the first sample (5 min). With low propranolol concentrations (1 μM and 10 μM) the stationary distribution ratio between the cells and the medium was reached at that time, while with higher concentrations the tracer continued to accumulate in the cells. With 0 1 mM of propranolol the approximate stationary distribution was reached after 90 min, the cell/medium concentration ratio of ^{14}C-propranolol being 28. When the propranolol concentration was still higher 0 4 mM, the tracer transiently reached an intracellular concentration higher than that at the end of the experiment.

The rate of inflow of ^{14}C-propranolol in cells treated with 0 4 mM propranolol was decreased when the pH of the medium was lowered from .5 to 7 0 After increasing the pH to 8.0 the uptake rate was enhanced

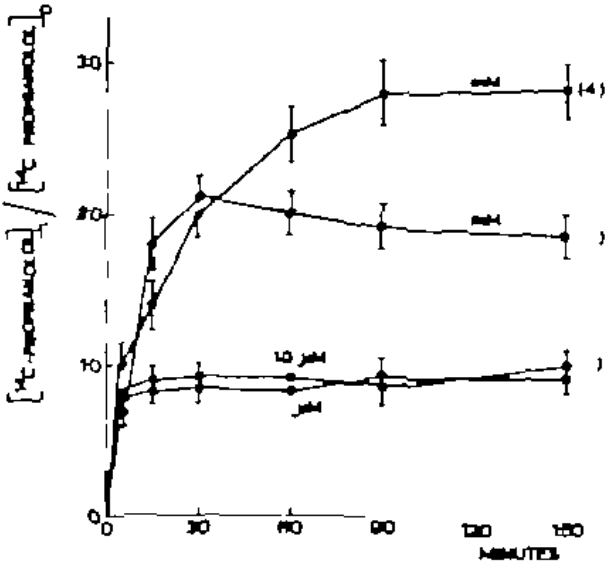


Fig. 3 Cell ( )/medium ( ) concentration ratios of ^{14}C-propranolol added to red cell suspension t 0 min with various total propranolol concentrations Numbers of experiments and means ± S.D

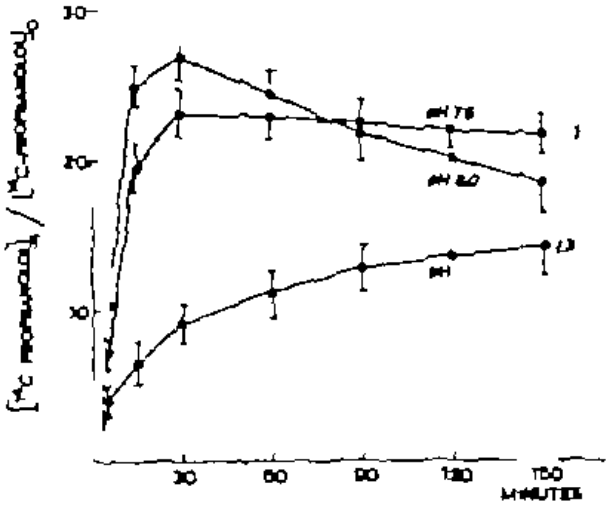


Fig. 4. Effect of the pH of the medium on the cell (i)/medium (o) concentration ratios of ^{14}C propranolol added to the cell suspension at 0 min with 0.4 mM non radioactive propranolol.

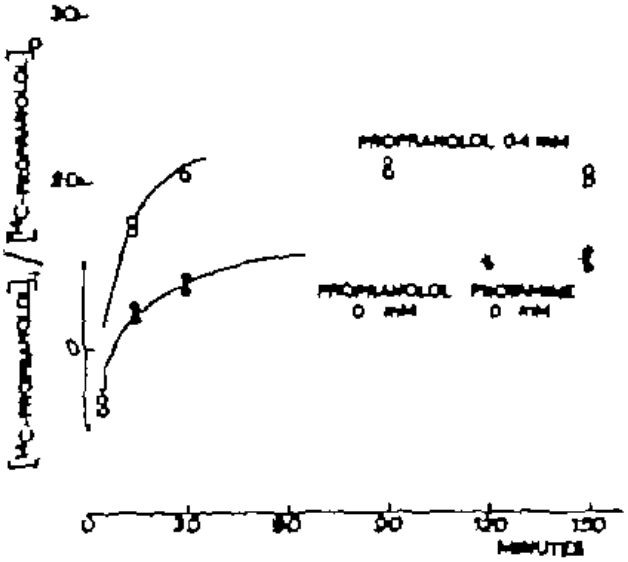


Fig. 5. Effect of protamine on the uptake of ^{14}C propranolol.

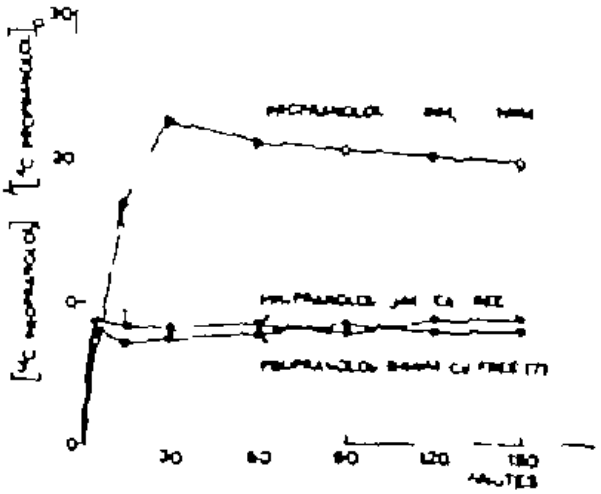


Fig. 6. Effect of calcium ions on the uptake of ^{14}C-propranolol.

(Fig 4). The height of the transient cellular accumulation as compared to that at the end of the experiment was also greater at pH 8.0

Addition of an equimolar amount of protamine reduced both the rate of ^{14}C-propranolol uptake and its stationary distribution ratio between the cells and the medium in cells with 0 4 mM propranolol (Fig 5)

When calcium ions were omitted from the medium there was no increased cellular accumulation of ^{14}C-propranolol when high concentrations of propranolol were present (Fig 6).

Outflow and binding by whole red cells

The cells were loaded with propranolol by incubation for 30 min in a medium containing 0 4 mM bulk propranolol and the propranolol tracer The fraction of the initial radioactivity remaining in the cells after washing for various times with propranolol free saline is shown semilogarithmically against time in Fig 7 Cells that were loaded with propranolol in medium containing calcium ions, contained 12 /o of their initial radioactivity after 6 washes in Ca-medium. The same cells when washed in Ca-free medium contained only 1.5 ^{0}/$_{0}$ of their initial radioactivity In Ca free medium the total cellular radioactivity as disintegrations per min per ml of cells accumulated during the 30 min loading procedure,

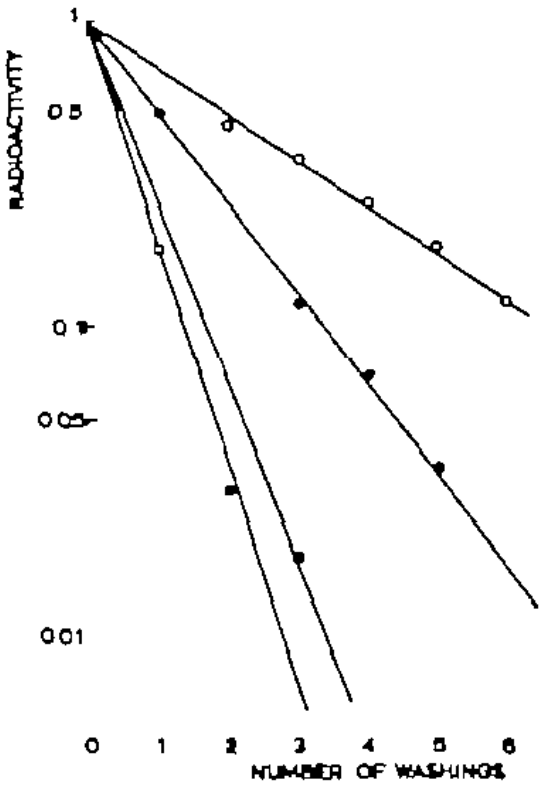


Fig. 7 Fraction f ^{14}C propranolol remaining in the cells after washing for various times in saline without propranolol The cells were initially loaded by the tracer with 0 4 mM propranolol. ○ cells loaded and washed in medium containing 0.5 mM calcium, ● loaded in Ca-containing and washed in Ca free medium, □ loaded in Ca free medium and washed in Ca-containing medium, ■ loaded and washed in Ca-free medium.

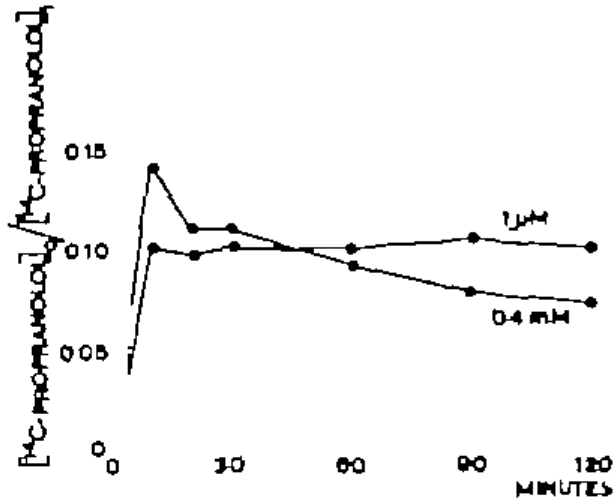


Fig. 8. Outflow of ^{14}C-propranolol from cells loaded at a propranolol concentration of 1 μM. The fractional cell volume during the experiment was 0.1.

was about 0.4 of that in cells incubated with calcium ions. In this case the tracer could easily be washed out irrespective of the calcium content of the medium.

The effective time for ^{14}C-propranolol outflow in each wash was 4 min. With its aid the outward rate constants, k_{out}, for ^{14}C-propranolol efflux could be calculated. For cells loaded with propranolol in the presence of calcium ions they were 5.2 hr^{-1} and 10.3 hr^{-1} in Ca-containing and Ca free washing media respectively. Those for cells loaded in Ca free medium were 20 hr^{-1} and 21 hr^{-1}.

In the other kind of experiments cells were loaded with propranolol tracer with a low (1 μM) total propranolol concentration. The outflow of ^{14}C-propranolol was then followed from cells suspended in a fractional cell volume of 0.1 in the usual medium containing 1 μM or 0.4 mM bulk propranolol. With the higher propranolol concentration the medium/cell concentration ratio of the propranolol tracer transiently attained a higher value than that at the end of the experiment (Fig. 8). The inward and outward rate

TABLE I. Rate constants of propranolol tracer and the initial total inflow of propranolol, 5 min after addition of propranolol to cell suspensions.

	Propranolol concentration	
	1 μM	0.4 mM
k_{in}, hr^{-1}	65	340
k_{out}, hr^{-1}	7.0	20
k_{in} [propranolol] — k_{out} [propranolol] mmole/litre cells/hr	0.063	85

constants for the propranolol tracer flow at the 5 min point, and the initial inflow of propranolol, are given in Table I. Interestingly the total inflow of propranolol by the higher propranolol concentration was enhanced more than in linear relation to the increase in propranolol concentration. The outward rate constant for the ^{14}C-propranolol efflux with 1 μM external propranolol was 7 0 hr^{-1} which fits fairly well with the rate constant of 5.2 hr^{-1} obtained earlier by a different method.

Uptake by red cell membrane stroma

After 30 min incubation with 1 μM or 0.3 mM propranolol red cell membrane stroma had ^{14}C-propranolol concentrations 2.48 ± 0.20 or 1.80 ± 0.17 times higher than the medium (means of five determinations ± S E.).

In one series of experiments where samples were taken after 5, 30 and 60 min, the ^{14}C-propranolol distribution ratios between stroma and medium were similar in all samples regardless of incubation time. Adding 1.5 mM ATP to the medium made the stroma viscous, but did not change the ^{14}C propranolol distribution ratio. Omission of calcium ions from the medium did not lead to visible changes in the distribution ratio of ^{14}C propranolol.

Movements of non radioactive potassium and sodium ions and their tracers in red cells during the propranolol induced non-stationary state

Different optical isomers of propranolol

Initially the mean cell and medium sodium concentrations were 7 mM and 145 mM, and those of potassium 95 mM and 4.8 mM respectively corresponding to a cell/medium concentration ratio of 0 05 for sodium and 20 for potassium. Adding 0.5 mM (±)-propranolol to the cell suspension increased the cell/medium concentration ratio of sodium and decreased that of potassium until a new time-independent state was reached (Fig. 9) The decrease in the cell/medium concentration ratio of potassium was even greater with (+)-propranolol than with (±)-propranolol, while with (—)-propranolol the decrease was less. Changes in the cell/medium concentration ratios of sodium were smaller than those of potassium with all the different optical isomers of propranolol.

At the end of the experiments the cell/medium concentration ratios of bulk potassium ions were 1 4, 2.6, and 1 6 with (+), (—) and (±)-propranolol (0 5 mM). Those of sodium were 0.20 0 16, and 0 19 respectively

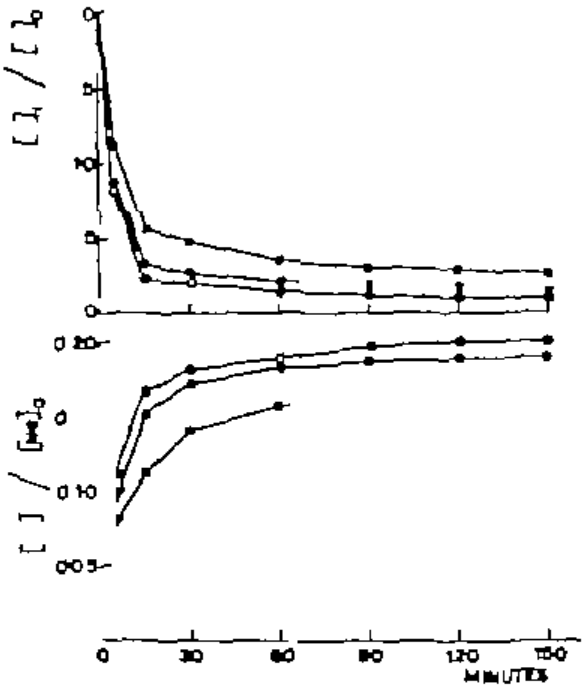


Fig. 9. Cell (i)/medium (o) concentration ratios of bulk potassium and sodium ions after addition of 0.5 mM propranolol of various optical forms to red cell suspension. ○ (+), ■ (−) and ● (±)-propranolol. In controls without propranolol the cell/medium concentration ratios of potassium and sodium bulk ions remained unchanged and were 20 and 0.03 respectively

The packed red cell volumes were reduced, and were 0.78, 0.88 and 0.80 times the initial volumes with (+), (−), and (±)-propranolol.

In the controls without propranolol the cell/medium concentration ratios of bulk potassium and sodium ions and the packed red cell volumes did not change noticeably during incubation.

The flows of bulk potassium and sodium ions as mmole/litre initial cells are shown as a function of time in Fig. 10. At the end of the experiments, when the flows were ceased, the outflow of potassium was 50 mmole/litre cells after treatment with 0.5 mM (+)-propranolol. The corresponding

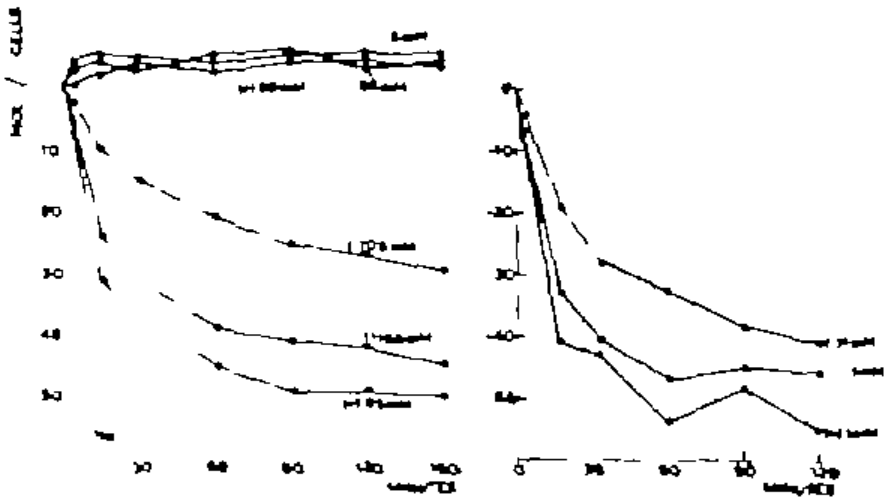


Fig. 10. Flows of bulk potassium and sodium ions as mmole/litre initial cells after exposure to various optical isomers of propranolol.

potassium flow produced by (—)-propranolol was much lower (27 mmole/litre cells) while that with (+)-propranolol was intermediate (45 mmole/litre cells). The concomitant inflow of sodium ions was low about 5 mmole/litre cells after 2.5 hr and no differences between the various forms of propranolol could be discerned.

The outflow of bulk potassium ions with 1 mM (—)-propranolol was similar to that with 0.5 mM (±)-propranolol (Fig 10) With 0.3 mM (—)-propranolol almost no shift of potassium ions was produced, while a clear outflow was observed when the (+)-propranolol concentration was 0.1 mM. Smaller concentrations down to 0.1 μM had no noticeable effect.

The rates of increases of the cell/medium concentration ratios of ^{42}K and ^{22}Na tracer ions were accelerated by all the forms of propranolol when the tracers were added together with 0.5 mM propranolol to the red cell suspension (Fig 11). With (+) and (±)-propranolol the potassium tracer transiently attained an intracellular concentration much higher than that at the end of the experiments. After 5 min the cell/medium concentration ratios of ^{42}K were 3.2 (+) and 2.7 (±), whilst the corresponding ratio for (—)-propranolol was 0 6, and 0 03 for controls without propranolol. The ^{42}K tracer then began to flow out of cells treated with (+)- or (±)-propranolol, and reached an

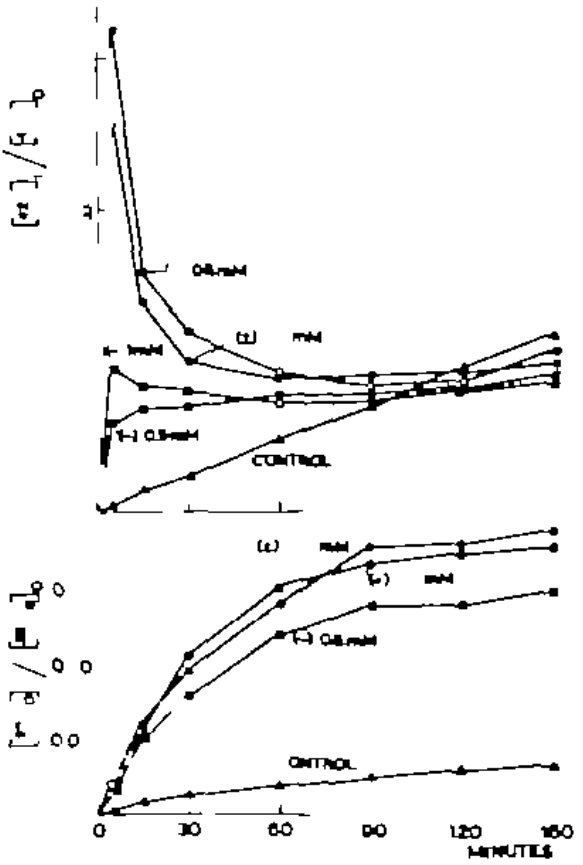


Fig. 11. Cell (*i*)/medium (*o*) concentration ratios of ^{42}K and ^{22}Na tracer ions added to the medium simultaneously with various optical isomers of propranolol.

approximate stationary state after about 60 min, when the cell/medium concentration ratios were near unity

Increasing the (—)-propranolol concentration from 0.5 mM to 1 mM somewhat accelerated the rate of uptake of the ^{42}K tracer However the cell/medium concentration ratio of ^{42}K did not exceed unity and in the first sample (5 min) it was almost the same as at the end of the experiment. With 0.3 mM (—)-propranolol the potassium distribution ratio during the experiments was near that of the controls. Even 0.1 mM (+)-propranolol was able to change the rate of ^{42}K uptake lower concentrations down to 0.1 μM had no noticeable effect

With 0.5 mM propranolol-treated cells the cell/medium concentration ratios of ^{22}Na tracer ions, measured simultaneously with ^{42}K, were somewhat lower with (—)-propranolol than with (+)- or (±)-propranolol (Fig. 11). However if the different shrinkage of the cells is taken into account, approximately the same amounts of ^{22}Na tracer were accumulated in all cases. At the end of the experiment the cell/medium ratios of the total amounts of ^{22}Na were 0.11 0 10 and 0 11 with (+)- (—)- and (±)-propranolol respectively while that of the controls without propranolol was 0.03.

The calculated potassium tracer rate constants and bulk ion flows 2.5 min after addition of 0.5 mM propranolol are given in Table II. The rate constants changed rapidly with time. In cells treated with (±)-propranolol the inward rate constant was reduced from 18 hr^{-1} to 4.5 hr^{-1} after 5 min, and the outward rate constant from 2.5 hr^{-1} to 2.3 hr^{-1} After 3 hr the respectiv rate constants are 0.66 hr^{-1} and 0.66 hr^{-1} (Ekman *et al.* 1969)

TABLE II. Rate constants of tracer potassium, concentrations and flows of bulk potassium ions in propranolol (0.5 mM)-induced non-stationary state 2.5 min after addition of various optical isomers of propranolol. Corresponding stationary state values of the control cells are given.

	Propranolol			Control
	dextro (+)	laevo (—)	racemic (±)	
k_{in}, hr^{-1}	20	4.2	18	0.37
k_{out}, hr^{-1}	3.2	0.8	2.5	0.019
$[K^+]_i$, mmole/litre cells	93	95	93	93
$[K^+]_e$, mmole/litre cells	7.5	5.5	7.0	4.5
$k_{in}[K^+]_e$, mmole/litre cells/hr	150	25	130	1.8
k_{out} $[K^+]_i$, mmole/litre cells/hr	300	80	240	1.8
Bulk ion flow k_{in} $[K^+]_e - k_{out}[K^+]_i$ mmole/litre cells/hr	—150	—55	—110	0

Effect of the pH of the medium

The outflow of bulk potassium ions induced by 0.5 mM (±)-propranolol at pH 7.0 was about half of that at pH 7.5 or 8.0 (Fig. 12). The inflow of bulk sodium ions was small at each pH tested and no significant differences could be discerned.

By the end of the experiments the packed red cell volumes had decreased to 0.89, 0.78, and 0.79 times the initial volumes at the three pH values increasing from pH 7.0 to pH 8.0.

After adding ^{42}K and ^{22}Na tracer ions with 0.5 mM propranolol to intact red cell suspensions the initial rate of ^{42}K uptake was high at pH 7.5 and 8.0 and the tracer transiently attained a higher intracellular concentration than the final one (Fig. 13). At pH 7.0 the uptake rate of ^{42}K was low. After 2.5 min the tracer potassium inward rate constants were 1.1 hr^{-1}, 17 hr^{-1}, and 19 hr^{-1} at pH 7.0, 7.5, and 8.0. The corresponding outward rate constants were 0.28 hr^{-1}, 2.2 hr^{-1}, and 2.5 hr^{-1}.

The rate of increase, and the stationary state cell/medium concentration ratio of ^{22}Na in the propranolol treated cells were lower at pH 7.0 than at pH 7.5 or 8.0 (Fig. 13).

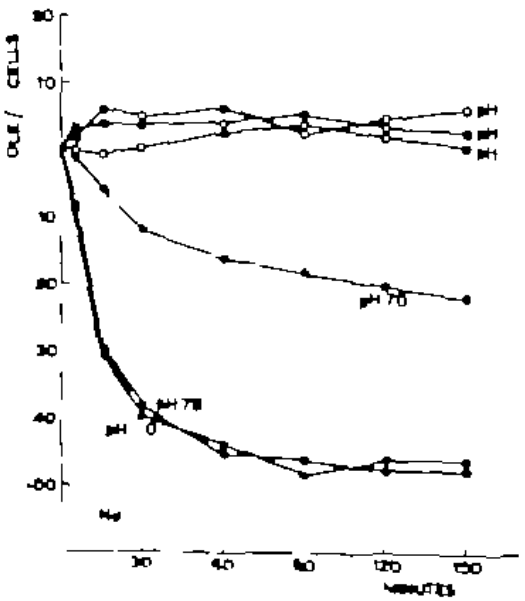


Fig. 12. Effect of pH on flows of bulk potassium and sodium ions in 0.5 mM propranolol treated cells.

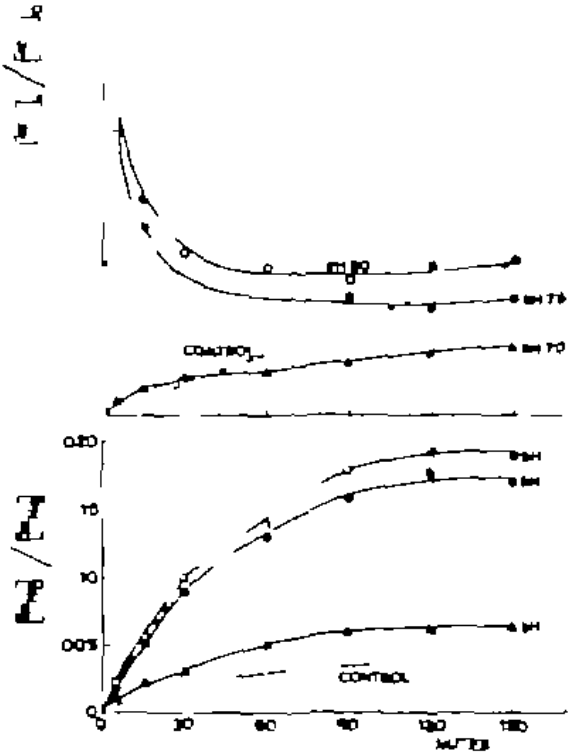


Fig. 13. Effect of pH on cell (I)/medium (o) concentration ratios of ^{42}K and ^{22}Na tracer ions added to the medium simultaneously with 0.5 mM propranolol. Control lines denote tracer uptakes at pH 7.5 in cells without propranolol.

In the control cells changing the pH had little effect on the behaviour of bulk potassium and sodium ions. At pH 7.0 the rate of uptake of both ^{42}K and ^{22}Na tracers was about 0.8 times that at pH 7.5. At pH 8.0 uptake of ^{22}Na was 1.5 times higher than at pH 7.5 whereas the uptake rate of ^{42}K was as low as at pH 7.0. The results obtained here for intact cells are in agreement with those at various pH values found by Pfleger, Rummel and Seifen (1967).

Effect of potassium and sodium ions

The height of the 0.5 mM propranolol induced rapid transient cellular accumulation peak of ^{42}K tracer ions above the final stationary concentration was sensitive to the bulk potassium ion concentration of the medium (Fig. 14). Table III shows the calculated potassium tracer rate constants and the

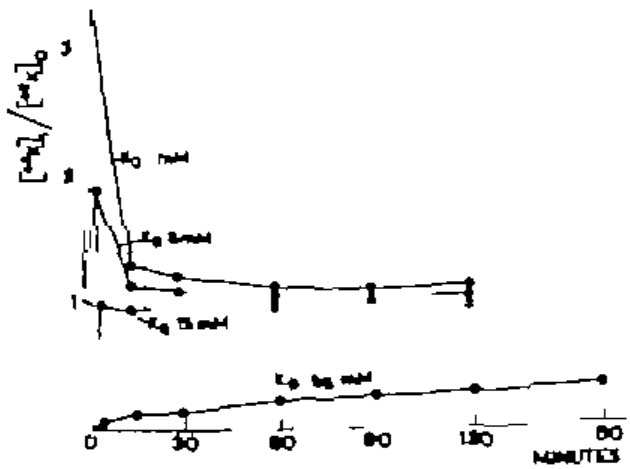


Fig. 14. Effect of external potassium ion concentration on cell (i)/medium (o) concentration ratios of ^{42}K added to the medium at 0 min simultaneously with 0.5 mM propranolol.

TABLE III. Effect of external potassium ion concentrations on potassium tracer rate constants and bulk ion flows in cells treated with 0.5 mM propranolol. Values 2.5 min after propranolol addition.

	Initial K+ concentration in the medium (mM)			
	4	8	15	93
k_{in}, hr^{-1}	31	15	8.5	1.0
k_{out}, hr^{-1}	2.7	2.6	2.8	1.0
$[K^+]_i$, mmole/litre cells	94	94	94	93
$[K^+]_o$, mmole/litre cells	6.5	10.6	17.4	95
k_{in} $[K^+]_o$, mmole/litre cells/hr	150	160	170	95
k_{out} $[K^+]_i$, mmole/litre cells/hr	250	260	260	93
Bulk ion flow $k_{in}[K^+]_o - k_{out}[K^+]_i$ mmole/litre cells/hr	−100	−100	− 90	0

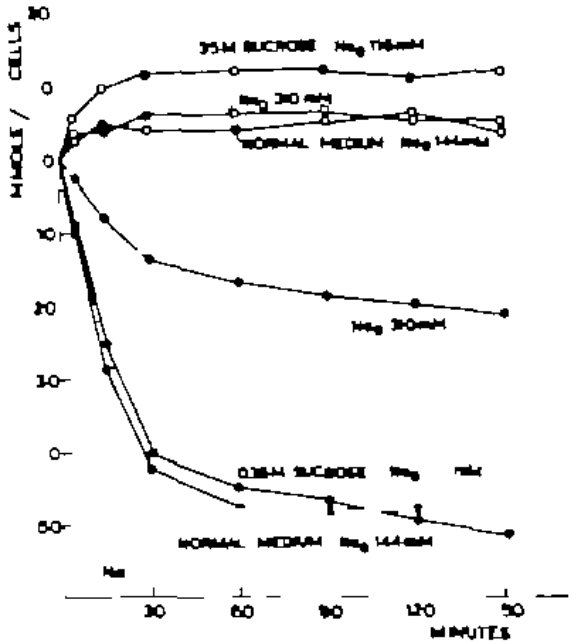


Fig. 15. Flows of bulk potassium and sodium ions in cells treated with 0.5 mM propranolol after increasing the medium sodium concentration to 310 mM, or adding sucrose to give a medium osmolality similar to that with 310 mM sodium.

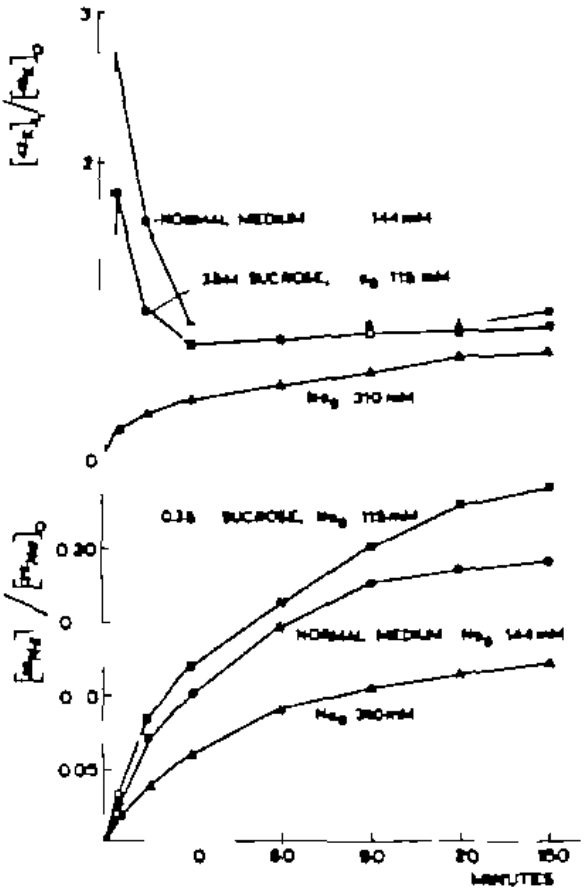


Fig. 16. Cell (i)/medium (o) concentration ratios of ^{42}K and ^{22}Na tracer ions in the same cells as in Fig. 15. Tracer ions were added at 0 min to the medium.

unidirectional fluxes of potassium ion. During the non-stationary state the inward and outward unidirectional fluxes of potassium ions were rather constant at different concentrations of external potassium ions.

When the sodium concentration of the medium was increased to 310 mM, the 0.5 mM propranolol-induced leakage of potassium ions was reduced (Fig 15) In the usual medium (144 mM sodium) the outflow of potassium ions was 46 mmole/litre cells after 2.5 hr while in the high sodium medium it was 21 mmole/litre cells. Where 0.35 M sucrose was added to make the osmolality of the medium the same as in the high sodium medium, the flow of potassium ions was about the same as that produced by propranolol in the usual medium without sucrose. Sucrose alone, without propranolol, induced a rather high outflow of potassium. The flow was 16 mmole/litre cells after 2.5 hr and was accompanied by a corresponding inflow of sodium ions. The inflow of sodium was small in propranolol treated cells suspended either

TABLE IV Rate constants of tracer potassium, concentrations and flows of bulk potassium ions 2.5 min after addition of 0.5 mM propranolol to usual, high sodium or sucrose-containing media.

	Medium		
	Usual [Na+] 144 mM	High sodium [Na+] 310 mM	Sucrose 0.35 M [Na+] 115 mM
k_{in}, hr^{-1}	18	2.3	16
k_{out}, hr^{-1}	2.3	0.25	1.4
$[K+]_i$, mmole/litre cells	95	159	155
$[K+]_o$, mmole/litre cells	7.2	4.0	6.6
k_{in} $[K+]_o$, mmole/litre cells/hr	130	10	105
k_{out} $[K+]_i$, mmole/litre cells/hr	220	40	220
Bulk ion flow k_{in} [K+] — k_{out} [K+] mmole/litre cells/hr	— 90	— 30	—115

in the usual or the high sodium medium. In the sucrose-treated cells propranolol did not increase the flow of sodium ions above that induced by sucrose alone.

After propranolol treatment the initial rate of uptake of ^{42}K tracer ions from the medium was somewhat lower after sucrose treatment than in the cells suspended in the usual medium (Fig. 16). In the high sodium medium the rate of uptake of ^{42}K tracer was much lower than in the other two groups and was near that of the control cells without propranolol. Table IV gives the rate constants of potassium tracer and flows of bulk potassium ions 2.5 min after addition of propranolol.

In control cells the uptake rate of ^{42}K was decreased by sucrose treatment, the cell/medium concentration ratios after 2.5 hr being 1.2 and 0.76 in the usual and sucrose-containing media respectively

Sucrose treatment increased the cell/medium concentration ratio of ^{22}Na in 0.5 mM propranolol treated cells, while in cells suspended in the high sodium medium the ratio was lower about half of that in the usual medium (Fig. 16) Without propranolol the cell/medium concentration rations of ^{22}Na at the end of the experiments were 0.092, 0.045, and 0.022 in sucrose-containing, usual and high sodium media respectively

Effect of calcium and magnesium ions

The outflow of bulk potassium ions produced by 0.5 mM propranolol was reduced when magnesium ion concentration in the medium was raised to 20 mM (Fig. 17). On the other hand, similar increase in the calcium ion con-

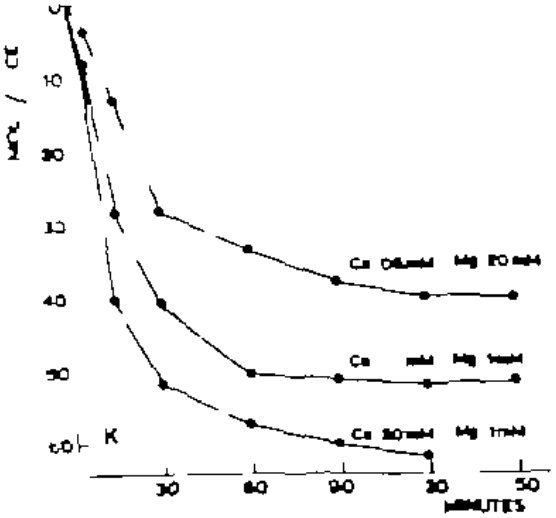


Fig. 17 Flows of bulk potassium ions after adding 0.5 mM propranolol to cell suspensions with different external calcium and magnesium ion concentrations.

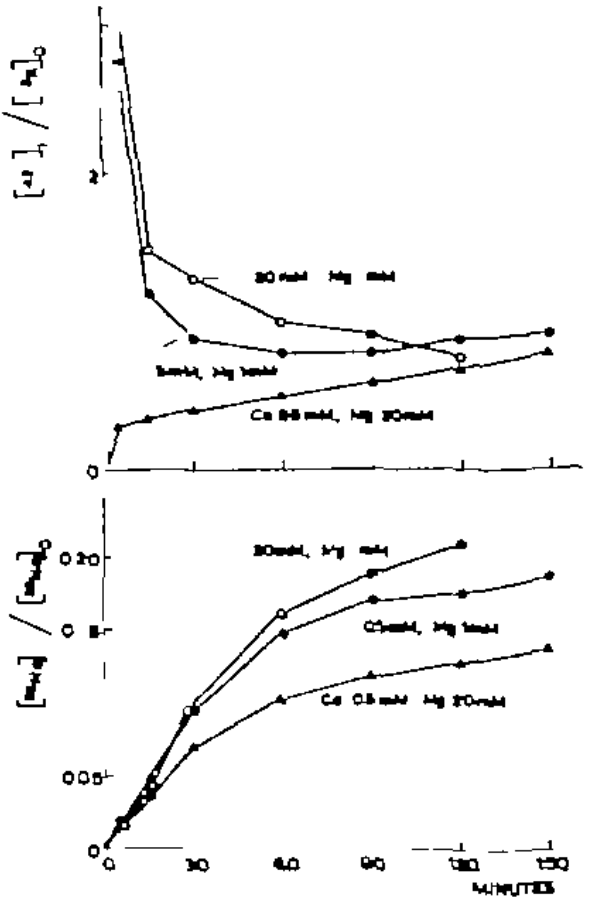


Fig. 18. Cell (I)/medium ( ) concentration ratios of ^{42}K and ^{22}Na tracer ions added simultaneously with 0.5 mM propranolol to cell suspensions with different external calcium and magnesium ion concentrations.

centration enhanced this flow The mean flows of bulk potassium after 2 hr were 52, 40 and 62 mmole/litre cells in the usual, high magnesium and high calcium media respectively The flows of bulk sodium ions were small, and were only slightly if at all, changed by increasing the calcium or magnesium ion concentrations in the medium. The corresponding packed red cell volumes after 2 hr were 0.81 0 86 and 0 75 times the initial ones.

Experiments with the high calcium medium were only continued for 2 hr since after that time considerable amounts of haemoglobin were released.

The increased initial cellular uptake of ^{42}K tracer ions produced 0.5 mM propranolol was greatly reduced when the magnesium ion concentration was raised to 20 mM (Fig 18). In high calcium medium the observed transient accumulation peak of ^{42}K at the beginning of the experiments was somewhat increased. After exposure to propranolol an approximate stationary distribution of ^{42}K between the cells and the medium was reached after 60 min in cells suspended in the usual medium. In the high calcium medium the cell/medium concentration ratio of ^{42}K continued to decrease even at the end of the experiment.

Increasing the medium magnesium concentration slightly decreased the cell/medium concentration ratios of ^{22}Na in propranolol-treated cells (Fig. 18).

However if the smaller shrinkage of these cells is taken into account, approximately the same amounts of ^{22}Na tracer were accumulated as in cells suspended in the usual medium. In high calcium medium the cell/medium concentration ratios of ^{22}Na were higher than those of cells in the usual medium. This elevation may be related to the increased tendency for cellular breakdown allowing a more free permeation of sodium ions. This was also manifested by the outflow of haemoglobin and potassium tracer

In control experiments without propranolol 20 mM magnesium did not influence the behaviour of bulk potassium and sodium ions or ^{42}K and ^{22}Na tracer ions, as compared to cells suspended in the usual medium, while in the high calcium medium there was some outflow of bulk potassium ions, and later of haemoglobin.

When the cells were initially suspended in a Ca, Mg-free medium the outflow of bulk potassium ions produced by 0.5 mM propranolol was only 14 mmole/litre cells at the end of the experiments, and there was a concomitant inflow of an equal amount (13 mmole/litre cells) of bulk sodium ions (Fig. 19) Addition of 1 mM magnesium had little effect on the flows of bulk potassium and sodium ions, but after addition of 1 mM calcium the characteristic rapid great selective outflow of bulk potassium ions (60 mmole/litre cells after 2 hr) became evident. The simultaneous inflow of

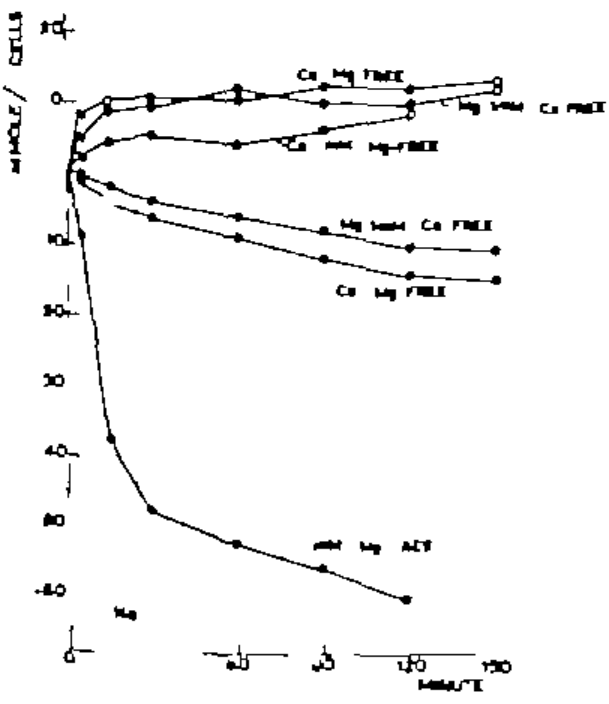


Fig. 18. Flows of bulk potassium and sodium ions in cells suspended in Ca, Mg free and Ca or Mg-containing medium after adding 0.5 mM propranolol.

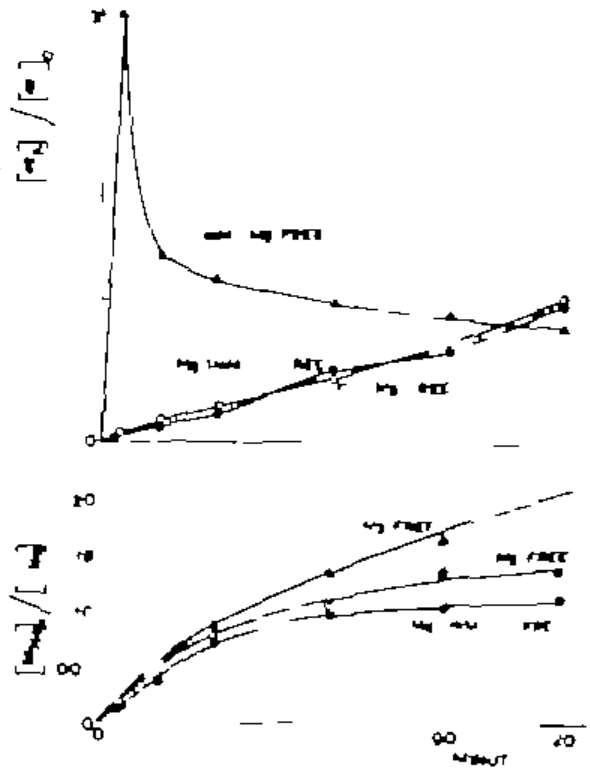


Fig. 20. Cell (i)/medium (o) concentration ratios of ^{42}K and ^{22}Na tracer ions added simultaneously with 0.5 mM propranolol to Ca, Mg free, and Ca or Mg-containing medium.

TABLE V Rate constants of tracer potassium, concentrations and flows of bulk potassium ions 2.5 min after addition of 0.5 mM propranolol to cell suspensions with varying external calcium and magnesium ion concentrations.

	Ca, Mg free	Ca 1 mM No Mg	No Ca Mg 1 mM	Ca 0.5 mM Mg 1 mM	Ca 20 mM Mg 1 mM	Ca 0.5 mM Mg 20 mM
k_{in}, hr^{-1}	0.24	19	0.24	18	20	3.1
k_{out}, hr^{-1}	0.092	2.5	0.050	2.3	2.7	0.68
[K+] mmole/litre cells	96	94	95	94	93	95
$[K^+]_o$ mmole/litre cells	4.9	7.3	4.8	7.3	7.5	6.1
$k_{in}[K^+]_o$ mmole/litre cells/hr	1.2	140	1.2	130	150	19
k_{out} [K+] mmole/litre cells/hr	8.7	240	4.6	220	250	65
Bulk ion flow k_{in} [K+] — k_{out}[K+] mmole/litre cells/hr	—7.5	—100	—3.4	—90	—100	—46

bulk sodium ions was only about 5 mmole/litre cells at 2 hr but later increased when the cells began to lyse. The packed red cell volumes after 2 hr were 1.0 1.0 and 0.74 times the initial volumes in Ca, Mg free, 1 mM Mg or 1 mM Ca-containing media.

The rapid initial uptake of ^{42}K tracer ions produced by 0.5 mM propranolol was abolished in a Ca, Mg-free medium or in Ca free medium with 1 mM magnesium but became evident in the presence of 1 mM calcium (Fig. 20) In this case, as with 20 mM Ca and 1 mM Mg, no stationary state for cell and medium ^{42}K ions was reached at the end of the experiments. These cells were prone to lysis at the time.

Table V shows the potassium tracer rate constants 2.5 min after adding 0.5 mM propranolol to cell suspensions with differing external calcium and magnesium ion concentrations.

The cell/medium concentration ratios of ^{22}Na are shown in the lower part of Fig. 20 In propranolol-treated (0.5 mM) cells the least ^{22}Na was accumulated at first in cells suspended in medium containing 1 mM calcium (Fig. 21) Together with the greatly accelerated exchange of potassium this shows that the initial selectivity between potassium and sodium ions was greatest in Ca-containing medium.

When enough EDTA Na_2 was added to chelate the magnesium and calcium ions in the usual medium, bulk potassium and sodium ions and ^{42}K and ^{22}Na tracer ions behaved, after treatment with 0.5 mM of propranolol, as they did in a Ca, Mg-free medium.

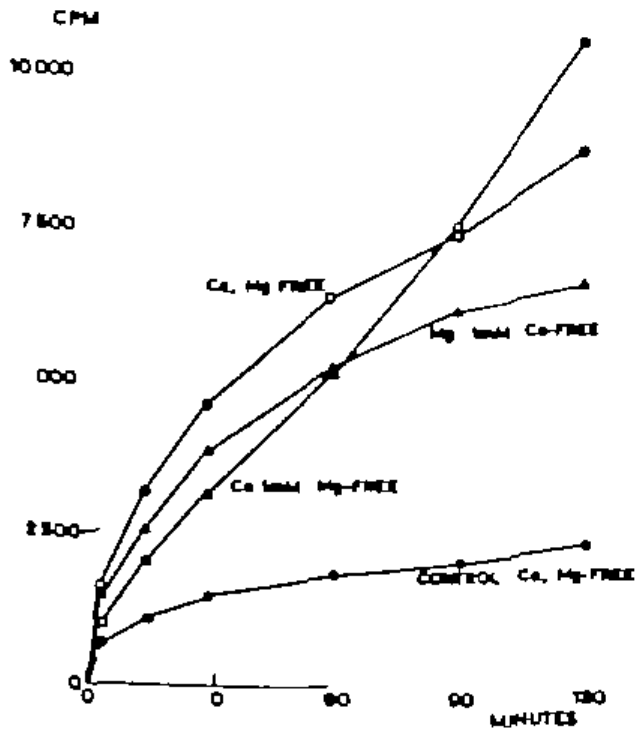


Fig. 21 Cellular ^{22}Na radioactivities (counts per minute per ml of cells) after adding ^{22}Na tracer ions to the medium at 0 min with 0.5 mM propranolol.

In experiments without propranolol omitting the calcium of magnesium ions from the medium (or chelation with EDTA Na_2) did not change the behaviour of bulk potassium and sodium ions or their tracer ions.

Ouabain and propranolol

After 2.5 hr incubation with 0 01 μM or 1 μM ouabain the outflow of bulk potassium ions was 3 0 mmole/litre cells or 3.4 mmole/litre cells. The corresponding inflow of bulk sodium ions was 2.5 mmole/litre cells or 3.7 mmole/litre cells (n = 10) The packed red cell volumes did not change from the initial ones.

Ouabain drastically reduced the rate of increase of the cell/medium concentration ratio of ^{42}K added simultaneously with ouabain to the cell suspension (Fig 22) At the end of the 2.5 hr experiment this ratio was 1 15 in the controls without ouabain, but 0.35 and 0.21 with 0.01 μM and 1 μM ouabain respectively Table VI shows the potassium rate constants and unidirectional fluxes in cells treated with 1 μM ouabain. Both the inward rate constant and unidirectional flux of potassium was markedly reduced by ouabain.

The uptake rate for ^{22}Na was only slightly influenced by ouabain (Fig 22) With 0 01 μM ouabain no differences between controls and ouabain-treated cells could be detected. In cells treated with 1 μM ouabain the cell/medium concentration ratios at the end of the experiments were slightly higher than in the controls.

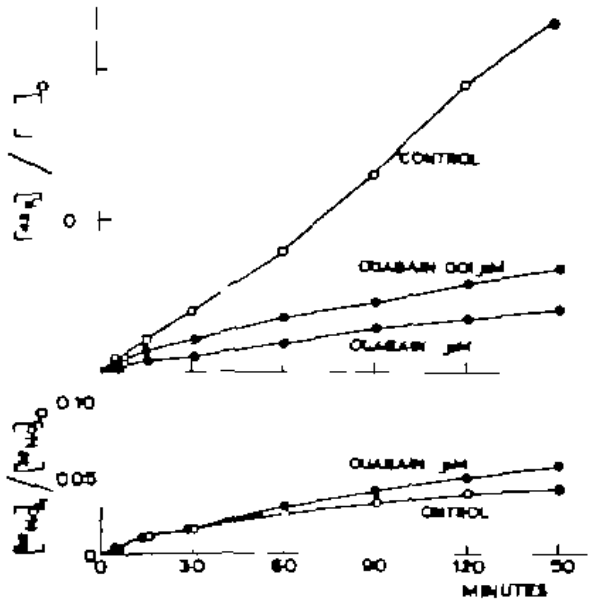


Fig. 22. Cell (i)/medium (o) concentration ratios of ^{42}K and ^{22}Na tracer ions added to the medium at 0 min simultaneously with ouabain.

TABLE VI Rate constants of tracer potassium, concentrations and flows of bulk potassium ions in ouabain-treated and control cells during the whol exper iment, and after 2.5 min in cells treated with propranolol alone or with propranolol and ouabain.

	Control	Ouabain (1 μM)	Propranolol (0.5 mM)	Ouabain+Propranolol (1 μM) (0.5 mM)
k_{in}, hr^{-1}	0.37	0.075	18	18
k_{out}, hr^{-1}	0.019	0.019	2.4	2.5
[K+] mmole/litre cells	95	94	94	94
$[K+]_e$, *mmole/litre cells*	4.8	5.2	7.5	7.5
$k_{in}[K+]_e$ mmole/litre cells/hr	1.8	0.39	135	135
k_{out} $[K+]_i$ mmole/litre cells/hr	1.8	1.8	230	235
Bulk ion flow k_{in} [K+] — k_{out} [K+] mmole/litre cells/hr	0	—1.4	— 95	—100

Adding 0.01 μM or 1 μM ouabain did not change the 0.3 mM or 0.5 mM propranolol-induced flow of bulk potassium ions (Fig 23). That of sodium was somewhat increased, for instance the inflow of bulk sodium after 2.5 hr was 4 mmole/litre cells with 0.5 mM propranolol and 10 mmole/ litre cells with 0.5 mM propranolol and 1 μM ouabain. The above difference, though small, seemed to be valid since it occurred in all six experiments performed. Ouabain did not change the propranolol induced shrinkage of the cells.

Neither did ouabain change the propranolol-induced rapid initial cellular uptake of ^{42}K tracer ions (Fig. 24) Table VI shows that the calculated potas-

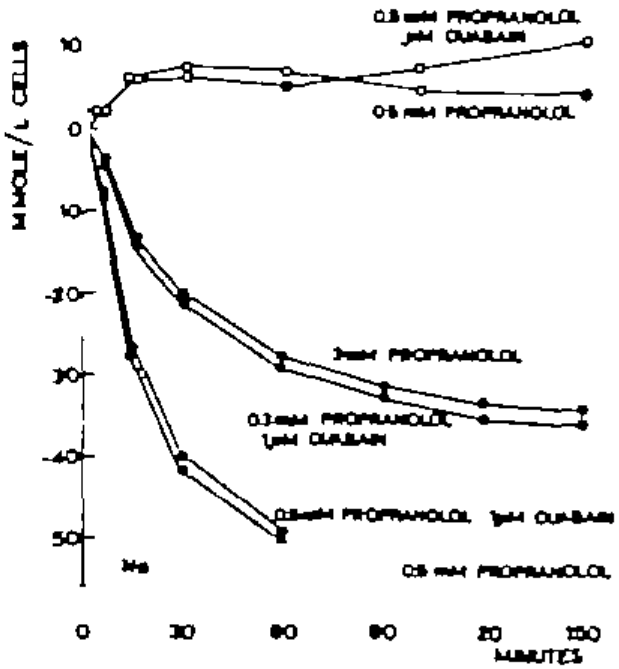


Fig. 23. Flows of bulk potassium and sodium ions in cells treated with propranolol and ouabain.

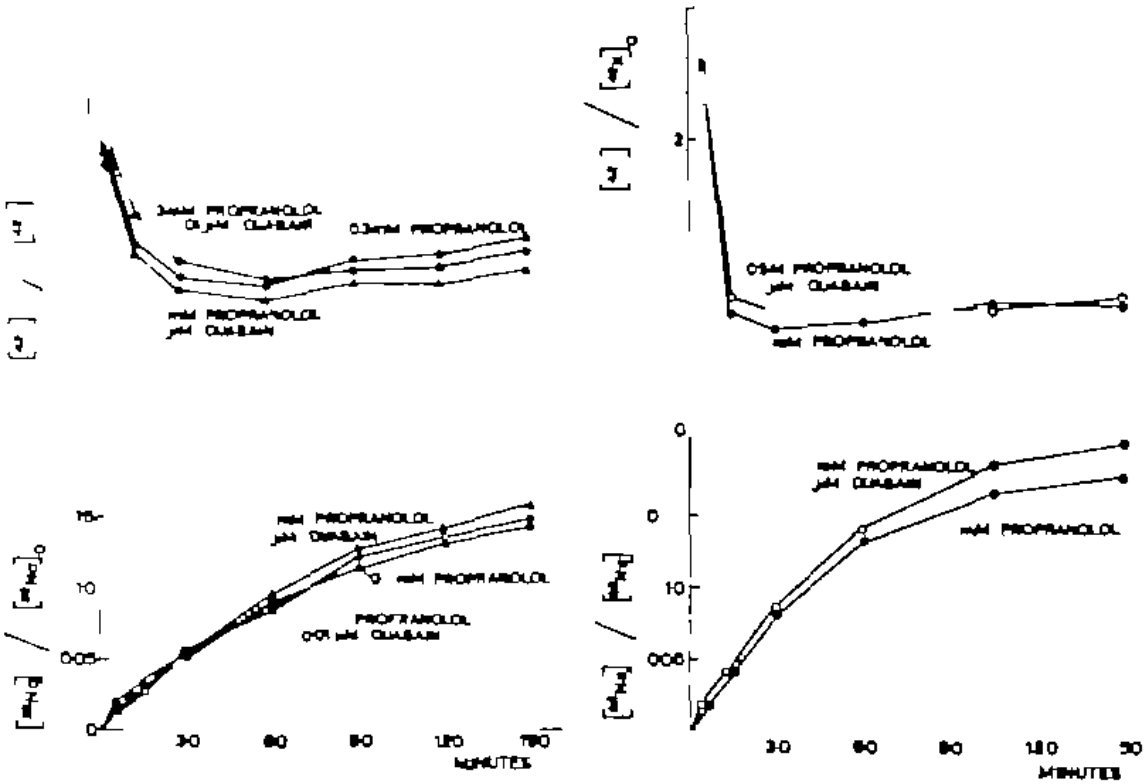


Fig. 24. Cell (i)/medium (o) concentration ratios of ^{42}K and ^{22}Na tracer ions added to the medium at 0 min with propranolol and ouabain.

sium rate constants and unidirectional fluxes were equal in cells treated either with propranolol alone or with propranolol and ouabain.

At the end of the experiments the cell/medium concentration ratios of ^{22}Na tracer ions were slightly higher when the cells were simultaneously trated with 0.5 mM propranolol and 1 μM ouabain than when only 0.5 mM propranolol was used (Fig. 24).

Effect of protamine

The outflow of bulk potassium induced by 0.5 mM propranolol was reduced when the cells were treated with equimolar protamine at the same time (Fig 25) The inflow of bulk sodium was slightly enhanced by protamine, but the difference may be erroneous since the uptake of ^{22}Na was not increased by simultaneous treatment with propranolol and protamine. The packed red cell volumes at the end of the experiment were 0 79 and 0.87 times the initial volumes in cells treated with propranolol alone and with propranolol plus protamine. The rate of the cell shrinkage was also lowered after treat ment with protamine.

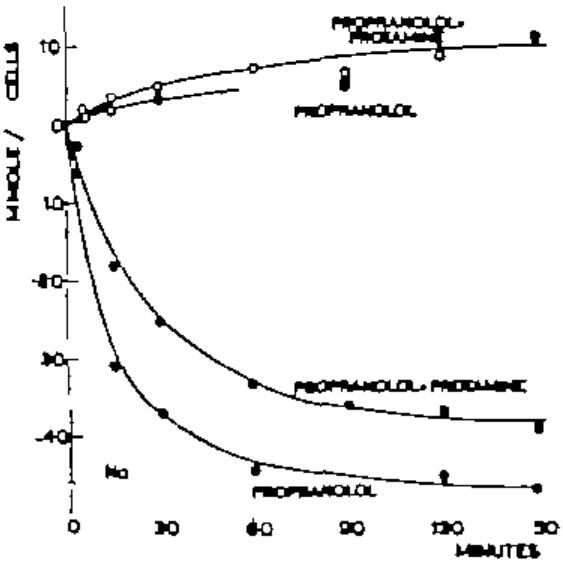


Fig. 25. Flows of bulk potassium and sodium ions after treatment with 0.5 mM propranolol and 0.5 mM protamine.

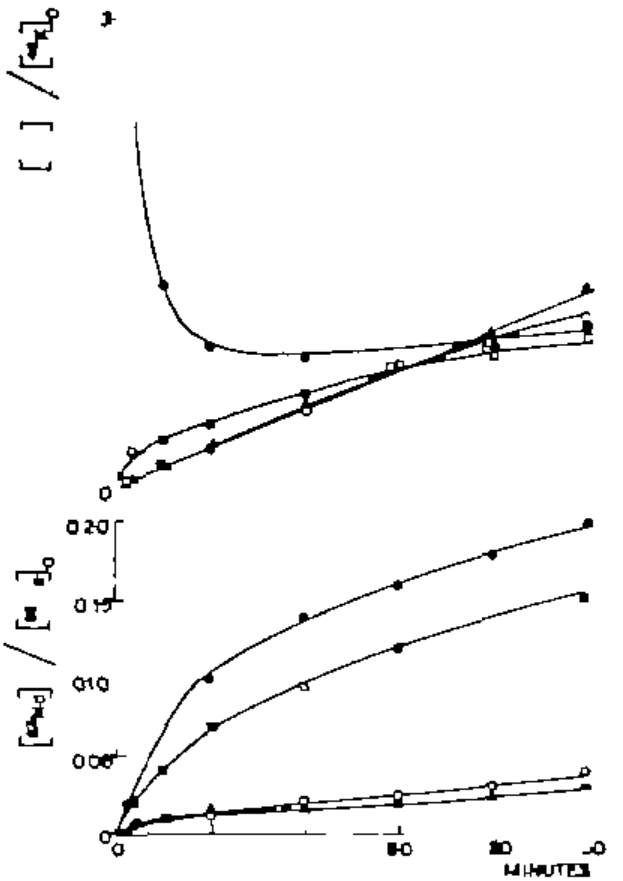


Fig. 26 Cell (i)/medium (o) concentration ratios of ^{42}K and ^{22}Na tracer ions added to the medium at 0 min with propranolol and protamine. ● propranolol 0.5 mM, □ propranolol 0.5 mM and protamine 0.5 mM, ○ protamine 0.5 mM, △ neither drug.

The rapid initial uptake of ^{42}K tracer ions produced by 0.5 mM propranolol was greatly reduced when 0.5 mM protamine was added simultaneously (Fig 26). In propranolol treated cells the inward and outward rate constants after 2.5 min were 17 hr^{-1} and 2.3 hr^{-1} while those in cells treated with propranolol and protamine were 2.1 hr^{-1} and 0.45 hr^{-1} respectively Maximal inhibition of the initial rate of ^{42}K uptake was observed when the molar concentrations of protamine and propranolol were equal, but some retardation of the ^{42}K uptake was still achieved when the protamine concentration was 10 μM.

The cell/medium concentration ratios of the ^{22}Na tracer ions were slightly lowered after protamine administration (Fig 26). However when the smaller shrinkage of the cells treated with propranolol and protamine is taken into account, approximately equal amounts of the ^{22}Na tracer ions were accumulated in both cases.

After adding protamine 15 min beforehand the flows of bulk potassium and sodium ions and of ^{42}K and ^{22}Na tracer ions were similar to those when propranolol and protamine were added simultaneously

Protamine alone in a concentration of 0.5 mM did not change the behaviour of bulk potassium or sodium ions, packed red cell volumes or ^{42}K and ^{22}Na tracer ions during the 2.5 hr experiments as compared to similar controls without protamine.

Reversibility of normal cation permeability after propranolol treatment

The results of a typical experiment (three were performed) show that the rapid uptake of ^{86}Rb added to intact cell suspensions with propranolol, was reduced in cells previously treated with propranolol and then washed 3 times in a tenfold volume of propranolol free saline (Fig. 27 A) This treatment reduced the outflow of bulk potassium during the 2 hr incubation from 48 mmole/litre cells to 19 mmole/litre cells. The cells still contained about 30—35 % of their initial propranolol content as calculated from experiments with ^{14}C-propranolol (Fig. 7).

When propranolol treated cells were washed for long enough to allow all the initially accumulated propranolol to flow out, the ^{86}Rb tracer accumulated at a similar rate in the control cells and the cells previously treated with propranolol (Fig. 27 B) The outflow of bulk potassium was negligible The rate of ^{86}Rb accumulation was the same as that found by Ekman *et al.* (1969)

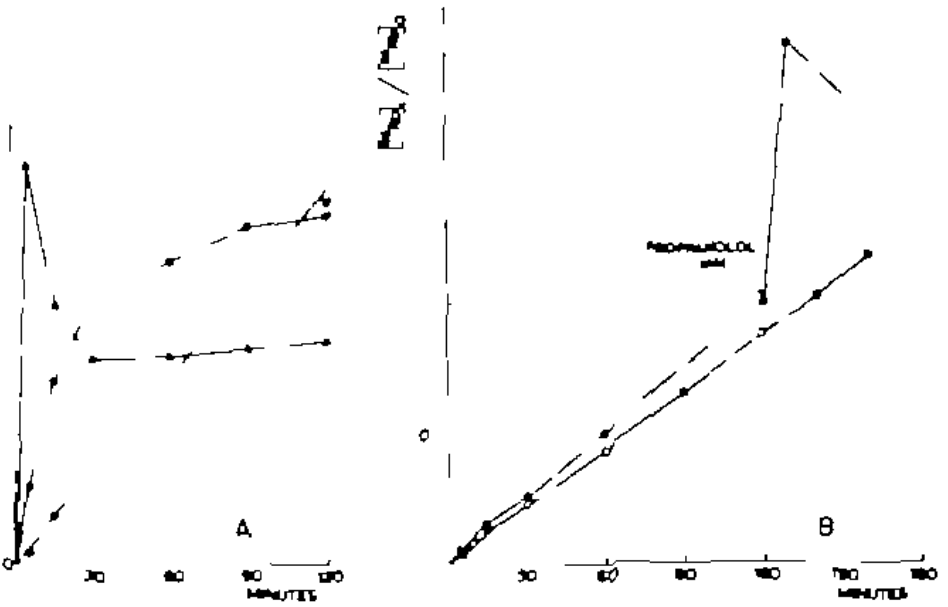


Fig. 27 Reversibility of normal uptake of ^{86}Rb tracer ions in red cells treated with 0.5 mM propranolol, then washed free of propranolol. Cell (i)/medium (o) *concentration ratios f ^{86}Rb added to the medium at 0 min. A)* ▲ *propranolol* and ^{86}Rb added to intact red cell suspension, ○ intact cells without propranolol, ● propranolol-treated cells washed 3 times in tenfold volume of saline. B) ● cells treated with propranolol and washed free of propranolol. After 120 min 0.5 mM propranolol was added, ○ washed as before but not treated with propranolol.

for fresh control cells, which shows that the cells had not suffered during the procedure. Readdition of propranolol after 120 min induced the characteristic rapid uptake of ^{86}Rb tracer ions. Simultaneously bulk potassium ions began to leak out and the packed red cell volumes began to decrease.

Sucrose permeability

After 3 hr the cell to medium concentration ratios of ^{14}C-sucrose, added to the medium, remained as low as 0 014 and 0.022 in cells treated with 0.5 *mM and 1 mM propranolol, while that of the controls was 0 011 (n = 3)* Thus, the low permeability of the red cell membrane to sucrose was not destroyed by propranolol.

Adenosine triphosphate concentration

The ATP concentration of control cells without propranolol was 0 64 ± S E 0.072 mmole/litre cells at the beginning of the experiments and 0.52 ± S. E 0.065 mmole/litre cells after 60 min incubation at 38 C (n = 6) In the first sample after addition of 0.5 mM propranolol (about 5 min) the ATP concentration was 1.06 ± S. E. 0.083 mmole/litre cells. After 60 min incubation it was 0.98 + S. E. 0 071 mmole/litre cells (n = 6)

Adenosine triphosphatase activity

In whole red cells the ATPase activity as inorganic phosphate (Pi) liberated in 60 min was increased by treatment with propranolol (Table VII). In control experiments the mean Pi production rate was 1.04 ± S. E. 0.039 mmole/litre cells/hr (n = 9). With 0.5 mM of propranolol the amount of Pi liberated during the 60 min incubation was 1 61 times that of the controls. Higher concentrations of ouabain, as used in experiments with isolated red cell membrane stroma, were not used with the whole cell suspension since they caused a considerable haemolysis.

With 0.5 mM propranolol plus 10 μM ouabain the Pi production rate was 1.33 times that of the controls, so that in this case the amount of Pi liberated was less than that with propranolol alone. During the 60 min incubation the decrease was 0.28 mmole/litre cells, which was the same as that produced by ouabain without propranolol.

When calcium was withdrawn the mean Pi production rate was 0 98 ± S. E. 0.050 mmole/litre cells/hr (n = 9) without propranolol, with 0.5 mM

TABLE VII. Relati e ATPase activity as inorgani phosphate liberated in 60 min, in propranolol and/or ouabain-treated whole red cells and isolated red cell membrane stroma. Activity in similar controls without drugs taken as unity. Mean values ± S.E. Numbers of determinations in parentheses.

	Propranolol (mM)					Ouabain (mM)		Propranolol + Ouabain (mM) (P) (O)	
	1	0.5	0.1	0.01	0.001	0.1	0.01	0.5(P) + 0.1(O)	0.5(P) + 0.01(O)
Whole cells		1.61					0.68		1.33
± S.E.		0.084					0.035		0.057
		(10)					(10)		(6)
Red cell membrane stroma	0.62	0.75	0.89	0.98	1.04	0.56		0.54	
± S.E.	0.058	0.035	0.037	—	—	0.028		0.030	
	(5)	(8)	(4)	(3)	(3)	(7)		(6)	

propranolol it was 1.20 ± S. E. 0.033 mmole/litre cells/hr and 0.72 ± S. E. 0.039 mmole/litre cells/hr with 10 μM ouabain.

In contrast to the results with whole red cells, higher concentrations of propranolol decreased Pi production in red cell membrane stroma (Table VII). In controls without propranolol the mean Pi production rate during the 60 min incubation was 0.65 ± S. E. 0.027 μmole/mg protein (n = 8). That with 0.5 mM propranolol was 0.49 = S. E. 0.030 μmole/mg protein (n = 8). When 0.1 mM ouabain was added the Pi production rate was 0.56 times that of the controls. With 0.5 mM propranolol plus 0.1 mM ouabain the Pi production rate was 0.54 times the control value, indicating that propranolol could not counteract the decrease caused by ouabain.

In the above experiments conditions were arranged so as to measure the $(Na^+ + K^+)$-activated, Mg^{++}-dependent, ATPase activity.

In additional experiments with 5 mM calcium inhibition of the Pi production rate by 0.1 mM ouabain was low, about 8 %. In 3 series of experiments with propranolol in a concentration range from 1 μM to 1 mM the Pi production rate was not detectably different from that of controls.

Experiments with isolated rat diaphragm

Cation and water contents

The potassium and sodium concentrations were 98.9 ± S. E. 2.8 and 31.6 ± S. E. 0.50 mmole/kg wet muscle in fresh diaphragms (n = 12). It is however difficult to keep isolated diaphragm muscles in this state. The muscle potassium content decreases, and the sodium content increases rather rapidly until a new stationary state is reached after a few minutes. After 15 min soaking the potassium concentration of the control muscles was reduced to 84.6 ± S. E. 1.9 mmole/kg (n = 6). Thereafter their potassium content remained rather constant throughout the 2.5 hr experiments, but with high concentrations of propranolol potassium ions continued to flow out (Fig. 28).

The sodium concentration in the control muscles was 53.4 ± S. E. 3.2 mmole/kg (n = 8) after 15 min soaking. There was a tendency for the sodium ion content to increase further during the course of the experiment, and after 2.5 hr the sodium concentration was 70.5 ± S. E. 2.9 mmole/kg (n = 8). Propranolol increased this process, so that muscle sodium ion concentrations were 88.4 ± S. E. 2.7 mmole/kg (n = 5) and 123.6 ± S. E. 3.6 mmole/kg (n = 5) after 2.5 hr with 0.1 mM and 1 mM propranolol.

The water concentration of the fresh muscle was 765 ± S. E. 4.9 g/kg (n = 7). After soaking for 2.5 hr the water concentration was 780 ± S. E. 4.6 g/kg (n = 8) in control muscles without propranolol and 834 ± S. E. 5.2 g/kg

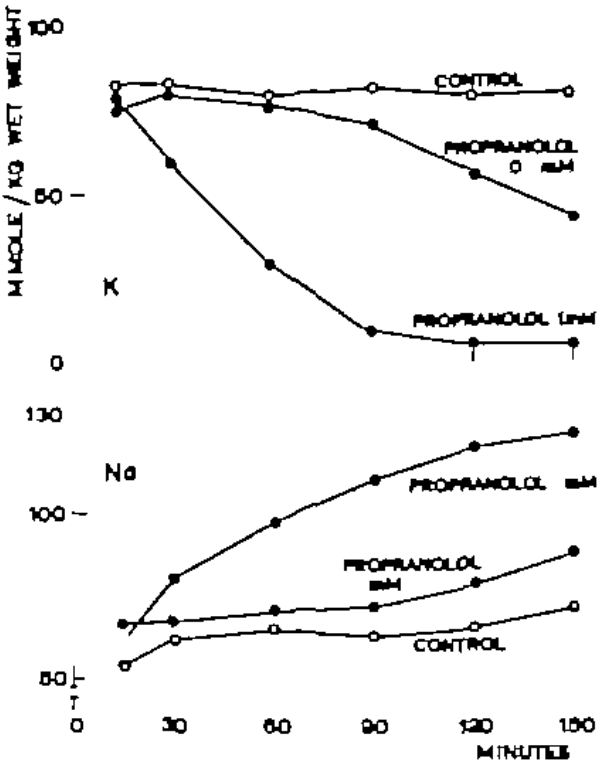


Fig. 28. Potassium and sodium ion concentrations of the rat diaphragm muscle as mmole/kg wet weight in propranolol treated and control muscles during incubation.

(n = 8) in muscles treated with 1 mM propranolol. The increase of in water content of the propranolol treated muscles is significant ($P < 0.001$)

Extracellular space

The extracellular space was determined with ^{14}C-sucrose. Fig 29 shows the uptake of ^{14}C-sucrose when the diaphragm muscles were immersed in enough ^{14}C-sucrose-containing saline to maintain a practically constant composition. The ^{14}C-sucrose that has entered the tissue is expressed as sucrose space, as defined by the ratio ^{14}C-sucrose per 100 g tissue/^{14}C-sucrose per ml of extracellular fluid. When a steady state was reached after 3 hr the

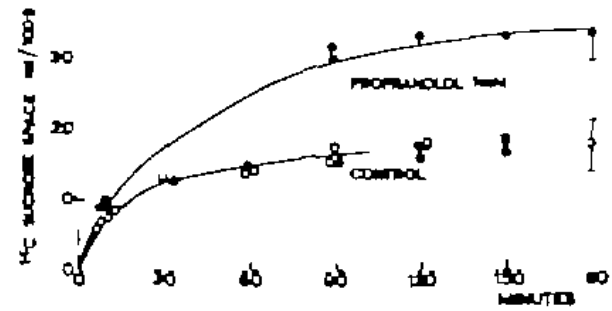


Fig. 29 Sucrose space in the diaphragm muscle. Means ±S D f 6 determinations after 180 min, other marks are individual values of 3 different experiments.

sucrose space, as means ± S E. of 6 determinations, was 18.6 ± 0.41 ml/100 g tissue and 34.8 ± 0.63 ml/100 g tissue with no and 1 mM propranolol respectively

Intracellular cation and water concentrations

The intracellular cation and water concentrations were calculated from the tissue analyses together with the values of the extracellular space (Table VIII). In fresh diaphragm muscle intracellular sodium ions represented 14.9 % of the total muscle sodium content. During the 2.5 hr incubation their amount increased to 62 %. The corresponding increment in the cellular sodium concentration as mmole/kg fibre water was from 8.1 to 73. There was also a slight loss in the intracellular potassium ion concentration.

With 1 mM propranolol the increase in the internal sodium concentration was even more prominent than in the controls. After 2.5 hr the intracellular sodium ion concentration was 147 mmole/kg fibre water in propranolol treated muscles, a value equal to the sodium concentration in the medium. The internal potassium concentration was concomitantly decreased, the ratio internal/external concentration remaining however above unity

Inflow of ^{22}Na and ^{42}K tracer ions

Fig. 30 shows the uptake of tracer ions as muscle/medium concentration ratios against time when the muscle strips were soaked in a large volume

TABLE VIII. Effect of propranolol on cation and water concentrations in diaphragm muscle.

		Initial	2.5 hr soaked	
			Control	Propranolol 1 mM
Internal concentration (mmole/kg fibre water)	N^+	8.1	73	147
	K^+	169	145	12
Ratio internal/ external concentration	N^+	0.06	0.50	1.0
	K^+	35	29	2.4
Internal water concentration (g/kg muscle)		579	594	496
Selectivity coefficient, $[Na^+]_e/[Na^+]_i \times [K^+]_i/[K^+]_e$		306	59	2.4

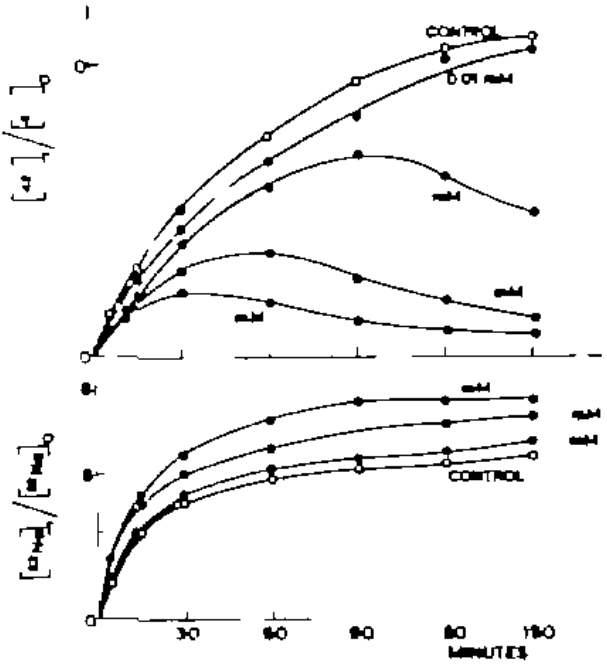


Fig. 30. Cell (i)/medium (o) concentration ratios of ^{42}K and ^{22}Na tracer ions in diaphragm muscles when added at 0 min with propranolol to incubation medium.

of buffered saline containing ^{42}K and ^{22}Na tracer ions. The accumulation of ^{42}K in the control muscles was mono-exponential towards a constant stationary state having an inward rate constant, k_{in}, of 10.2 hr^{-1}. Treatment with high concentrations of propranolol lowered the muscle/medium concentration ratios of ^{42}K. As with red cells, the tracer transiently attained concentrations in the muscles that were higher than those in the final propranolol induced stationary state. With the highest propranolol concentration used (1 mM) the muscle/medium concentration ratio of ^{42}K was 1.1 after 2.5 hr. When the ^{42}K concentrations were calculated per volume of water the cell/medium concentration ratio was 2.2, which is near the 2.4 obtained from the bulk potassium ion concentrations.

Propranolol increased the muscle/medium concentration ratios of ^{22}Na tracer ions (Fig. 30). The rate constant for ^{22}Na inflow was 1.4 hr^{-1} in the control muscles, and 1.8 hr^{-1} and 2.4 hr^{-1} in mucles treated with 0.1 mM and 1 mM propranolol.

Outflow of ^{22}Na, ^{42}K, and ^{86}Rb tracer ions

The diaphragms were loaded with tracers by intraperitoneal injections given 12—36 hr beforehand. Fig. 31 shows the ^{22}Na and ^{86}Rb activities in the diaphragms plotted semi logarithmically against time after soaking in a succession of samples of inactive saline. The rate of outflow of ^{86}Rb from the control muscles was rather constant with a half life of 59 min, the corresponding outflow rate constant, k_{out}, was 0.70 hr^{-1}. After a few minutes

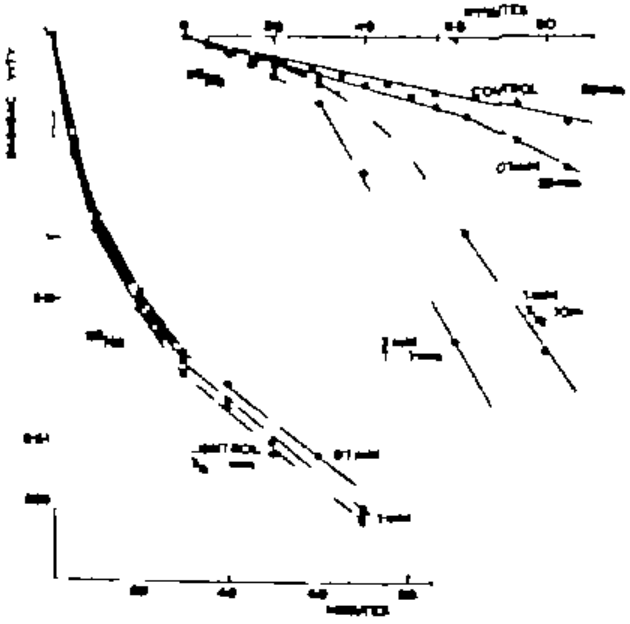


Fig. 31. Effect of propranolol on the outflow of ^{22}Na and ^{86}Rb tracer ions from isolated hemidiaphragms. Fraction of the initial radioactivity remaining in the diaphragms when soaked in a succession of samples of non radioactive saline is plotted semilog. against time.

propranolol increased the rate of ^{86}Rb efflux. When the new constant values were reached, the rate constants were 1.4 hr^{-1} 4.1 hr^{-1} and 5.9 hr^{-1} with 0.1 mM, 1 mM, and 3 mM propranolol respectively With 0 01 mM propranolol the efflux rate was similar to that in controls.

The outflow of ^{42}K in two series of experiments with various concentrations of propranolol from 0.01 mM to 3 mM was qualitatively similar to that of ^{86}Rb. The rate constant for the ^{42}K outflow in control muscles without propranolol was 0 65 hr^{-1} The ^{86}Rb tracer was used in most experiments instead of ^{42}K, because its half life is more suitable for these prolonged experiments.

The outflow of ^{22}Na tracer ions can be divided into two parts, one rapid, the other slow representing the flow from the extracellular and from the cellular space. After 20 min the outflow from the rapidly exchanging portion

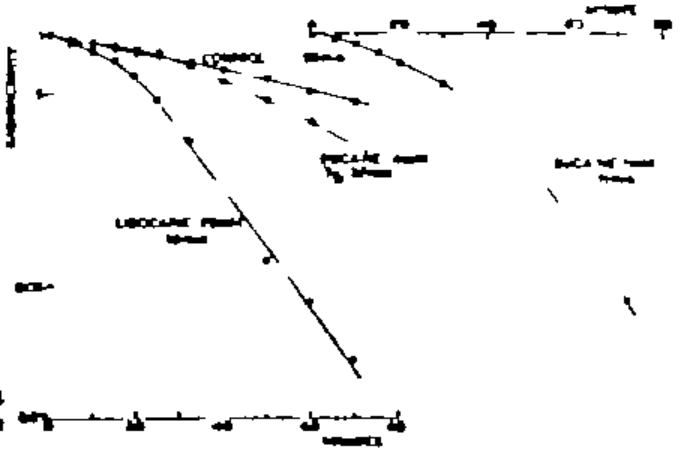


Fig. 32. Outflow of ^{86}Rb tracer ions from diaphragms exposed to lidocaine or dibucaine.

was practically finished. After that the semi logarithmic plot of the remaining radioactivity in the muscles against time gave a straight line. At this time the outflow rate constant for ^{22}Na tracer in the control muscles was 3.2 hr^{-1} No apparent differences could be discerned between the controls and the propranolol-treated muscles. The zero time intercept of the slowly exchanging part of the ^{22}Na indicated that about 10—15 % of the total ^{22}Na content in the muscles was composed of the slowly exchanging, i.e. intracellular ^{22}Na ions. This is the same as that found by the bulk sodium ions.

Effect of lidocaine and dibucaine on ^{86}Rb tracer outflow

Like propranolol, lidocaine and dibucaine increased the rate of ^{86}Rb outflow though higher concentrations were needed (Fig 32) After a few minutes the outflow reached a new constant rate the rate constants were then 1.5 hr^{-1} and 4.1 hr^{-1} with 4 mM and 20 mM lidocaine, and 3 8 hr^{-1} with 1 mM dibucaine.

DISCUSSION

Accumulation and exchange of propranolol

When ^{14}C-propranolol was added to the medium the radioactivity in the cells after 5 min was 7—10 times higher than that in the medium. When the total propranolol concentration was so low that no shifts of bulk alkali ions were produced, the final stationary distribution of propranolol between the cells and the medium was reached at 5 min. With high concentrations of propranolol and in the presence of calcium ions propranolol continued to accumulate in the cells. Approximate stationary distribution was reached after 30 min, the cell/medium concentration ratios of propranolol being 20—30. Potter (1967) previously showed that cells accumulate propranolol. He found 30 times higher propranolol concentrations in the cells of isolated rabbit atria than in the medium.

The great accumulation of propranolol was accompanied by outflow of bulk potassium ions, and increased exchange as judged by experiments with radioactive tracer ions. Experiments in which the inflow and outflow of ^{14}C propranolol was studied with total concentrations of propranolol that brought the system into a non-stationary state showed that propranolol itself, as well as alkali ions, was exchanging at bi-directionally accelerated rates across the membrane. During the non-stationary state the inflow of propranolol, suprisingly increased more than in linear proportion to the increase in the propranolol concentration.

Addition of protamine or lowering of the pH decreased both the uptake of propranolol and the accelerated cation exchange. The free base form of local anaesthetics can diffuse freely across lipid barriers normally impermeable to charged molecules (Ritchie and Greengard 1966). Lowering the pH decreases the amount of the uncharged form of propranolol, which could be one reason for the lowered uptake of propranolol at low pH. However reduction in the amount of uncharged propranolol due to a pH drop from 7.4 to 7.0 can hardly be the sole explanation since even at pH 7.4 only a small fraction of propranolol, having pKa 9.45 is in the uncharged form. Changes in charged groups of the membrane seem to be important factors in these events.

Binding of propranolol by membrane stroma was less than the uptake into whole cells, the stroma/medium concentration ratios were about 2. In contrast to the results with whole cells, increasing the propranolol concentration lowered the stroma/medium concentration ratio, indicating an ordinary saturable process. Binding to the membrane was not influenced by ATP or calcium ions.

A simple possibility for the high uptake of propranolol by whole cells is that propranolol might be bound to haemoglobin. The lowered uptake of propranolol at pH 7.0 in whole cells could be due to the fact that the negative charge of haemoglobin is near zero at pH 7.0 (Harris 1960). Uptake of lidocaine and procaine in frog nerves is also lower at pH 7.2 than at pH 8.2 (Strobel and Bianchi 1970). The phenomenon is, however further complicated by the finding that calcium ions did not increase propranolol uptake by isolated membranes while they did increase uptake by whole cells.

Effect of calcium, magnesium potassium, and sodium bulk ions on propranolol induced cation shifts

In the absence of calcium ions the propranolol induced outflow of potassium ions was low and was accompanied by a similar inflow of sodium ions. The rate of exchange of potassium ions, determined with tracers, was not greatly increased without calcium ions. Magnesium ions alone had no influence, but when added with calcium they opposed the effects of calcium. In this respect propranolol differs from fluoride, which can accelerate potassium movements in the presence of either calcium or magnesium ions (Gardos 1959, Lepke and Passow 1960).

The role of calcium and magnesium ions in propranolol induced accelerated exchange of cations is difficult to elucidate. Without calcium ions in the incubation medium the epithelial cells of toad bladder became separated and wide aqueous channels are opened. The bladder becomes permeable to inulin (Hays, Singer and Malamed 1965). It has been shown that calcium ions decrease the movements of water and sodium in epithelial cells (Curran, Herrera and Flanigan 1963), and also decrease the permeability of the red cell membrane to cations (Maizels 1959, Bolingbroke and Maizels 1959, Solomon 1960). If red cells were rendered very permeable to alkali ions by treatment with lactose, calcium ions could restore the low permeability. Extracellular calcium ions could thus bind cells to one another and bind the membrane components of individual cells together more tightly in other words narrow the membrane pores, leading to decreased membrane permeability.

On the other hand Hoffman (1962) found that calcium ions produce a considerable increase in the permeability of red blood cell ghosts to potassium ions, while magnesium ions stabilized the membrane and were needed for

the retention of potassium in the ghosts. Similarly in the present work, cells treated with calcium and propranolol released haemoglobin as well as increased amounts of potassium ions. Magnesium ions decreased lysis of cells. The increased cellular fragility in the presence of calcium ions might have some relevance to the finding of Ponder (1953) that cells that have gained calcium ions in exchange for potassium ions are easily broken by mechanical disturbance. This suggests that intracellular calcium ions favour cellular breakdown.

Various cations may compete for the same sites in the membranes. The amount of each cation retained should then depend on the relative affinity of the membrane sites for the particular cation. Thus the access of a given cation to the membrane could be restricted by other cations (Mazia 1940 Gent, Trounce and Walser 1964, Sanui and Pace 1963 and 1965 Carvalho 1968 Magnuson, Shelton and Magnuson 1970). This is probably the reason for the lowered exchange of potassium ions in the high sodium medium Direct competition between sodium and calcium ions has also been suggested (Lüttgau and Niedergerke 1958, Gent et al. 1964, Whittam 1964) Similarly magnesium ions, when added in large amounts, are known to occupy most of the membrane binding sites for cations (Ginzburg, Friedlander and Pouchovsky 1967), which could explain inhibition in the propranolol induced accelerated uptake of potassium tracer ions in the high magnesium medium. Interestingly increasing the magnesium concentration did not alter the exchange of sodium ions.

In a medium of low ionic strength, in which sodium is replaced by the non-penetrating non-electrolyte sucrose, human red cells lose potassium (Parsow 1964, Donlon 1968) In the present experiments, without propranolol but with 115 mM sodium chloride and 0.35 M sucrose, there was a marked outflow of cellular potassium matched by an equimolar entry of sodium. Thus sucrose treatment renders the cell membrane permeable to both potassium and sodium ions. This probably contributes to the decrease in the selective acceleration of potassium exchange by propranolol, the high potassium — potassium exchange being partly changed to potassium — sodium exchange.

Different optical isomers of propranolol

Dextro (+)-propranolol induced a greater outflow and exchange of potassium ions than the laevo (—)-form. The inward rate constant for potassium tracer 2.5 min after addition of propranolol was increased 50-fold by (+)-propranolol and 10-fold by (—)-propranolol. The corresponding outward rate constants were increased 200 and 40-fold. At this time the inward unidirectional fluxes of potassium were 150 mmole/litre cells/hr with (+)-propranolol

and 25 mmole/litre cells/hr with (—)-propranolol. The corresponding outward unidirectional fluxes were 300 mmole/litre cells/hr and 80 mmole/litre cells/hr.

The potassium tracer rate constants were not increased by (—)-propranolol to the same extent as by (+)-propranolol, even when the (—)-propranolol concentration was increased to give a similar outflow of bulk potassium to that with (+)-propranolol.

The inflow of sodium ions was almost equal with (+)- and with (—)-propranolol. Together with the above results this shows that the selectivity between sodium and potassium ions was lower in cells treated with (—)-propranolol than with (+)-propranolol.

The various optical forms of propranolol differ in many other properties. As an adrenergic beta-blocking agent (+)-propranolol is 100 times weaker than (—)-propranolol (Vaughan Williams 1970). Dextro-propranolol, however possesses the non-specific actions of propranolol, for instance local anaesthetic activity, quinidine-like actions on cardiac muscle action potentials, and has the anti-digitalis properties (Howe and Shanks 1966; Dohadwalla et al. 1969; Vaughan Williams 1970). In these cases the doses of (+)- and (—)-propranolol are near one another but the doses are 100 times higher than the doses of (—)-propranolol needed to induce adrenergic beta-blockade. One distinct difference between the optical isomers of propranolol is that in skeletal muscle sarcoplasmic reticulum the total ATPase activity is increased by (+)-propranolol and decreased by (—)-propranolol. In cardiac muscle sarcoplasmic reticulum both isomers of propranolol decrease ATPase activity (Scales and McIntosh 1968).

Propranolol and ouabain

Both cardiac glycosides and propranolol decreased the non-equilibrium distribution of sodium and potassium ions between the cell and the medium. The mechanism of this decrement seems to be different in the two cases. Thurber and Thompson (1967) showed that logarithm of $[Na]_o/[Na] \times [K]_i/[K]_o$, which shows the deviation of the distribution of sodium and potassium ions from the equilibrium is directly related to the flow of lactic acid produced by metabolic reactions. The rate of lactic acid flow per unit of effective membrane area may considered to be the decisive factor in this non-equilibrium. However only the rate of lactic acid flow per volume of cells is known. Decrement of the non-equilibrium may be due to either decreased production of lactic acid or increased effective membrane area. Propranolol did not decrease the rate of lactic acid production when expressed per unit volume of cells (Ekman et al. 1969). The greatly accelerated rate constants of cationic tracers suggest that propranolol would have increased the effective area of the membrane.

In contrast, cardiac glycosides decrease the rate of cation exchange (Glynn 1957). The area accessible for cardiac glycosides in red cell membranes is extremely low about 100—200 molecules are bound per cell (Hoffman and Ingram 1968, Baker and Willis 1969 Ellory and Keynes 1969) Thus it is tempting to suggest that the decreased exchange and non-equilibrium distribution of sodium and potassium ions in red cells treated by cardiac glycosides are related to decreased flow of lactic acid and not to decreased effective area of the membrane. The rate of lactic acid production, as expressed per unit volume of cells, is indeed found to be decreased by 15—50 /s by ouabain (Murphy 1963 Minakami et al. 1964, Whittam and Ager 1965)

The mechanism by which propranolol suppresses cardiac arrhythmias induced by cardiac glycosides is not wholy elucidated. Since depressed sympathetic drive after surgical intervention or after catecholamine depletion by reserpine pretreatment (Méndez, Aceves and Méndez 1961 a and b, Roberts et al. 1963) was found to reduce cardiac glycoside-induced ventricular tachycardias, the protection against these arrhythmias offered by beta-adrenergic blocking drugs was first ascribed to the decreased sympethetic drive on the heart (Stock and Dale 1963 Erlij and Méndez 1964) It was, however found that also (+)-propranolol, a weak adrenergic beta-blocking drug, readily antagonized cardiac arrhythmias caused by toxic doses of ouabain (Howe and Shanks 1966) On the other hand, INPEA (N isopropyl p-nitrophenyl-ethanolamine) and MJ 1999 (isopropylamino-hydroxyethyl-methanesulphonanilide) did not provide protection against cardiac glycoside-induced arrhythmias, even though both were effective against adrenaline-induced arrhythmias (Somani and Lum 1965 1966, Somani and Watson 1968) It was concluded that beta receptor inhibition by propranolol, or other beta-adrenergic blocking drugs, was not the mechanism through which they prevented cardiac arrhythmias induced by cardiac glycosides.

Addition of ouabain together with propranolol did not prevent the propranolol-induced accelerated cation exchange. This, combined with the opposite effects of propranol and ouabain on the rates of cation exchanges, favours an idea that the propranolol reduction of the digitalis-induced cardiac arrhythmias might somehow be coupled to the different movements of cations induced by these agents. It is known that decreased potassium permeability in cardiac muscle contributes to the changes in electrical behaviour and thus also to the arrhythmias, caused by cardiac glycosides (Lyon and DeGraff 1966 Weatherall 1966). There is a close relationship between the decreased potassium permeability of red cells and the lethal doses of various cardiac glycosides (Solomon et al. 1956, Machová 1960) In addition to propranolol, pronethalol (Ekman et al. 1969), butydrine and alprenolol (Manninen 1969) all of which are adrenergic beta receptor-blocking drugs possessing anti-digitalis properties

(Botti, Visioli and Ambanelli 1966 Turner 1966 Duce, Garberg and Johansson 1967), could increase the rate of cation exchange in red cells. However INPEA and MJ 1999 which do not posses the anti-digitalis properties (Somani and Lum 1965 1966 Somani and Watson 1968) did not accelerate the rate of cation exchange in red cells (Ekman *et al.* 1969 Manninen 1969)

ATP and ATPase activity

Since many theories concerning ion transport in red cells involve splitting ATP to ADP and inorganic phosphate, ATP content and ATPase activities seemed worth studying

In whole red cells the ATP concentration was almost immediately doubled by propranolol. In isolated rat liver mitochondria the ATP content is also increased by propranolol (Wickström 1970) ATP turnover and probably also net synthesis of ATP are increased in red cells in which alkali ions are flowing down their concentration gradients (Garrahan and Glynn 1967 Glynn 1968, Lant and Whittam 1968 Glynn and Lew 1970). Thus is is tempting to associate the observed increase in ATP concentration by propranolol with the outflow of bulk potassium salts along their concentration gradient. Chlorpromazine, which also leads to a leak of cations, increases the ATP concentration of rabbit red cells (Ahtee 1966) However lead salts, which have similar effect upon the outflow and exchange of potassium in red cells to propranolol, decrease the ATP content of red cells due to ATP outflow (Lindemann and Passow 1960).

Simultaneously with the ATP increase, propranolol increased the ATPase activity of whole red cells by a factor of 1 6 ATPase activity can also be increased by freezing the cells (Scharff and Vestergaard Bogind 1966) which is also supposed to lead to an altered state of the red cell membrane. Propranolol could not inhibit the ouabain induced diminution in the rate of the ATP breakdown, which suggests that propranolol and ouabain affect red cell ATPase activity independently

The opposite results, that is, propranolol decreased ATPase activity were obtained with isolated red cell membrane stroma, through which no net salt flow occurred. This decrease was similar to that caused by ouabain. The experimental conditions were arranged so as to measure $(Na^+ + K^+)$-activated, Mg^{++}-dependent, ATPase activity which is inhibited by ouabain, and is commonly ascribed to the cation pump ATPase (Post *et al.* 1960 Dunham and Glynn 1961 Jarnefelt 1962, Post 1968). Similar inhibition by propranolol and ouabain, regardless of their opposite effects on the cation exchange rates in whole cells, deserves consideration in regard to suggestions that this ATPase is related to the cation pump.

In isolated red cell membrane stroma there were no gradients of potassium and sodium ions across the membrane, and then increased cation shifts down

ther gradients would not stimulate the adenosine nucleotide metabolism. This might be one reason for the different behaviour of whole cells and the membrane stroma.

Usually higher concentrations of ouabain are needed to decrease ATPase activity than to decrease the uphill fluxes of sodium and potassium ions. In experiments with isolated red cell membrane stroma the doses of ouabain used to suppress ATPase activity are usually 0.1 mM. Similar concentrations were used, for instance, by Whittam (1958, 1964) with whole red cells. In the present experiments this concentration of ouabain induced a considerable outflow of haemoglobin, showing that cell constituents other than the cation pump were affected. The packed red cell volumes, however remained unchanged after ouabain treatment. The lysis was of the non-colloidosmotic type, which may be accompanied by the appearance of rather large holes or pores in the membrane (Jandl 1965).

Low concentrations of ouabain, that are still enough to decrease the rate of cation exchange, have sometimes been found to increase the ATPase activity (Repke 1963, Repke 1965, Palmer Lasseter and Melvin 1966).

ATPase activity is decreased by N-ethylmaleimide (Skou 1965 b, lead salts (Hasan, Vihko and Hernberg 1967 Hernberg 1967), and oxidized glutathione (Dick, Dick and Tosteson 1969) All these agents are known to increase the rate of cation movements in red cells (Passow 1964).

The present results are somehow confusing since propranolol and ouabain both decreased similarly the cation pump ($Na^+ + K^+$)-ATPase activity in isolated red cell membrane stroma, but had opposite effects on cation exchange rate in whole red cells. It is tempting to conclude, at least, that the effect of propranolol on this ATPase activity cannot explain the accelerated exchange of cations in whole red cells after exposure to propranolol.

Isolated rat diaphragm

The present results with isolated rat diaphragms show that propranolol can cause increased cation leakage in cells other than red cells. In the diaphragms the propranolol selectivity for potassium ions as against sodium ions was not so great as in red cells. This may be because, even in control muscles without propranolol, the potassium and sodium ions did not deviate from the non-equilibrium state as much as in the red cells.

Recently van Zwieten (1969) has observed that propranolol may decrease the rate of efflux of ^{86}Rb ions from electrically driven guinea-pig atrial muscle slices. This is quite opposite to the present findings with diaphragm muscle slices. In rabbit atrial muscle fibres the only effect of propranolol in electrical potential measurements is to decrease the rate of the rise of the action potential (Vaughan Williams 1966) According to the customary

principles of electrical potential analysis (Hodgkin 1958, Noble 1966) this means that the sodium conductivity is decreased, and suggests that the potassium permeability is not altered. In the present work with diaphragm muscle propranolol did not change the rate of ^{22}Na tracer outflow but the rate of ^{22}Na tracer uptake and hence the stationary distribution ratio of sodium between the cells and the medium was increased.

Two local anaesthetics, lidocaine and dibucaine, increased the outflow rate of ^{86}Rb tracer ions from the diaphragms as did propranolol. It is tempting to believe that the local anaesthetic property of propranolol is based on the same mechanism as the increased leakage of cations.

Possible effect of propranolol on the membrane structure

The dried red cell membrane is composed of 55 / protein, 35 /o lipids, and 10 % carbohydrate. Various models for the organisation of the membrane components have been put forward (Gorter and Grendel 19..5 Danielli and Davson 1935 Lenard and Singer 1966, Robertson 1967 Zahler 1969) Knowledge of the intimate membrane architecture, especially with respect to the interrelations between various chemical components and their functions, still remains vague. Therefore attempts to explain alterations in cell function in terms of changes in membrane conformation should be regarded as over simplifications.

Since the time-honoured work of Overton and Collander and their co-workers (reviewed by Wartiovaara and Collander 1960) there is reason to believe that membrane lipids participate in transport across cell membranes. The total lipid content of human red cells is about 5 mg/ml cells, made up of cholesterol, phospholipids, and neutral lipids in molar ratios of 1 0.8 0 1 (for review see e.g van Deenen and De Gier 1964, Panow 1969).

Some results suggest that membrane lipids could participate in propranolol induced accelerated exchange of cations. Propranolol has a high chloroform/ water partition ratio (Almirante *et al.* 1966) It also has a high affinity for cell constituents, as revealed by the high uptake of propranolol, not only by whole red cells, but also by isolated red cell membrane stroma. Propranolol inhibits the swelling of mitochondria (i.e. lysis) induced by phospholipase A (Saris and Seppälä 1970) Propranolol also inhibits the lipid facilitated transport of calcium ions from the aqueous phase to chloroform (Nayler 1966)

Recently artificial bimolecular lipid leaflets have proved to be useful models to study the membrane transport phenomena, although their properties differ in some respect from the cell membranes from which the lipids used in their formation were extracted. For example the electrical resistance and the cation and anion selectivities are altered from the original cell membranes

(Passow 1969). In natural membranes interactions between lipids and proteins also contribute to the permeability properties (Wallach and Gordon 1968)

Valinomycin and certain other natural and synthetic polypeptides and polyethers, that increase the potassium permeability of red cells, also selectively decrease the resistance of artificial bimolecular lipid leaflets to potassium and rubidium ions more than to sodium ions (Andreoli *et al.* 1967 Lev and Buzhinsky 1967 Mueller and Rudin 1967 Lardy 1968 Tosteson 1968 Henderson *et al.* 1969) In bimolecular leaflets made of calf brain lipids propranolol does not decrease the membrane resistance for cations, but the valinomycin induced potassium over sodium selectivity is decreased by propranolol (Lev and Grinfeldt, unpublished results). In the case of erythrocyte lipid leaflets they found that concentration asymmetry of propranolol leads to a potential difference positive potential arising on the side of higher concentration of propranolol.

The effect of valinomycin on red cells and thin lipid membranes is explained by the formation of pores of low resistance (Chappell and Crofts 1965), or the formation of selective ring channels in which the valinomycin molecule is the main part of the ring through which the cations would pass selectively (Tosteson *et al.* 1967) Valinomycin might also act as a mobile carrier (Eisenman 1968, Haynes, Kowalsky and Pressman 1969) The first suggestion is general enough to include many kinds of mechanisms. It does not seem likely that such small and structurally simple chemicals as propranolol, fluoride, or lead ion alone could produce increased selective cation leakage by the two other suggested means.

Some detergents dissolve the membrane lipids making the red cell membrane leaky to one or several species of ions (Ponder 1948, Passow 1964, Jacob 1967 1968) In hereditary spherocytosis cellular cholesterol is more loosely bound than in normal cells. This may be related to the increased permeability of spherocytes to sodium ions (Jacob 1967 1968 Cooper and Jandl 1968). Propranolol does not, however elute cholesterol or phospholipids from isolated red cell membrane stroma (Manninen, unpublished results) Further if propranolol eluted some essential membrane component, the damage would be irreversible. However normal behaviour towards potassium bulk ions and rubidium tracer ions could be restored to red cells that were previously treated with propranolol. Readdition of propranolol again initiated accelerated cation exchange. These results show that the presence of propranolol in the membrane is needed to lower the resistance of the red cell membrane to cation passage.

Decreasing the negative charge of the cell by adding protamine, a strong polycation, or lowering the pH from 7.5 to 7.0 reduced the propranolol-induced uptake of ^{14}C-propranolol and ^{42}K tracer ions. The effects differed in that reducing pH also decreased the inflow of sodium ions. The out

SUMMARY

Propranolol was found to induce a massive outflow of bulk potassium ions from human red cells. Tracer potassium was rapidly taken up into the cells against this flow The potassium inward and outward rate constants just after addition of dextro (+)-propranolol were as high as 20 hr^{-1} and 3.2 hr^{-1} The corresponding rate constants for intact control cells were 0.37 hr^{-1} and 0 019 hr^{-1} The potassium shift produced by laevo (—)-propranolol was far less than that with the (+)-isomer

The concomitant net uptake of bulk sodium ions was low The red cell volumes were decreased.

Propranolol in all instances was readily taken up into the red cells. Exposing cells to concentrations of propranolol that induced an outflow of potassium, still increased the uptake of propranolol more than in a linear proportion to the increase in propranolol concentration. Uptake of propranolol by isolated red cell membrane stroma was less than that by the whole red cells.

Calcium ions were needed for the accelerated potassium potassium exchange and for the increased uptake of propranolol. Magnesium ions alone had no influence, but opposed the effect of calcium. In a high sodium medium potassium exchange was reduced.

Cation shifts and the uptake of propranolol were reduced by addition of protamine or lowering the pH from 7.5 to 7.0

Ouabain did not abolish propranolol induced accelerated exchange of potassium ions.

Normal cation permeability could be restored after propranolol treatment by washing out propranolol. Readdition of propranolol again induced accel erated exchange of cations.

Sucrose permeability was not increased by propranolol.

ATP concentration and ATPase activity in whole red cells were increased by propranolol, while in isolated red cell membrane stroma ($Na^+ + K^+$)-ATPase activity was decreased. Ouabain decreased ($Na^+ + K^+$)-ATPase activity in red cell membrane stroma as did propranolol.

Propranolol induced cation shifts, similar to those in red cells, also occurred in isolated rat diaphragm muscle.

It is suggested that an accelerated carrier-mediated exchange of cations across the membrane, driven by the flow of bulk ions down their gradients, would explain the observed bi-directionally accelerated movements of tracer ions. Based on models systems, described in the literature, it is supposed that circulation of water across the membrane would act as the carrier A non-homogeneous membrane is a prerequisite for such a theory

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ACTA PHYSIOLOGICA SCANDINAVICA
SUPPLEMENTUM 356

Pharmacological Properties of Dehydroascorbic Acid and Ascorbic Acid

Effects on the Central and Peripheral Nervous Systems in Experimental Animals.

BY

SVEN ERIK SJÖSTRAND

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From the Department of Pharmacology Royal Veterinary College
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Contents

Introduction 3

CHAPTER I

Pharmacological effects of dehydroascorbic acid and ascorbic acid in conscious and anaesthetized animals 9

Part 1 General materials and methods 9

Preparation of substances 9

Chromatographic investigations 10

Animals and diet 11

Anaesthesia 11

Part 2. Effects of dehydroascorbic acid and ascorbic acid on motor activity salivation and lacrimation in conscious and anaesthetized animals 12

Materials and methods 12

Results 12

Discussion 13

Part 3 Effects of dehydroascorbic acid and ascorbic acid on the nictitating membrane in cats 14

Materials and methods 14

Results 15

Discussion 18

Part 4 Effects of dehydroascorbic acid and ascorbic acid on neuromuscular transmission 19

Materials and methods 19

Results 20

Discussion 20

Part 5. Effects of dehydroascorbic acid and ascorbic acid on the blood pressure in cats and rabbits 22

Materials and methods 22

Results 23

Discussion 28

Part 6. Effects of dehydroascorbic acid following decerebration, spinal cord section and bilateral adrenalectomy 30

Materials and methods 30

Results 41

Discussion 53

Chapter II
Effects of various compounds on the pharmacological response to dehydroascorbic acid of the blood pressure in cats and rabbits 35
Materials and methods 35
Results 37
Discussion 44
Chapter III
Effects of various compounds on the accumulation of ^{14}C-dehydroascorbic acid and ^{1} C-ascorbic acid in brain and heart tissues of mice *in vivo* 47
Materials and methods 47
Results 50
Discussion 51
Chapter IV
Effects of dehydroascorbic acid and ascorbic acid on levels of endogenous noradrenaline and dopamine in the brain and of noradrenaline in the heart of mice 53
Materials and methods 53
Results 54
Discussion 57
Chapter V
Effects of dehydroascorbic acid and ascorbic acid on cholinesterases *in vitro* 58
Materials and methods 58
Results and comments 59
General Discussion 61
Summary 70
Acknowledgements 73
References 74

Introduction

Vitamin C was apparently the first individual nutrient to be widely associated with disease. Primitive people in many parts of the world knew of the necessity for fresh food, particularly from plant sources to prevent and cure the disease now recognized as scurvy Among all the animals studied so far only man and other primates, guinea-pigs and a few other species are dependent on food sources for the vitamin (Roy and Guha 1958 a, b)

In 1928 Szent-Györgyi isolated a substance with the chemical formula $C_6H_8O_6$ from the adrenal cortex of the guinea-pig. This substance was called hexuronic acid and was found to cure scurvy Later it was given the name of ascorbic acid[1] (Szent-Gyorgyi and Haworth 1933) Since its identification

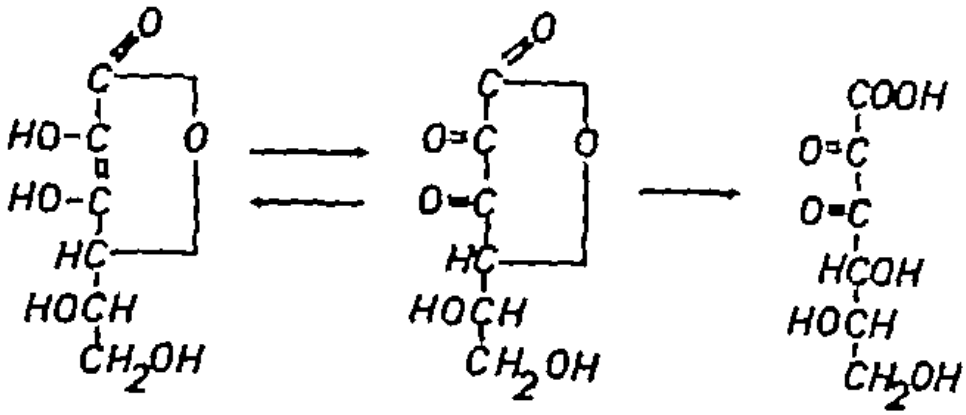


Fig. 1 Structural formulae of ascorbic acid, dehydroascorbic acid and 2,3-diketogulonic acid.

numerous, mainly chemical, investigations have been performed on ascorbic acid. However the biochemical role of this substance in the organism is not yet clear and a variety of functions have been suggested (see Baker 1967) During the biochemical reactions ascorbic acid is oxidized to dehydroascorbic acid which easily reduces back to ascorbic acid in the tissues both *in vivo* and *in vitro* (Fig. 1) (Johnson and Zilva 1934 Fox and Levy 1936,

[1] Ascorbic acid refers throughout this study to the biologically active levo-ascorbic acid.

Roe and Barnum 1936 Borsook *et al.* 1937 Schulze *et al* 1938 Penney and Zilva 1943 Todhunter *et al.* 1950, Dayton *et al.* 1959 1966 Patterson and Mastin 1951 Martin 1961 Martin and Mecka 1961)

Dehydroascorbic acid[1] is considered to be a normal constituent in the organism. It has been reported that human plasma contains dehydroascorbic acid in variable amounts comprising on average about 20 % of the ascorbic acid content (Stewart *et al.* 1952)

Little work has been done on the physiological relationship between ascorbic acid — dehydroascorbic acid and the central nervous system. Both decreased and increased concentrations of ascorbic acid appear to affect normal functions of the brain. Crandon *et al.* (1940) reported that in experimental ascorbic acid deficiency in man, extreme fatigue develops at an early stage. Mental and emotional changes have also been observed. Hodges *et al.* (1969) reported symptoms of depression in man. In scorbutic guinea pigs an impaired nervous activity has been noticed (Follis 1958) On the other hand, if large amounts of ascorbic acid are given to man, insomnia, irritability and anxiety develop (see Glatzel 1966) If the concentration of ascorbic acid in the brain of guinea pigs is increased by injection of dehydroascorbic acid, a rise in the higher nervous activity (producing general neuromotor excitement) lasting for several days has been observed (Lyhs and Tegeler 1963)

Administration of dehydroascorbic acid to rats is followed by some pronounced acute pharmacological responses, e. g. a rise in the blood pressure, increased salivation lacrimation and motor activity (Patterson and Mastin 1951) Hypertension following injection of dehydroascorbic acid has also been observed in cats and guinea pigs (Wegman 1958) Both adrenergic and cholinergic mechanisms seem to be activated by injected dehydroascorbic acid. It has been suggested that most of the pharmacological responses are evoked in the central nervous system.

As dehydroascorbic acid is rapidly reduced to ascorbic acid in the tissues, it was suggested by Patterson and Mastin (1951) that the responses recorded after injection of dehydroascorbic acid were triggered by metabolically formed ascorbic acid. However no definite conclusion can be drawn.

Defects in the circulatory system have been observed in scorbutic humans and animals Investigations have shown that ascorbic acid is probably involved in the maintenance of the tone of the blood vessels (Lee 1961) When tested on the blood pressure different responses have been noticed. Kreitmair (1934) and Wegman (1958) observed that injection of ascorbic acid into cats resulted in a transient decrease in the blood pressure due to its acidic properties (Weg

[1] Dehydroascorbic acid refers throughout this study to levo-dehydroascorbic acid.

man 1958) Reiser (1938) on the other hand noticed a slight hypertensive action of injected ascorbic acid in spinalized cats. It has also been reported that ascorbic acid induces increased pressor reflexes and decreased depressor reflexes in dogs (Ungar and Ungar 1938)

Ascorbic acid has been shown to act as a co-factor in hydroxylation reactions (see review by Schneider and Staudinger 1964) Model systems for aromatic hydroxylation where ascorbic acid functions as an electron donor have been described (Udenfriend *et al* 1954 Breslow and Lukens 1960) Ascorbic acid has also been shown to act as a co-factor in dopamine β-hydroxylation *in vitro* (see review by Kaufman and Friedman 1965) Furthermore, it has been suggested that ascorbic acid participates in dopamine β-hydroxylation *in vivo* Izquierdo *et al* (1968) reported that ascorbic acid injected intraperitoneally into rats caused an increase of the endogenous noradrenaline level and a decrease of the dopamine level in various parts of the brain. These observations were associated with an increase of the dopamine β-hydroxylase activity caused by ascorbic acid.

Autoradiographic studies have revealed that injected ascorbic acid and dehydroascorbic acid are distributed fairly equally in the body (Hammarström 1966) A specific accumulation of both substances to sites of catecholamine synthesis was noticed.

In the present study the pharmacological properties of dehydroascorbic acid and ascorbic acid on the central and peripheral nervous systems have been extensively investigated. Special attention has been drawn to the effects of injected dehydroascorbic acid.

The aim of the present study was to ascertain

1 whether injected dehydroascorbic acid and ascorbic acid cause pharmacological responses in mice, cats and rabbits (conscious and anaesthetized) as earlier observed in rats (Patterson and Mastin 1951) The following parameters were studied motor activity salivation, lacrimation tone of the nictitating membrane, sympathetic ganglionic transmission, neuromuscular transmission and blood pressure.

2 whether the pharmacological responses to injected dehydroascorbic acid were influenced by prior decerebration, spinal cord section or bilateral adrenalectomy.

3 whether the pressor response to injected dehydroascorbic acid could be affected by compounds known to interfere with adrenergic mechanisms, and if dehydroascorbic acid could interfere with the blood pressure response to acetylcholine.

4 whether the so called "membrane pump blocking agents and reserpine influenced the tissue uptake of ^{14}C-labelled dehydroascorbic acid and ascorbic

acid in brain and heart tissues *in vivo*

5 whether injected dehydroascorbic acid and/or ascorbic acid influenced the levels of some endogenous catecholamines in brain and heart tissues *in vivo*

6 whether dehydroascorbic acid and/or ascorbic acid was/were able to inhibit cholinesterases *in vitro*

CHAPTER I

Pharmacological Effects of Dehydroascorbic Acid and Ascorbic Acid in Conscious and Anaesthetized Animals

In the following experiments dehydroascorbic acid or ascorbic acid was injected into cats, rabbits and mice. Some of the previously observed effects were confirmed (Patterson and Martin 1931 Wegman 1958) Effects of the two substances on other parameters, e. g. the tone of the nictitating membrane the sympathetic ganglionic transmission and the neuromuscular transmission were also studied.

The pharmacodynamic actions of dehydroascorbic acid were studied more extensively than previously and some new pharmacological properties of this compound ha ve been observed.

Part 1 General materials and methods

Preparation of substances

Ascorbic acid obtained commercially in crystalline form (Pharmacopoea Nordica) was dissolved in redistilled water at a concentration of 50 mg/mL The pH of this solution was 2.2.

Dehydroascorbic acid was prepared in two different ways by oxidation of ascorbic acid as described by Patterson (1950) and by preparation from a dehydroascorbic acid — methanol complex.

One gram of ascorbic acid dissolved in 20 ml redistilled water was shaken with an equal volume of diethylether containing 0.61 g benzoquinone. This amount of benzoquinone is equivalent to the amount of ascorbic acid. After shaking for 15 minutes the ether phase was removed and the water solution was washed 5 times w ith an equal olume of ether The final concentration was estimated to be 50 mg dehydroascorbic acid per ml[1] Since there was some residual ether after this procedure the sample was placed under vacuum for about 30 seconds. After this a little ether still remained but gradually evaporated in the open air during the experiment. thus cooling the sample

[1] According to the hromatographic investigations (page 10) minute amounts of impurities were detected However throughout the present study the doses of dehydroascorbic acid given were alculated as if the oxidation exchange from ascorbic acid or release from the methanol complex was 100 %

Dehydroascorbic acid is very easily decomposed at high temperatures but this can be prevented by keeping the solution cool.

By adding redistilled water at +60°C to crystalline dehydroascorbic acid — methanol complex, dehydroascorbic acid was released from the complex. The complex was obtained from Mann Research Laboratories, New York, USA and its preparation and properties are described by Pecherer (1951) The dehydroascorbic acid released from the complex is reported to give all the normal chemical reactions of dehydroascorbic acid. Within two minutes the crystals had completely dissolved and the solution was immediately cooled to about +4°C. Before injection this solution was warmed to room temperature. No adverse effects have been reported from the small amount of methanol present in the solution (Clayton *et al.* 1954 see also Chapter I Part 5 Fig 10)

The solutions of dehydroascorbic acid were freshly prepared before each experiment and were used within 4 hours. The pH of freshly prepared and 4 hours old solutions of dehydroascorbic acid prepared in either way varied between 2.2 and 2.5

2,3-diketogulonic acid[1] was prepared by oxidation of dehydroascorbic acid obtained from ascorbic acid oxidation. Ascorbic acid (3.5 g) was dissolved in 35 ml redistilled water and shaken with 2.1 g benzoquinone dissolved in ether After being shaken for 15 minutes the ether phase was removed. The water phase was washed 5 times with ether After the final washing the ether was evaporated under vacuum. Ten ml 2 N NaOH was added to the water phase and shaken for 10 minutes to adjust the pH for an optimal oxidation reaction. Five ml 4 N HCl was then added and the solution was shaken again for 5 minutes. The final concentration of the 2,3-diketogulonic acid solution was estimated to be 50 mg/ml. This preparation has previously been described in detail by von Euler and Hasselquist (1952) The pH of the prepared solution was 1.1

Chromatographic investigations

Dehydroascorbic acid decomposes easily at neutral or alkaline pH. According to Prochazka (1958) and Heimann and Heimann (1965) however it is fairly stable at pH —3

Ascorbic acid is also easily decomposed, especially when heavy metal ions, i.e. copper and iron, are present.

In order to check the purity of the ascorbic acid solution and that of dehydroascorbic acid obtained by ascorbic acid oxidation and from the methanol complex, thin layer chromatography was performed (see Chapter

[1] The levo-form.

III and Hammarström 1966) The chromatograms showed that negligible amounts of impurities were present in fresh as well as 4 hours old solutions of dehydroascorbic acid. A very small spot with the same R_f value as ascorbic acid could be detected. According to Patterson (1950) ascorbic acid is oxidized to 95—98 % with benzoquinone.

Animals and diet

Cats (1.5—4.0 kg body weight) and rabbits (2.0—3.0 kg) of both sexes and female mice (NMRI strain with an average body weight of 20 g) were used in the investigations.

The cats were fed with "Mjau" (purchased from Tre Kök AB, Sweden) This is a commercial canned cat food made from fresh fish and primary products of beef and pork. Vitamins added per kilo are vitamin A 5,000 I. U., vitamin D 500 I. U vitamin E 5 mg, vitamin B_1 1 mg and vitamin B_2 1 mg The cats were also given water or milk *ad lib*

The rabbits were kept on a diet consisting of carrots, Swedish turnips and cabbage *ad lib*

The mice were fed a complete pelleted mouse diet and water *ad lib* The pellets were supplied by AB Ewos, Södertälje, Sweden and were composed of whey powder steamed cereals, soybean powder Prot Animal (defatted fish protein concentrate) dry yeast, soybean oil lime stone bentonite, vitamin concentrate, and trace elements. Average analysis of dry matter crude protein 22.5 % crude fat 5.0 % fiber 3.0 % NaCl 0.5 % Ca 0.8 % P 0.6 % water 8.0 % Vitamins added (per kilo) vitamin A 7,200 I U vitamin D_3 720 I. U vitamin E 84 mg, vitamin B 3 mg, vitamin B_2 6 mg, Ca-pantothenic acid 10 mg Trace elements added (per cent) Fe 0.0017 Cu 0.00017 Co 0.000017 I 0.00006, Mn 0.0017 Zn 0.0125

Anaesthesia

The *cats* were anaesthetized either by intraperitoneal injection of pentobarbitone, (Mebumalnatrium® ACO) (35 mg/kg) or by intramuscular injection of a solution containing chloralose and urethane. The doses were 64 mg chloralose/kg and 214 mg urethane/kg.

The *rabbits* were anaesthetized by intravenously administered 20 % urethane solution. The dose was 1.4 g urethane/kg

6 % pentobarbitone in propyleneglycol solution.

in the conscious and anaesthetized animals are probably expressions of the same mechanism(s)

Following dehydroascorbic acid injection, certain atropine-sensitive effects have been observed i.e. increased salivation and lacrimation which corresponds with observations on rats by Patterson and Mastin (1951) In the present study also ear twitching was observed and this is interpreted as the result of a central cholinergic activation (cf Armitage *et al.* 1966) by dehydroascorbic acid (see General Discussion)

Injected ascorbic acid, on the other hand, did not seem to produce any behavioural changes or to affect the salivation and lacrimation in cats and rabbits.

Part 3 Effects of dehydroascorbic acid and ascorbic acid on the nictitating membrane in cats

Materials and methods

In this investigation 8 chloralose-urethane and 14 pentobarbitone anaesthetized cats were used.

The nictitating membrane was connected by a silk thread to a force-displacement transducer (Grass FT 03) and the tension was adjusted to 2 grams.

In 4 chloralose urethane and 10 pentobarbitone anaesthetized cats the tone of the non-stimulated nictitating membrane was studied before and after injection of dehydroascorbic acid or ascorbic acid. In 4 cats (2 chloralose-urethane and 2 pentobarbitone anaesthetized) the sympathetic trunk was first severed above the cervical sympathetic ganglion and later on during the experiment the ganglion was removed.

In 4 of the pentobarbitone and 4 of the chloralose-urethane anaesthetized cats the cervical sympathetic trunk was cut and a bipolar platinum electrode was applied to the peripheral end (preganglionic) Stimulus currents were supplied by a Grass model S 4 stimulator The stimulation consisted of square wave pulses of ?0 msec duration and an intensity of 1.5 V Stimulation was given in the form of trains of such pulses. The duration of the individual trains was 5 sec and the frequency within the trains was varied between 1 and 20 imp sec The train repetition rate was 95 sec. After obtaining reproducible contractile responses the substances to be tested were injected.

Recordings were made on a Grass ink writing oscillograph model 5 (Grass Instrument Co. Quincy Mass. USA) The paper speed was 0.25 mm/sec.

A polythene tubing (Portex PP 90 Portland Plastics Ltd., Hythe, Kent England) was inserted in one of the femoral veins. The tubing was provided with a cannula. All injections were made through this.

The doses of dehydroascorbic acid (obtained by ascorbic acid oxidation) used were 5 10 25 50 100 and 150 mg/kg (8 chloralose urethane and 11 pentobarbitone anaesthetized cats)

Ascorbic acid was tested on the non-stimulated nictitating membrane in 6 of the cats (3 pentobarbitone and 3 chloralose urethane anaesthetized) and on the stimulated nictitating membrane in 3 of the cats (pentobarbitone anaesthesia) Doses of ascorbic acid tested were 25 50 100 and 200 mg/kg

Atropine (1—2 mg/kg) was given intravenously to 3 cats (non-stimulated nictitating membrane) prior to injection of dehydroascorbic acid. In 2 cats 2 mg/kg of propranolol (Inderal® ICI) and 1 mg/kg of dihydroergotamine (Orstanorm® Sandoz) were injected intravenously prior to administration of dehydroascorbic acid.

Results

Non stimulated nictitating membran

The effect of dehydroascorbic acid on the non-stimulated nictitating membrane was different in pentobarbitone and chloralose-urethane anaesthetized cats. When the former anaesthetic was used the responses were faint and not uniform. Injection of 5 mg/kg caused a small contraction whereas injection of 10 mg/kg always caused a small, fairly long lasting relaxation (Fig. 2) The relaxation increased with a higher dose (Fig 3) Preganglionic sympathectomy or removal of the cervical ganglion did not alter the response. Atropine treatment, however reversed the relaxation to a small contraction when the nerve supply was intact.

In the chloralose-urethane anaesthetized cats injection of dehydroascorbic acid caused a dose dependent contraction of the nictitating membrane (Fig.4) After premedication with propranolol and dihydroergotamine the response to injected dehydroascorbic acid was markedly reduced. After preganglionic sympathectomy no effect at all was observed (Fig. 4)

In some of the cats injection of ascorbic acid resulted in a small contraction of the nictitating membrane. In others no effect at all could be seen. The response appeared to be independent of the type of anaesthetic used.

Stimulated nictitating memb ane

Administration of dehydroascorbic acid influenced electrically stimulated contractions of the nictitating membrane. The effects were not uniform, how-

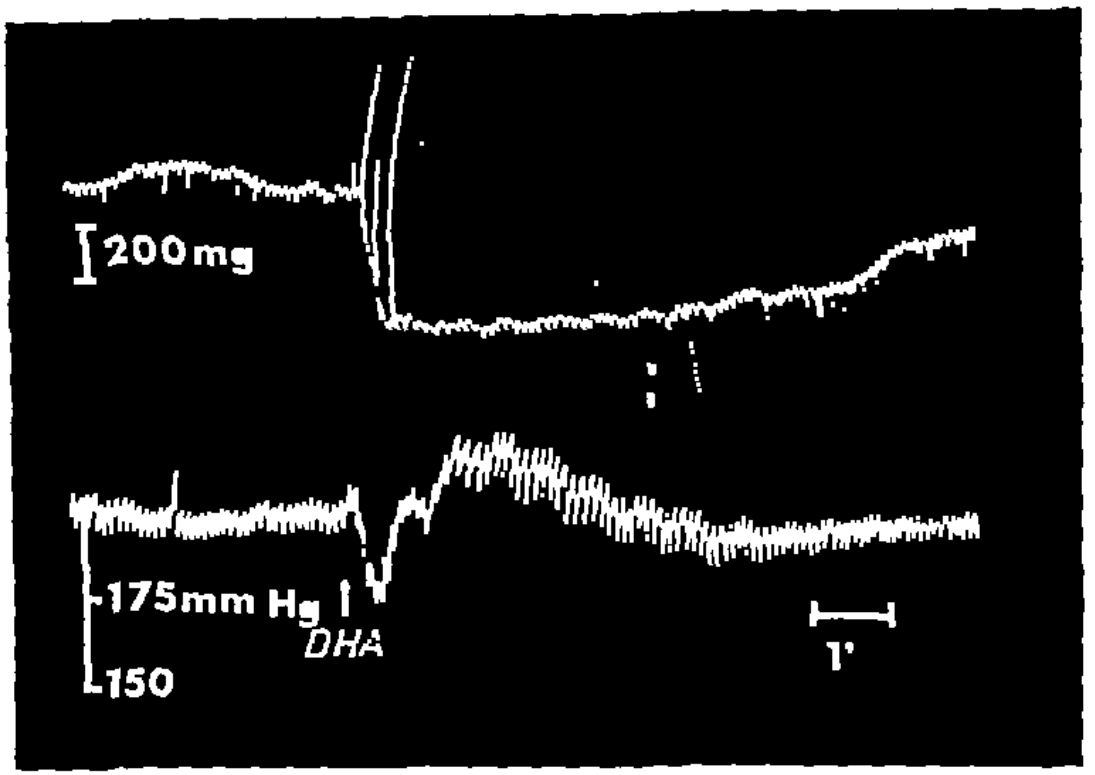


Fig. 2. Cat, pentobarbitone anaesthesia. The effect of intravenous injection of 10 mg/kg dehydroascorbic acid (DHA) on the tone of the nictitating membrane (above) and on the blood pressure (below)

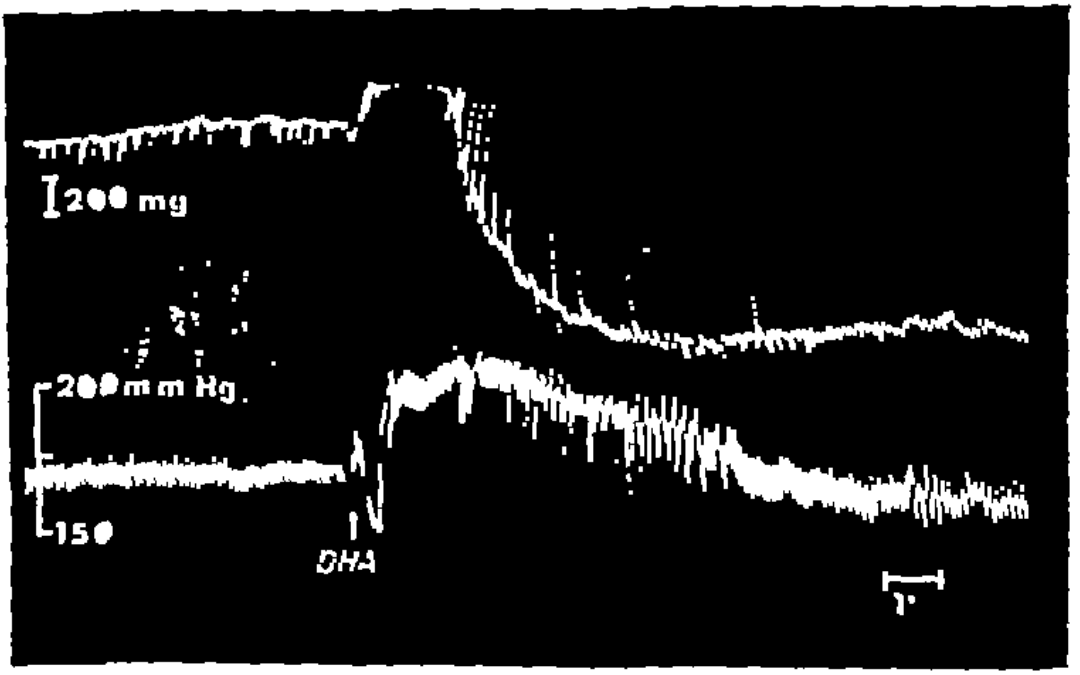


Fig. 3 Cat, pentobarbitone anaesthesia. The effect of intravenous injection of 100 mg/kg dehydroascorbic acid (DHA) on the tone of the nictitating membrane (abov) and on the blood pressure (below)

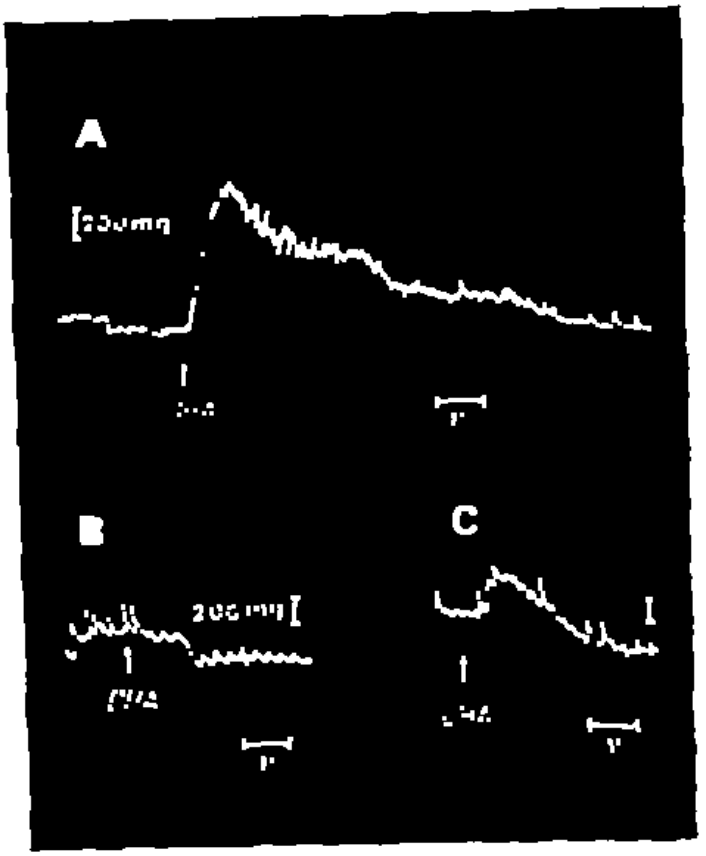


Fig. 4. Cat, chloralose-urethane anaesthesia. The effect of intravenous injection of 50 mg/kg dehydroascorbic acid (DHA) on the tone of the nictitating membrane. A normal response. B the response after sympathectomy C the response after premedication with propranolol (2 mg/kg) and dihydroergotamine (1 mg/kg)

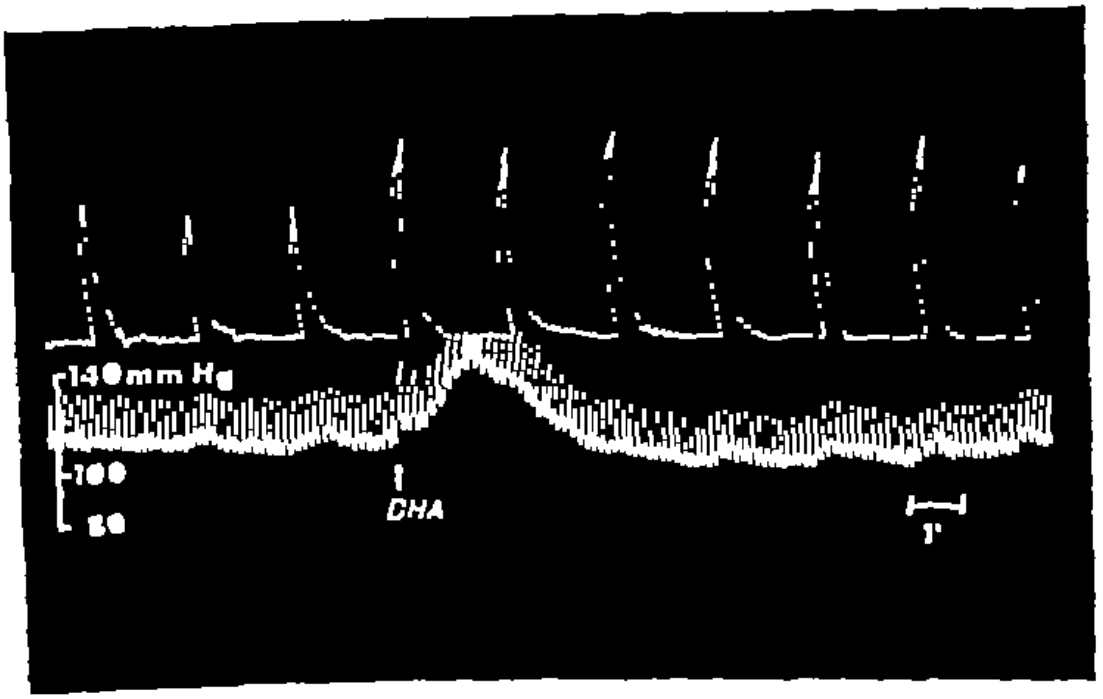


Fig. 5. Cat, chloralose-urethane anaesthesia. The effect of intravenous injection of 10 mg/kg dehydroascorbic acid (DHA) on the stimulated nictitating membrane (see text) (above) and on the blood pressure (below). Note the increase in amplitude of the response to stimulation of the nictitating membrane immediately after the injection.

ever Injection of ascorbic acid had no effect whatever on the response to stimulation at any of the doses tested

When *low frequency stimulation* (up to 10 imp/sec) was applied, injection of 5 mg/kg dehydroascorbic acid did not change the response to stimulation. If the dose was increased to 10 and 25 mg/kg the amplitude of the response from the electrically induced contractions increased by about 50 % (Fig. 5) This augmented response was, however only observed in 3 of the tested cats (chloralose urethane anaesthesia) The other 5 animals either did not respond at all or demonstrated a small reduction in amplitude. When higher doses (50 and 100 mg/kg) of dehydroascorbic acid were given a decrease in amplitude lasting for about 5—10 minutes was always observed (Fig 6) After injection of 100 mg/kg the response to stimulation immediately after injection was reduced to about 75 % of the normal response.

When *high frequency stimulation* (10—50 imp/sec) was applied any of the doses of dehydroascorbic acid tested caused an inhibition of the response to stimulation.

Discussion

Since dehydroascorbic acid activates central sympathetic centres causing blood pressure rise (Part 6 Chapter II) it was thought that, analogous to sympathomimetic agents with direct or indirect adrenergic effects, administration of dehydroascorbic acid would result in a constriction of the smooth muscle

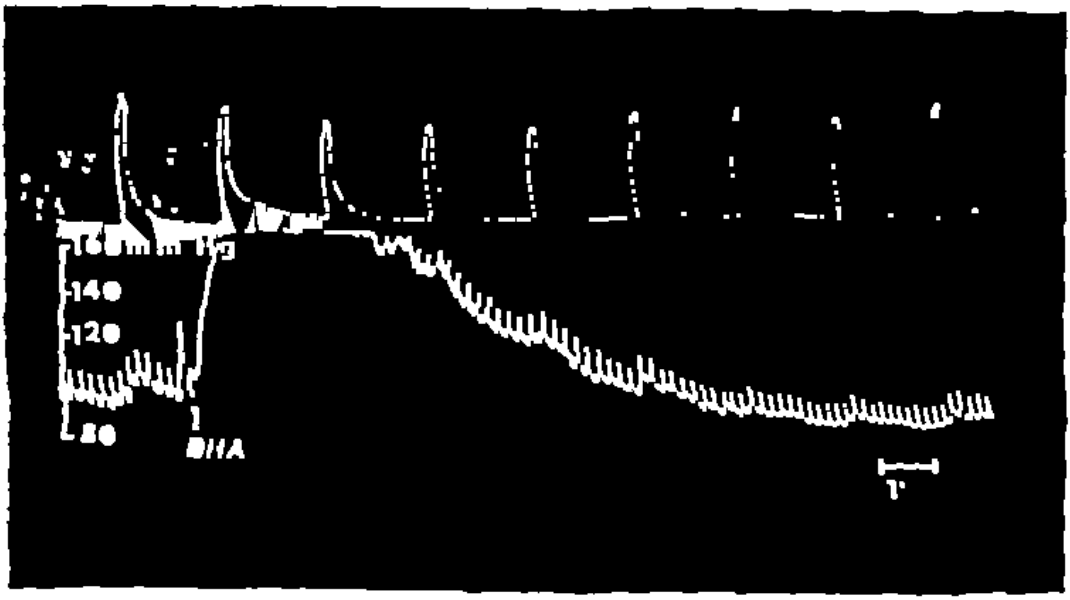


Fig. 6. Cat, chloralose-urethane anaesthesia. The effect of intravenous injection of 100 mg/kg dehydroascorbic acid (DHA) on the stimulated nictitating membrane (see text)

of the non-stimulated nictitating membrane. However two different types of response were observed. In the pentobarbitone anaesthetized cats injection of dehydroascorbic acid was followed by relaxation of the nictitating membrane. This effect still remained after cutting the sympathetic nervous supply above or below the superior cervical ganglion. Furthermore it was shown that the relaxation was inhibited by premedication with atropine. This suggests that the relaxation was due to activation of cholinergic mechanisms.

In the chloralose urethane anaesthetized cats the nictitating membrane constricted after administration of dehydroascorbic acid, demonstrating a sympathomimetic effect. It was observed that the constrictor response was reduced by premedication with propranolol and dihydroergotamine, indicating an effect via adrenergic receptors. Following sympathetic denervation no constriction at all was observed after injection of dehydroascorbic acid, indicating a central site of action.

After injection of ascorbic acid a small constrictor response was occasionally observed. However further experimental work is required to clarify whether injected ascorbic acid really affects the nictitating membrane or not.

The effects of injected dehydroascorbic acid on the stimulated nictitating membrane are thought to be due to activation of adrenergic mechanisms at ganglionic level and/or at postganglionic sites (see General Discussion). The response appears to be dependent on the anaesthetic used (cf. above).

Part 4 Effects of dehydroascorbic acid and ascorbic acid on neuromuscular transmission

Materials and methods

In this investigation 10 pentobarbitone or chloralose-urethane anaesthetized cats were used.

Induced contractions of the gastrocnemius muscle were recorded. The tendon of the muscle was connected via a thread to a force-displacement transducer (Grass FT 10). The muscle was excited by stimulation of the sciatic nerve. A bipolar platinum electrode was applied to the peripheral end of the free-dissected and cut nerve. Stimulus currents were supplied by a Grass model S 4 stimulator. Single pulses were given every 5th second. The duration of each pulse was 50 msec and the intensity 1 V.

Recordings were made on a Grass ink writing oscillograph model 5. The paper speed was 0.5 mm/sec.

In one of the femoral veins a polythene tubing (Portex PP 90) was in-

troduced. The tubing was provided with a cannula. Intravenous injections were made through this catheter in 6 cats. In 4 cats injections were made through a cannula inserted in the femoral artery close to the branch supplying the gastrocnemius muscle.

The doses of dehydroascorbic acid given intravenously were 5, 10, 25, 50, 100 and 150 mg/kg (4 cats). Two doses, 10 and 25 mg/kg of dehydroascorbic acid were injected intraarterially (? cats).

Ascorbic acid (50, 100 and 150 mg/kg) was given intravenously to ? cats and intraarterially to another ? cats.

Results

Intravenous injection

Low doses (5—25 mg/kg) of intravenously injected dehydroascorbic acid did not influence the muscular response elicited by sciatic stimulation. From 50 mg/kg an enhanced excitability was observed. The amplitude of the contraction recordings increased gradually with the dose. At 100 and 150 mg/kg the response to stimulation was increased by almost 100 % and 200 % respectively (Fig. 7). The effect lasted for about 10 minutes.

Ascorbic acid given intravenously in equivalent doses to dehydroascorbic acid did not produce any changes of the electrically induced contractions (Fig. 8).

Close intraarterial injection

Intraarterial injection of 10 and 25 mg/kg of dehydroascorbic acid caused an increased amplitude of the response to stimulation (25 % and 100 % respectively). This effect lasted for 5—10 minutes.

After an intraarterial injection of ascorbic acid a slight dose-dependent inhibition of the response to stimulation was observed.

Discussion

The facilitatory effect of dehydroascorbic acid on neuromuscular transmission in the gastrocnemius muscle seems to be due to a local action in the muscle since the response was produced equally well by intravenous injection and by close intraarterial injection of low doses. This augmented response might probably be attributed to the slight cholinesterase-inhibiting action of dehydroascorbic acid (Chapter V). This is supported by the fact that injection of ascorbic acid, which seems to be devoid of cholinesterase inhibiting action

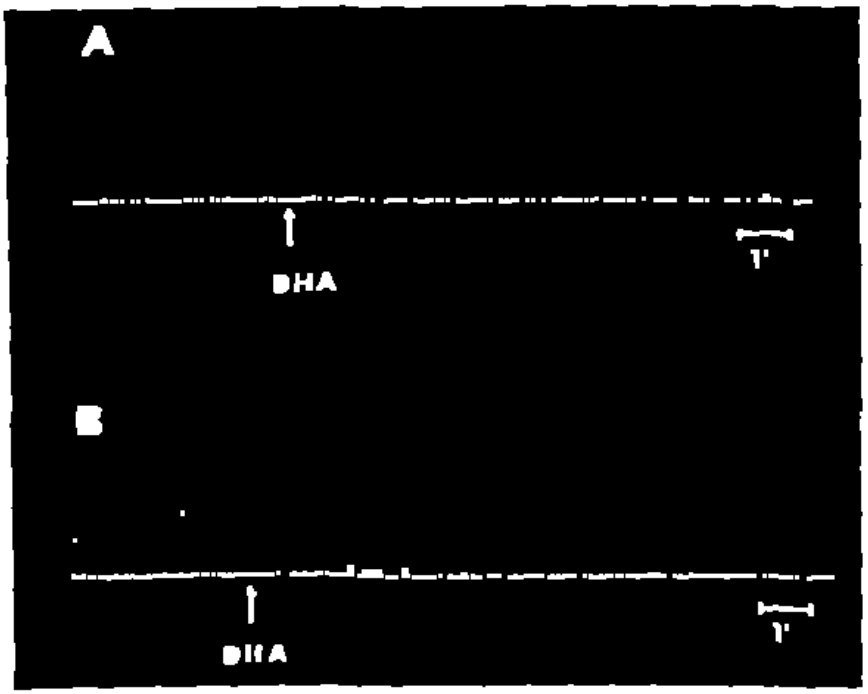


Fig. 7 Cat, pentobarbitone anaesthesia. The effect of intravenous injection of 100 mg/kg (A) and 150 mg/kg (B) dehydroascorbic acid (DHA) on the stimulated gastrocnemius muscle. Note the increase of the amplitude immediately after the injection.

(Chapter V) does not cause facilitation of the neuromuscular transmission. The cause of the slight inhibitory effect observed after intraarterial injection of ascorbic acid is not known.

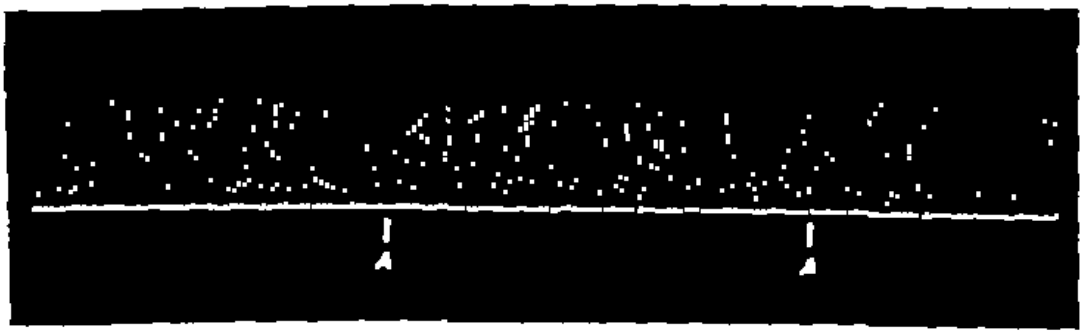


Fig. 8. Cat, pentobarbitone anaesthesia. The effect of intravenously injected ascorbic acid (↓) on the stimulated gastrocnemius muscle. The first arrow indicates injection of 100 mg/kg and the second arrow injection of 150 mg/kg ascorbic acid.

Part 5 Effects of dehydroascorbic acid and ascorbic acid on the blood pressure in cats and rabbits

Materials and methods

The experiments were carried out on 24 chloralose-urethane and 28 pentobarbitone anaesthetized cats and on 70 urethane anaesthetized rabbits.

One of the common carotid arteries was cannulated with a polythene tubing (Portex PP 90) which was connected to a Stratham P 23 Ac transducer for measuring the systemic blood pressure.

In 3 of the pentobarbitone anaesthetized cats the cervical vagi and sinus nerves were cut.

Recordings were made on a Grass ink writing oscillograph model 5. The paper speed was 0.25 mm/sec.

In one of the femoral veins a polythene tubing (Portex PP 90) was inserted. The tubing was provided with a cannula. In 2 of the rabbits one of the common carotid arteries was cannulated with a cranially orientated polythene tubing for intraarterial injections. In these animals the blood pressure recordings were registered from one of the femoral arteries.

Dehydroascorbic acid, obtained by ascorbic acid oxidation, was injected intravenously. The doses employed were 5, 10, 25, 50 and 100 mg/kg. Dehydroascorbic acid prepared from the methanol complex (10—100 mg/kg) was given to 2 cats (1 pentobarbitone and 1 chloralose-urethane anaesthetized cat) and 1 rabbit.

Ascorbic acid (5—150 mg/kg) was injected intravenously to 5 pentobarbitone and 3 chloralose-urethane anaesthetized cats and to 1 rabbit.

In one chloralose-urethane anaesthetized cat 2,3-diketogulonic acid (see Part 1) was injected intravenously (50 and 100 mg/kg).

Lactic acid (Pharmacopoea Nordica) solution was prepared by diluting the concentrated lactic acid with redistilled water to a final concentration of 3 N (*pH* 1.6). This solution was injected intravenously into 3 rabbits in volumes equivalent to those used for dehydroascorbic acid. In 2 rabbits lactic acid was also injected into the common carotid artery.

A solution was made in the same manner as that employed for preparing dehydroascorbic acid omitting the ascorbic acid. To the solution was added a small quantity of hydroquinone (approximately 30 mg) which is the product formed from benzoquinone during ascorbic acid oxidation. This solution (reagent blank) was injected intravenously into 2 pentobarbitone and 2 chloralose-urethane anaesthetized cats and into 2 rabbits. The administered volumes were equivalent to those used for dehydroascorbic acid.

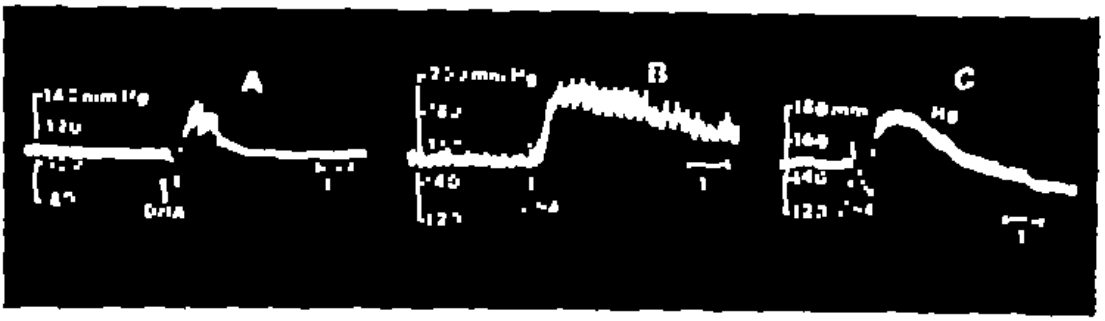


Fig. 9 The blood pressure response to intravenous injection of 50 mg/kg dehydroascorbic acid (DHA) in a pentobarbitone anaesthetized cat (A) a chloralose-urethane anaesthetized cat (B) and a urethane anaesthetized rabbit (C)

Redistilled water was also injected intravenously in volumes up to 3 ml/kg into 2 pentobarbitone and 2 chloralose-urethane anaesthetized cats and into 2 rabbits.

Results

Injection of dehydroascorbic acid often resulted in two different effects, an initial transient depressor response immediately followed by an increase of the blood pressure. The depressor response was more pronounced in the rabbits and the pentobarbitone anaesthetized cats than in the chloralose-urethane anaesthetized cats. It was observed that this decrease in blood pressure (about 20—30 mm Hg) did not change appreciably with the amounts injected. The depressor response rapidly decreased with the number of subsequent injections. In most experiments no depression was visible at the 3rd or 4th injection.

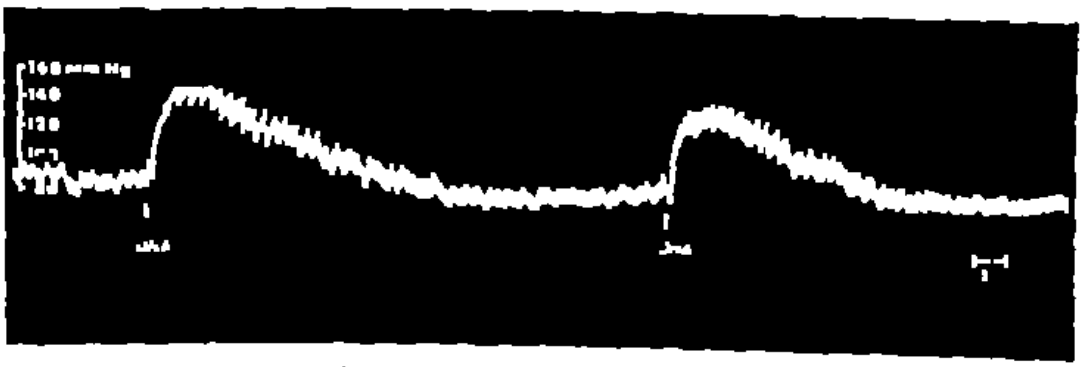


Fig 10. Cat, chloralose-urethane anaesthesia. The blood pressure response to intravenous injection of 50 mg/kg dehydroascorbic acid (DHA) prepared by ascorbic acid oxidation (first arrow) and from methanol complex (second arrow) For details see text.

TABLE 2. The average pressor response (B.P increase) ± S.E.M. (standard error of the mean) after single intravenous injection of various doses of dehydroascorbic acid into anaesthetized cats and rabbits. The values refer to the first injection of dehydroascorbic acid into each animal.

Pentobarbitone anaesthetized cats

Dose mg/kg	B.P increase mm Hg	No. of animals
5	9±1	4
10	19±11	4
25	30±5	3
50	45±4	4
100	51±5	4

Chloralose-urethane anaesthetized cats

Dose mg/kg	B.P increase mm Hg	No. of animals
5	13±3	4
10	20±18	3
25	27±3	4
50	60±4	5

Urethane anaesthetized rabbits

Dose mg/kg	B.P increase mm Hg	No. of animals
5	22±14	4
10	32±9	6
25	36±8	16
50	40±8	38

Pressor responses were always obtained after injection of dehydroascorbic acid into the rabbits as well as into the cats (independent of the type of anaesthetic used) (Fig. 9). This pressor effect increased with the dose. Table 2 shows the dose-dependent increase of the pressor response following a single injection of dehydroascorbic acid to anaesthetized cats and rabbits. The values refer to the first injection of dehydroascorbic acid to each animal.

After injection of dehydroascorbic acid prepared from the methanol complex no differences in blood pressure responses were seen from those obtained after injection of dehydroascorbic acid prepared by ascorbic acid oxidation (Fig. 10).

When dehydroascorbic acid was given in repetitive doses (50 mg/kg) to cats or rabbits there was a difference in response between the first and subsequent injections. If several injections with 10—15 minutes intervals were made, the pressor response gradually increased and reached a maximum after

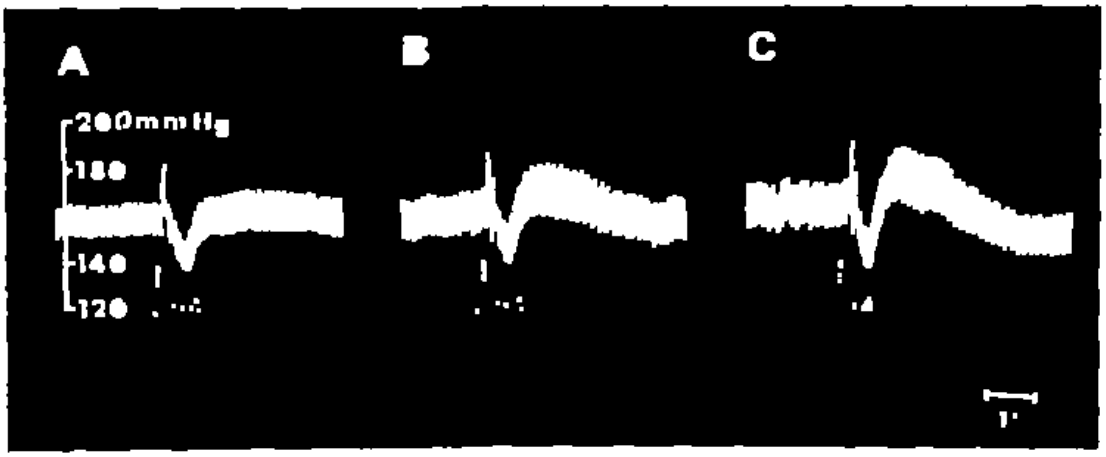


Fig. 11 Cat, chloralose-urethane anaesthesia. The blood pressure response to repeated intravenous injections of 50 mg/kg dehydroascorbic acid (DHA) with 10—15 minutes intervals. Note the successiv increase of the pressor response.

2—3 injections (Fig. 11) After 5—6 injections or more the blood pressure never completely returned to the initial level but remained slightly elevated. This level rose gradually following each subsequent injection.

When 50 mg/kg dehydroascorbic acid was given repeatedly to rabbits at 2 minute intervals the depressor and pressor effects gradually diminished, while after 3—6 injections the depressor response was abolished and after 15—20 injections no pressor response was obtained.

The dehydroascorbic acid solution obtained by ascorbic acid oxidation contains apart from oxidized ascorbic acid also redistilled water minute amounts of ether and possibly benzoquinone and hydroquinone. In order to check if the vehicle contributed to the observed effects a reagent blank was prepared. When injected intravenously in volumes equivalent to those used for dehydroascorbic acid only transient depressor responses were obtained (Fig. 12)

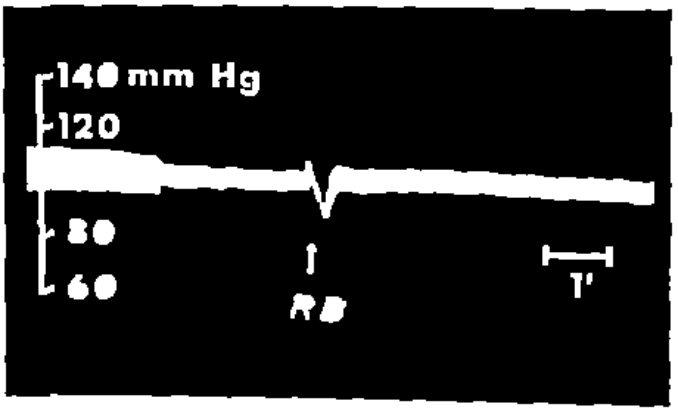


Fig. 12 Cat, pentobarbitone anaesthesia. The blood pressure response to intravenous injection of a reagent blank (RB ml/kg) For details see text.



Fig. 13. Cat, pentobarbitone anaesthesia. The blood pressure response to intravenous injection of 1.0 ml/kg redistilled water.

As the injected volumes were rather large (up to 3 ml/kg) the influence of corresponding volumes of redistilled water on the blood pressure was tested. When the redistilled water was injected at the same speed as the dehydroascorbic acid solution a transient fall in blood pressure was observed (Fig. 13) which was very similar to the initial depressor response observed after dehydroascorbic acid administration.

Dehydroascorbic acid is easily oxidized at neutral pH and it was thought that oxidative products might be formed in the blood. The first oxidative step from dehydroascorbic acid, 2,3-diketogulonic acid, could perhaps be responsible for or contribute to the observed pressor effects. This oxidation product

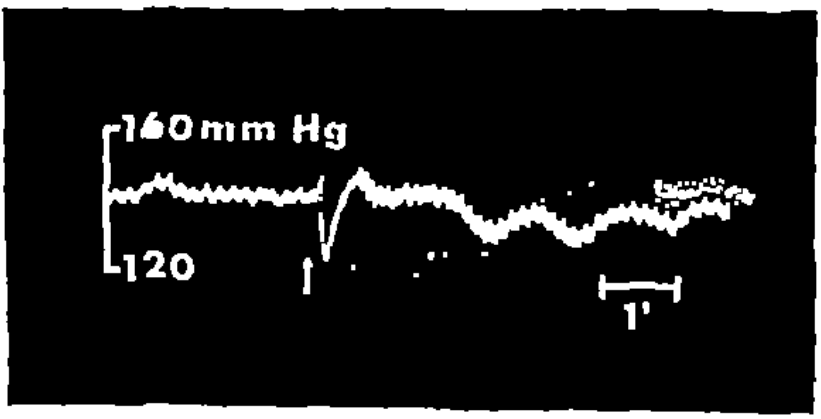


Fig. 14. Cat, chloralose-urethane anaesthesia. The blood pressure response to intravenous injection of 50 mg/kg 2,3-diketogulonic acid.

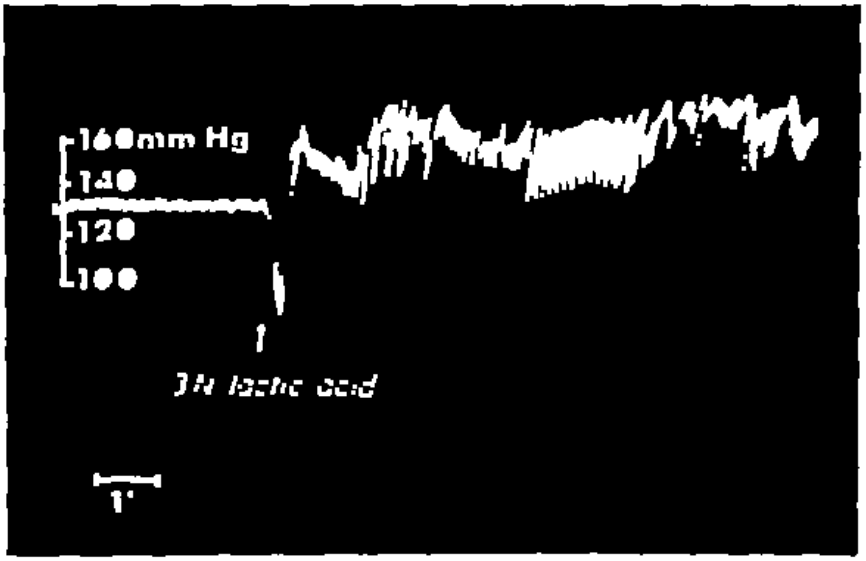


Fig. 15. Rabbit, urethane anaesthesia. The blood pressure response to intraarterial injection (carotid artery) of 3 N lactic acid (2 ml)

was synthetized (Part 1) and when injected intravenously into a chloralose-urethane anaesthetized cat only a transient depressor response was observed (Fig. 14)

Although the blood is known to possess a strong buffer capacity it was believed that when large amounts of the acidic dehydroascorbic acid solution (Part 1) were injected intravenously this buffer effect might be exceeded. To compare the effects of an acidic compound and those of dehydroascorbic acid on the blood pressure, lactic acid was chosen to induce acidosis. The strongest concentration it was possible to inject intraarterially into the carotid of the rabbit without immediately killing the animal was found to be 3 N (2 ml). The blood pressure response then recorded was a transient, fairly marked depressor effect followed by a long lasting increase in the blood pressure to a level slightly above normal (Fig. 15). When given intravenously the same concentration of lactic acid caused a fall in the blood pressure.

Following injections of different doses of ascorbic acid, only transient depressor responses were observed (Fig. 16). In 3 cats the cervical vagi and the sinus nerves were cut. When dehydroascorbic acid was injected into these cats (5 and 10 mg/kg) a pronounced hypertensive response was recorded in contrast to the small rise in the blood pressure often preceded by a transient depressor response obtained with the same doses prior to the nerve section. Fig. 17 illustrates the blood pressure response to 10 mg/kg dehydroascorbic acid before and after nerve section in one of the experiments.

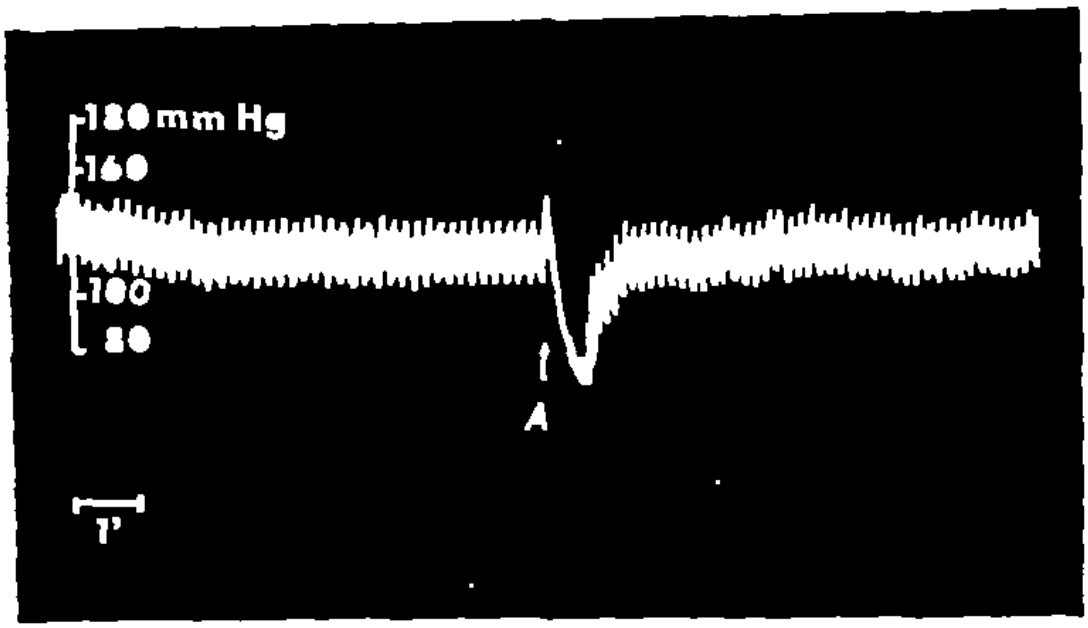


Fig. 16. Cat, chloralose-urethane anaesthesia. The blood pressure response to intravenous injection of 50 mg/kg of ascorbic acid (A)

Discussion

In the present investigation it was shown that intravenous injection of dehydroascorbic acid, prepared by ascorbic acid oxidation or from a dehydroascorbic acid—methanol complex, into anaesthetized cats and rabbits caused changes in the blood pressure. Very often this response presented a biphasic character depressor response followed by pressor response. These results are in agreement with observations by Wegman (1958) who found that injection

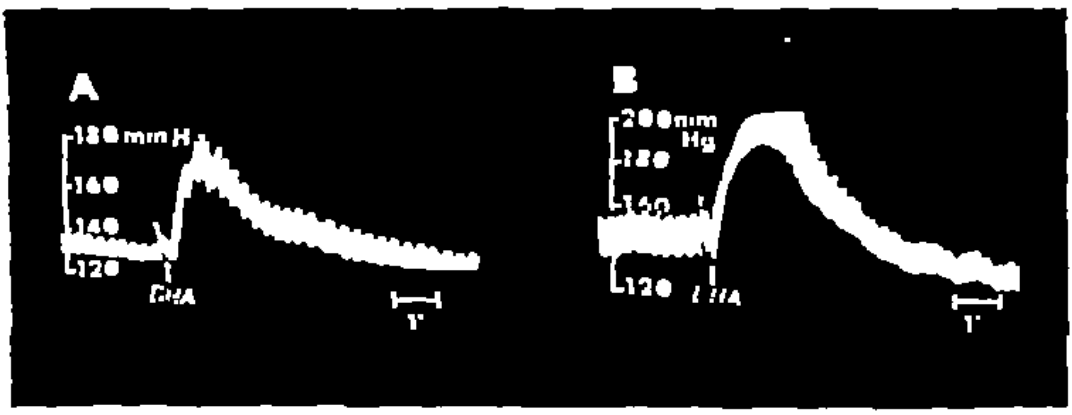


Fig. 17 Cat, pentobarbitone anaesthesia. The blood pressure response to intravenous injection of 10 mg/kg dehydroascorbic acid (DHA) before (A) and after (B) the cervical *ag* and *sinus nerves were cut*. Note the pronounced increase of the pressor response after the nerve section.

of dehydroascorbic acid into nembutal anaesthetized cats resulted in a similar biphasic response. It has previously also been shown that injection of dehydroascorbic acid causes pressor response in the rat (Patterson and Martin 1951) and the guinea pig (Wegman 1958)

The hypotensive response observed after dehydroascorbic acid administration in the present study is interpreted as mainly being due to an activation of baroreceptors caused by the large amount of liquid injected. Similar depressor responses were observed after injection of equivalent volumes of either redistilled water or reagent blank. The acidity of the injection solution (pH 2.2—2.5) may be a contributory factor

Section of the vagi and the sinus nerves altered the blood pressure response to injected dehydroascorbic acid. In normal cats a low dose caused an initial depressor response followed by a small rise in blood pressure. In animals where the nerves were sectioned the same dose of dehydroascorbic acid caused pronounced pressor responses. No hypotensive response was observed. This shows that when the baroreceptor mechanism is inactivated by nerve sectioning the depressor response disappears.

Injection of ascorbic acid always caused depressor responses, which is in agreement with observations by Kreitmair (1934) and Wegman (1958). Reiser (1958) however observed that injection of ascorbic acid into spinalized cats resulted in a small increase of the blood pressure. In anaesthetized dogs injection of ascorbic acid enhances the baroreceptor reflex causing an increase of the pressor reflex (Ungar and Ungar 1938) In the same investigation it was observed that the blood pressure response to electrical stimulation of the sinus nerve was depressed following injection of ascorbic acid.

The depressor effect of ascorbic acid in the present investigation is probably the result of an activation of baroreceptors and/or the acidic properties of ascorbic acid as shown by Wegman (1958)

The pressor effect of dehydroascorbic acid in cats and rabbits was shown to be increased with the dose. This pressor action is reported to be independent of the pH of the injected solution (Wegman 1958)

After repeated injections of the acidic dehydroascorbic acid solution the buffer capacity of the blood is most probably exceeded. This would result in a gradually increasing acidemia as well as a corresponding increase in stimulation of chemoreceptors hence a rise in blood pressure with successive injections. A similar rise in blood pressure was observed after intraarterial injection of lactic acid.

It was observed that the first 3 injections of dehydroascorbic acid, administered with long time intervals between the injections, resulted in gradually increasing pressor responses in the cats as well as in the rabbits. This phe-

nomenon has earlier been observed in rats by Patterson and Mastin (1951) and in cats by Wegman (1958)

It is known that dehydroascorbic acid is readily taken up by blood corpuscles and reduced to ascorbic acid (Hendry *et al* 1964 Hughes and Kilpatrick 1964 Hughes and Maton 1968) Injected dehydroascorbic acid is also widely distributed in the organism and easily taken up and reduced by various tissues (Hughes 1964 Hammarström 1966) These factors might contribute to a decrease in the amounts of dehydroascorbic acid in the blood thus reducing the amount of the substance reaching the brain. These depots will presumably become saturated with increasing amounts of injected dehydroascorbic acid, thus resulting in an increased amount of the active substance in the blood so that a larger amount would reach central effector sites (see Chapter I, Part 6)

Repeated injections of dehydroascorbic acid into rabbits at short time intervals caused a gradually decreasing pressor response thus demonstrating tachyphylaxis. This is consistent with observations in rats by Patterson and Mastin (1951) The direct mechanism of tachyphylaxis is not completely understood. It is possible that dehydroascorbic acid tachyphylaxis is due to a gradual saturation of the effector sites with metabolically formed ascorbic acid thus gradually reducing further uptake of dehydroascorbic acid. Further work is required before more definite conclusions can be drawn.

Part 6 Effects of dehydroascorbic acid following decerebration spinal cord section and bilateral adrenalectomy

Materials and methods

The experiments were carried out on anaesthetized cats (pentobarbitone or chloralose urethane anaesthesia) and rabbits.

Blood pressure recordings were performed as described in Part 5 The gross behaviour of the animals during anaesthesia was also observed.

Dehydroascorbic acid (obtained by ascorbic acid oxidation) was injected intravenously (see Part 5) in doses varying between 5 and 150 mg/kg.

Other drugs used were adrenaline (10 μg base) and noradrenaline (10 μg base) These substances were given by rapid intravenous injection.

Decerebration

Four cats and 9 rabbits were decerebrated. The cranium was exposed and the whole roof above the cerebrum removed. After incision of the brain membranes



Fig. 18. Cat, pentobarbitone anaesthesia. The blood pressure response to intravenous injection of 50 mg/kg dehydroascorbic acid (DHA) in (A) a normal animal, (B) after decerebration of the same animal and (C) after spinal cord section in the same animal.

the brain stem was cut directly in front of the tentorium in the cats and immediately in front of the cerebellum in the rabbits. All parts of the brain (including the pituitary gland) in front of cerebellum and pons were removed. A cotton cloth was pressed into the hole in the skull in order to prevent excess bleeding and the skin was closed. Twenty minutes were allowed to pass before recordings were made.

Spinal cord section

Seven cats (pentobarbitone or chloralose-urethane anaesthesia) and 2 rabbits were spinalized by cutting the spinal cord between C 1 and C 2. At the same time artificial respiration (Starling pump) was started.

Bilateral adrenalectomy

The adrenals were removed in 4 cats.

Results

At the beginning of each experiment (i.e. prior to operation) dehydroascorbic acid was injected 3—4 times with 10—15 minutes intervals in order to achieve uniform pressor responses. In some animals the pressor response was preceded by a transient fall of the blood pressure. Lacrimation, mucous salivation and an arousal effect were observed after each injection in both cats and rabbits.

Decerebrated cats

Immediately after the decerebration the blood pressure dropped from 140—160 mm Hg to about 60—100 mm Hg and became stabilized at this level.

After injection of dehydroascorbic acid (50 mg/kg) a rise in the blood pressure was obtained. The intensity and duration of this pressor response were

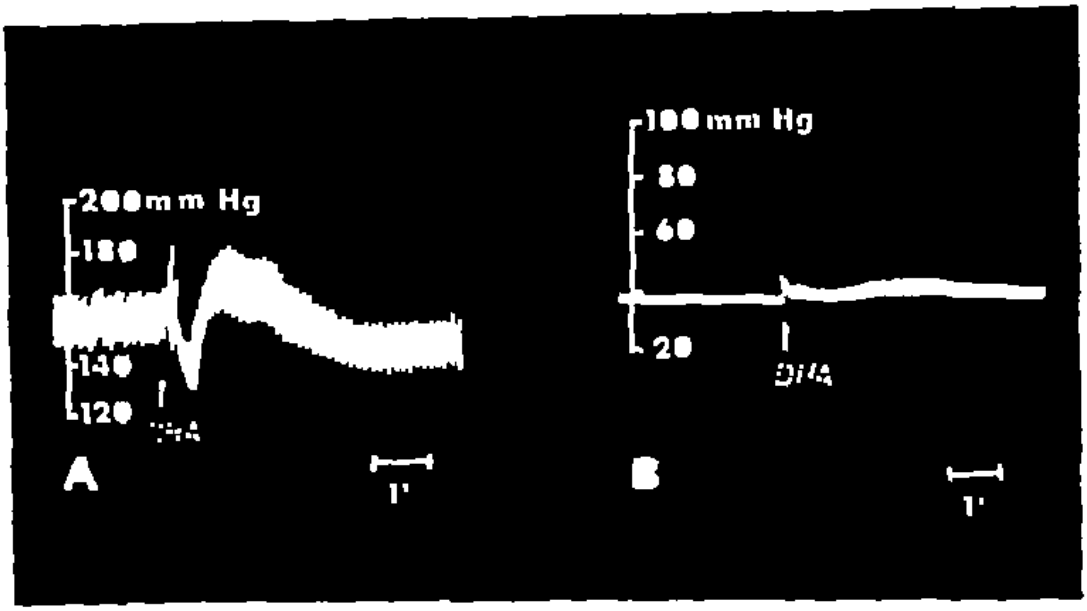


Fig. 19 Cat, chloralose-u ethane anaesthesia. The blood pressure response to intra venous injection of 50 mg/kg dehydroascorbic acid (DHA) before (A) and after (B) spinal cord section.

the same as before decerebration. In one cat the pressor response was even larger than before the operation (Fig. 18)

In most experiments the blood pressure never returned to the original level (80—100 mm Hg) but remained slightly elevated until the next injection of dehydroascorbic acid.

Neither increased salivation and lacrimation nor arousal effect were observed in the decerebrated cats.

Spinal cord section in cats and rabbits

After sectioning of the spinal cord in the cats and rabbits the blood pressure dropped to about 40—60 mm Hg and remained constant at this level. Intravenous injection of dehydroascorbic acid (50—150 mg/kg) into either of the species resulted exclusively in a slight pressor effect independent of the dose injected. This pressor effect consisted mainly of an increase in the systolic pressure (Figs 18 and 19). This effect lasted for about 10 minutes.

Neither increased salivation and lacrimation nor arousal effect were observed in the spinalized cats and rabbits.

Adrenaline and noradrenaline were given in order to check the variation ability of the blood pressure following decerebration and spinal cord section. These injections resulted in pronounced pressor effects, thus indicating a normal function at the peripheral adrenergic α-receptor sites.



Fig 20. Cat, chloralose-urethane anaesthesia. The blood pressure response to intravenous injection of 50 mg/kg dehydroascorbic acid (DHA) before (A) and after (B) bilateral adrenalectomy

Bilateral adrenalectomy in cats

Following this operation, injection of dehydroascorbic acid (50 mg/kg) resulted in a pressor response which was somewhat smaller than before the operation (Fig. 20)

Removal of the adrenals did not change the effect of dehydroascorbic acid on salivation and lacrimation. The arousal effect was also observed as prior to the operation.

Discussion

In the present investigation it was shown that the pressor effect of dehydro ascorbic acid remained unchanged after decerebration in cats. Injection of dehydroascorbic acid into decerebrated rabbits also caused a pressor response although it was somewhat reduced compared to that obtained prior to the operation. This suppressed response is probably due to partial damage to the vasomotor areas in the reticular formation caused by the operation.

Following spinal cord section the pressor effect of dehydroascorbic acid was markedly reduced in cats as well as in rabbits, presenting another character These results are in agreement with earlier observations in spinalized rats (Patterson and Mastin 1951) and cats (Wegman 1958)

Based on the present observations in decerebrated and spinalized cats and rabbits it is suggested that th pressor effect of dehydroascorbic acid is mainly evoked from caudal parts of the brain stem, most probably from the so called vasomotor centre in the medulla oblongata.

In the present experiments it was noticed that following bilateral adrenalectomy in cats, injection of dehydroascorbic acid caused a somewhat smaller

pressor response than prior to the operation. This may be an indication of a release of catecholamines from the adrenals by dehydroascorbic acid or metabolically formed ascorbic acid (McLean and Cohen 1963)

Adrenalectomy in rats, however does not appear to affect the pressor response of dehydroascorbic acid (Patterson and Martin 1951)

The increased salivation and lacrimation and the arousal effect observed after administration of dehydroascorbic acid into normal anaesthetized cats and rabbits were not seen after decerebration and spinal cord section. These results indicate that the effects mentioned are evoked in the central nervous system.

CHAPTER II

Effects of Various Compounds on the Pharmacological Response to Dehydroascorbic Acid of the Blood Pressure in Cats and Rabbits

Injections of dehydroascorbic acid into cats and rabbits result in both pressor and depressor responses. The depressor response is considered to be caused by the large volume injected and/or due to the acidity of the injection solution (Chapter I Part 5) and the pressor response is attributed to an effect of dehydroascorbic acid. This pressor response is similar to that obtained after injection of adrenergic nerve stimulants, e.g. tyramine or amphetamine.

It has also been observed that dehydroascorbic acid facilitates neuromuscular transmission (Chapter I Part 4)

The aim of the present experiments was to determine whether the pressor effect of dehydroascorbic acid could be affected by compounds known to interfere with adrenergic mechanisms and whether dehydroascorbic acid would interfere with the response to acetylcholine on the blood pressure.

Materials and methods

In these experiments anaesthetized cats (pentobarbitone or chloralose-urethane anaesthesia) and rabbits were used. For details of animals and anaesthesia see Chapter I Part 1

The animals were prepared for blood pressure recording as described in Chapter I Part 5. The number of animals, species, substances and doses tested in the various experiments are listed in Table 3.

Dehydroascorbic acid (obtained by ascorbic acid oxidation see Chapter I Part 1) was given intravenously in the following doses: 10, 25, 50, 100 and 150 mg/kg. In most experiments dehydroascorbic acid was injected 3—4 times initially at 10—15 minutes intervals in order to obtain uniform pressor effects.

All the drugs were administered intravenously (see Chapter I Part 5) unless otherwise stated.

In some experiments adrenaline, noradrenaline and tyramine were administered as control substances in order to ascertain normal response and to check the capacity for variation of the blood pressure

pressor response than prior to the operation. This may be an indication of a release of catecholamines from the adrenals by dehydroascorbic acid or metabolically formed ascorbic acid (McLean and Cohen 1963)

Adrenalectomy in rats, however does not appear to affect the pressor response of dehydroascorbic acid (Patterson and Mastin 1951)

The increased salivation and lacrimation and the arousal effect observed after administration of dehydroascorbic acid into normal anaesthetized cats and rabbits were not seen after decerebration and spinal cord section. These results indicate that the effects mentioned are evoked in the central nervous system.

Noradrenaline Free base	0.01[2]
Tyramine Tyramine hydrochloride	2—5

[1] Administered intraperitoneally daily for 4 consecutive days.
[2] Amount injected independent of body weight.

Results

Before treatment with any of the drugs used injection of dehydroascorbic acid always caused a pressor response sometimes preceded by a transient lowering of the blood pressure (compare Chapter I Part 5)

Ganglion blocking agents

Two different ganglion blocking agents were used, hexamethonium and tetra ethylammonium (TEA) In animals intravenously premedicated with either of these compounds the pressor response after dehydroascorbic acid injection was changed. When treated with hexamethonium (1—5 mg/kg) a reduction of the pressor effect was observed, but it was never completely eliminated (Fig. 21)

In experiments where TEA was used instead of hexamethonium a more pronounced action on the pressor response of dehydroascorbic acid was observed. After administration of a low dose of TEA (1 mg/kg) intravenously

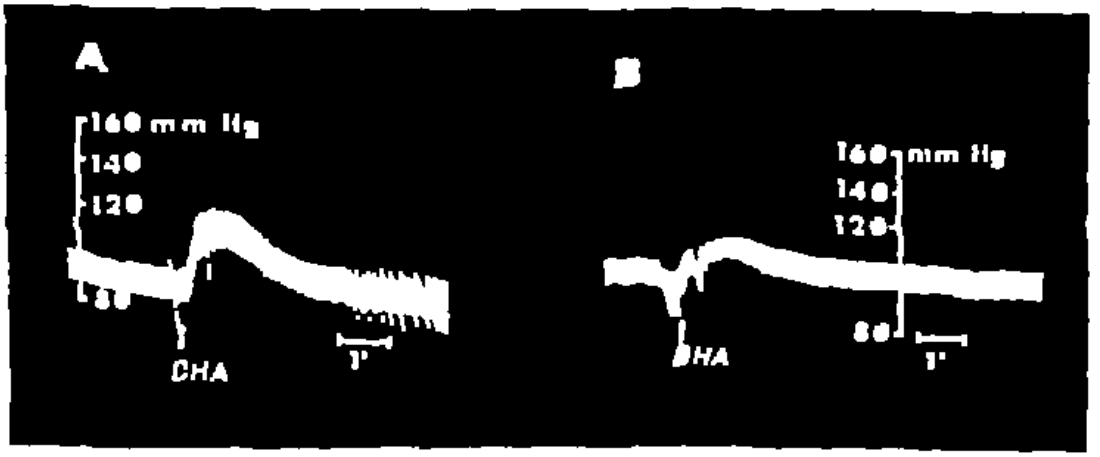


Fig. 21 Rabbit, urethane anaesthesia. The blood pressure response to intravenous injection of 50 mg/kg dehydroascorbic acid (DHA) before (A) and after (B) treatment with hexamethonium (2 mg/kg intravenously)

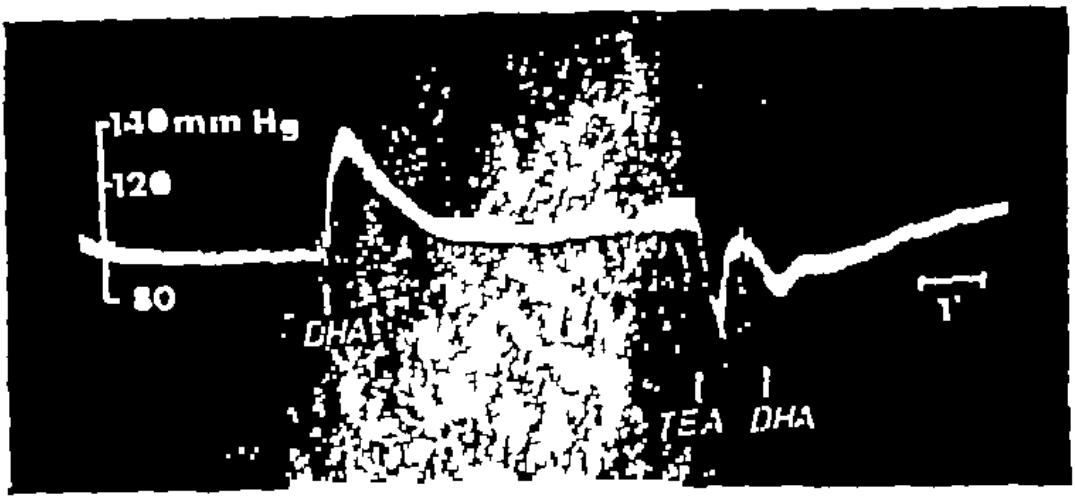


Fig. 22. Rabbit, urethane anaesthesia. The blood pressure response to intravenous injection of 50 mg/kg dehydroascorbic acid (DHA) before and after treatment with tetraethylammonium (TEA 1 mg/kg i.v.)

the pressor effect of dehydroascorbic acid was completely eliminated (Fig. 22).

Guanethidine and bretylium

The blood pressure response following dehydroascorbic acid was markedly changed after guanethidine and bretylium treatment. The pressor response to dehydroascorbic acid was strongly reduced, and in some experiments completely abolished following treatment with either of the two compounds (Figs. 23 and 24).

Vasodepressor agents

These experiments were carried out on chloralose-urethane anaesthetized cats as this type of anaesthesia and animal are most suitable for such experiments.



Fig. 23. Rabbit, urethane anaesthesia. The blood pressure response to intravenous injection of 50 mg/kg dehydroascorbic acid (DHA) before and after treatment with bretylium (Br 5 mg/kg i.v.)

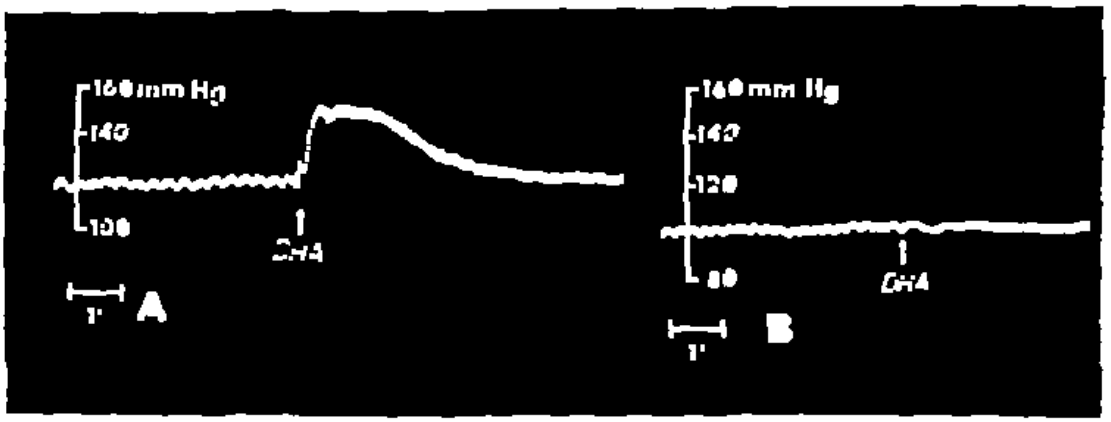


Fig. 24. Rabbit, urethane anaesthesia. The blood pressure response to intravenous injection of 50 mg/kg dehydroascorbic acid (DHA) before (A) and after (B) treatment with guanethidine (1 mg/kg I.V.).

Dihydroergotamine and phenoxybenzamine were used in different experiments in order to block the sympathetic α-receptors. Propranolol was used for blocking the adrenergic β-receptors.

After treatment with the α-receptor blocking agents the blood pressure response of injected dehydroascorbic acid changed. Instead of the usual pressor response a prominent depression was recorded (Fig. 25). In some experiments, however the pressor effect of dehydroascorbic acid was blocked but no depressor response occurred.

In experiments where injection of dehydroascorbic acid resulted in a fall of the blood pressure after α-receptor blocking (3 out of 7 tested cats) propranolol was given intravenously. When dehydroascorbic acid was given after the propranolol treatment faint pressor and depressor responses were observed (Fig. 26). When both adrenergic α- and β-receptors were blocked the response

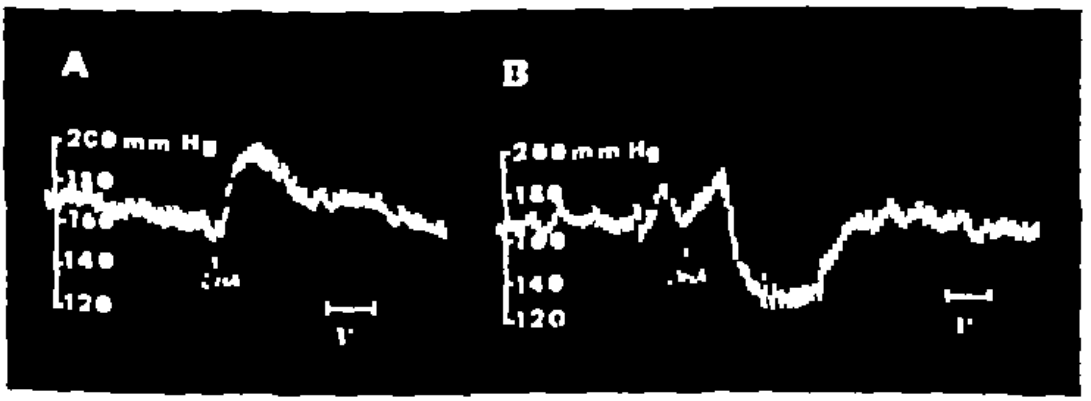


Fig. 25. Cat, chloralose-urethane anaesthesia. The blood pressure response to intravenous injection of 50 mg/kg dehydroascorbic acid (DHA) before (A) and after (B) treatment with dihydroergotamine (1 mg/kg I.V.). Note the reversal of the blood pressure response.

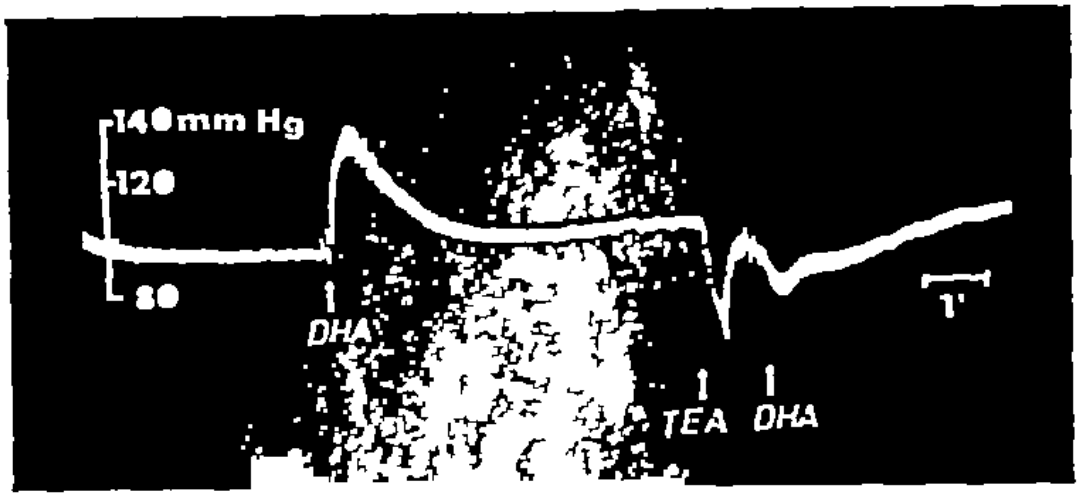


Fig. 22. Rabbit, urethane anaesthesia. The blood pressure response to intravenous injection of 50 mg/kg dehydroascorbic acid (DHA) before and after treatment with tetraethylammonium (TEA) 1 mg/kg i.v.

the pressor effect of dehydroascorbic acid was completely eliminated (Fig. 22).

Guanethidine and bretylium

The blood pressure response following dehydroascorbic acid was markedly changed after guanethidine and bretylium treatment. The pressor response to dehydroascorbic acid was strongly reduced, and in some experiments completely abolished following treatment with either of the two compounds (Figs. 23 and

Acute experiments following ganglion blocking agents

These experiments were carried out on chloralose-urethane anaesthetized cats and rabbits. Deep anaesthesia and animal are most suitable for such experiments.



Fig. 23. Rabbit, urethane anaesthesia. The blood pressure response to intravenous injection of 50 mg/kg dehydroascorbic acid (DHA) before and after treatment with bretylium (Br 5 mg/kg i.v.).

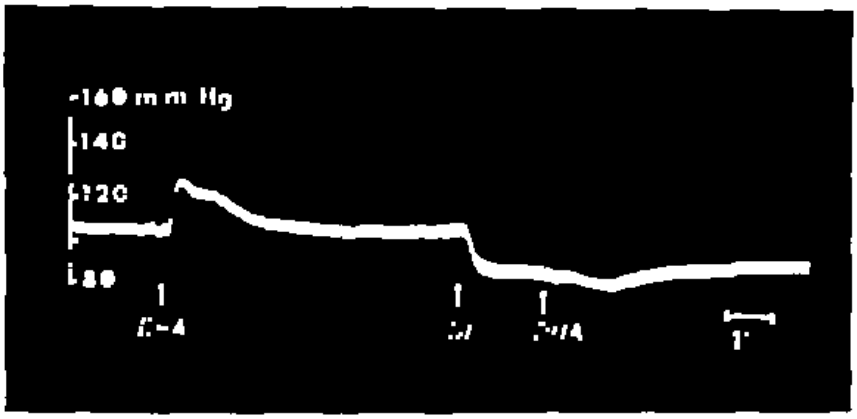


Fig. 28. Rabbit, urethane anaesthesia. The blood pressure response to intravenous injection of 50 mg/kg dehydroascorbic acid (DHA) before and after treatment with desmethylimipramine (DI 2 mg/kg I.v.)

To demonstrate the established effect of reserpine, tyramine (2—5 mg/kg) was also given to the reserpine treated animals. A slight rise in the blood pressure could be detected when the larger dose was given. The tyramine response was, however much smaller than that obtained in non reserpinized rabbits.

Imipramine desmethylimipramine and cocaine

Imipramine desmethylimipramine and cocaine affected the pressor response elicited by dehydroascorbic acid injection in rabbits. Pretreatment with low doses of imipramine and desmethylimipramine (0.5 mg/kg) and cocaine (1 mg/kg) reduced the pressor response to injected dehydroascorbic acid. When repeated low doses or single high doses (2 mg/kg) of either Imipramine or desmethylimipramine were given, the pressor response to dehydroascorbic acid was completely abolished (Figs 28 and 29). This was also observed after

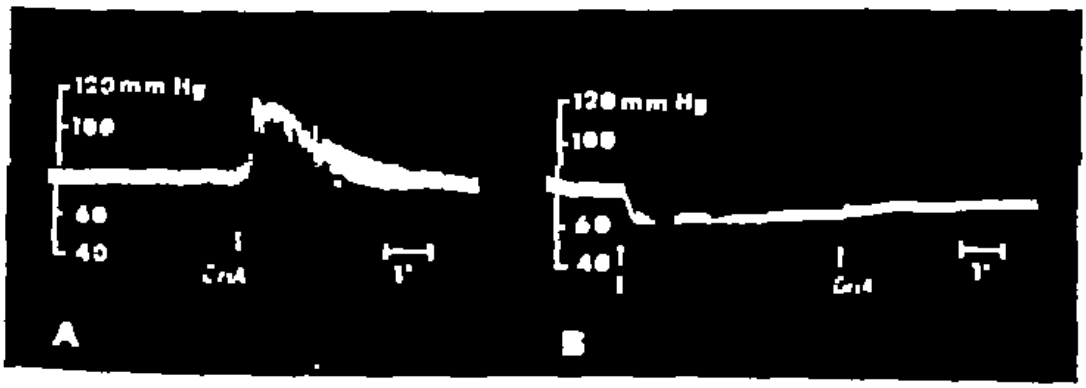


Fig. 29. Rabbit, urethane anaesthesia. The blood pressure response to intravenous injection of 50 mg/kg dehydroascorbic acid (DHA) before (A) and after (B) treatment with imipramine (I 2 mg/kg I.v.)



Fig. 30. Rabbit, urethane anesthesia. The blood pressure response to intravenous injection of 50 mg/kg dehydroascorbic acid (DHA) before (A) and after (B) treatment with cocaine (20 mg/kg i.

premedication with high doses (40 mg/kg) of intravenously administered cocaine (Fig. 30). The cocaine effect, however, was of rather short duration compared to that of the other 3 substances tested. In 1 cat dehydroascorbic acid was given after intravenous premedication with desmethylimipramine. In this experiment it was shown that the pressor response to dehydroascorbic acid was strongly reduced.

Acetylcholine

In the cat experiment it was observed that administration of dehydroascorbic acid in doses up to 50 mg/kg did not noticeably affect the depressor response caused by acetylcholine injection. However, when the dose was increased to 100 or 150 mg/kg the depressor response of acetylcholine was significantly enhanced. The level of the depression was increased and the duration was prolonged (Fig. 31). When 5 minutes had passed after dehydroascorbic acid administration the acetylcholine response was identical with that obtained before the dehydroascorbic acid injection.

In the rabbit injection of 50 mg/kg or more of dehydroascorbic acid changed the response of the blood pressure to acetylcholine. Prior to dehydroascorbic acid injection, administration of acetylcholine caused a fall in blood pressure which rapidly returned to normal. However, after the dehydroascorbic acid injection, when the pressor response had returned to the normal level, injection of acetylcholine caused a hypotensive response of the same magnitude as before but the blood pressure returned more slowly to the normal level. The duration of this depressor response of acetylcholine increased with the dose of dehydroascorbic acid injected. Acetylcholine injected during the pressor phase

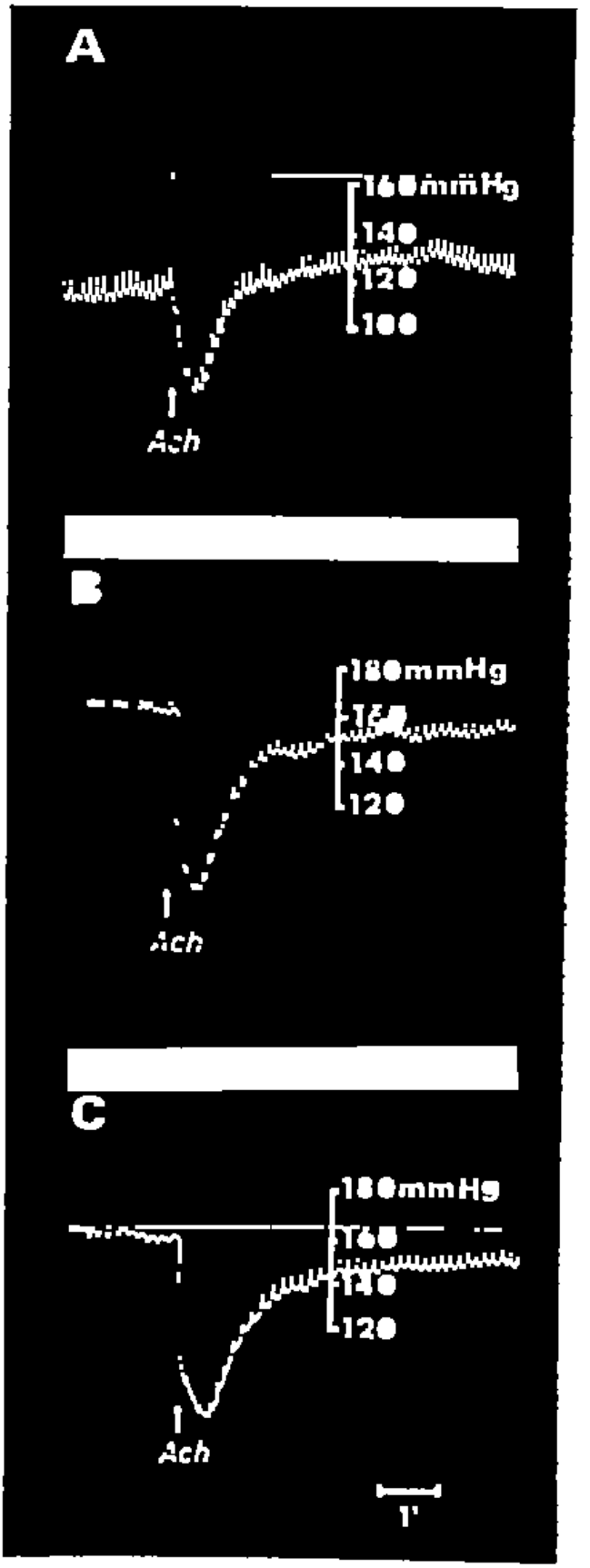


Fig. 31 Cat, chloralose-urethane anaesthesia. The effect of dehydroascorbic acid on the depressor response to acetylcholine. A the response to intravenous injection of 1 µg acetylcholine (Ach.) B the response to the same dose of acetylcholine after intravenous injection of 100 mg/kg dehydroascorbic acid. C the response to the same dose of acetylcholine after intravenous injection of 150 mg/kg dehydroascorbic acid.

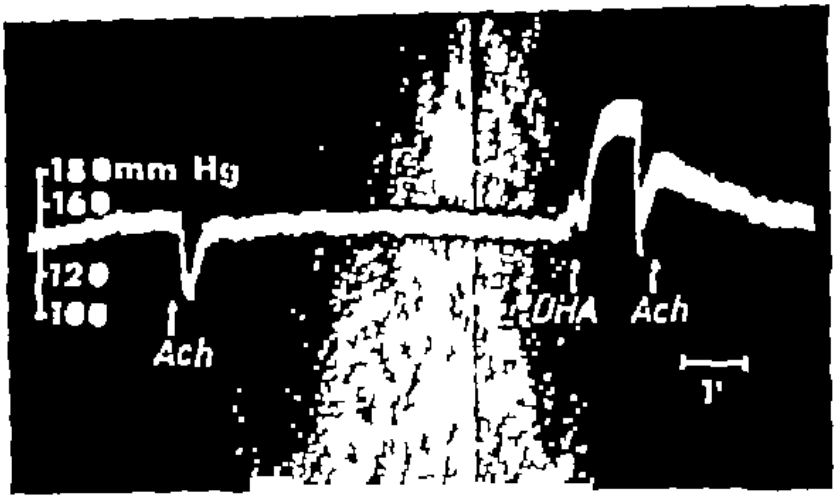


Fig. 32. Rabbit, urethane anaesthesia. The effect of dehydroascorbic acid (DHA 50 mg/kg i. v.) on the depressor response to acetylcholine (Ach 1 µg i.v.) the latter injected during the pressor phase of dehydroascorbic acid

of dehydroascorbic acid (150 mg/kg) caused a significantly larger fall than both before the administration of dehydroascorbic acid and after the pressor response had returned to the normal level (Fig. 32).

The response to dehydroascorbic acid on the blood pressure was not changed when given immediately after a single injection or after repeated administrations of acetylcholine.

Discussion

In the present investigation it was found that several compounds interfering with adrenergic mechanisms at different sites and by different actions changed the pressor response to injected dehydroascorbic acid.

After ganglion blocking with hexamethonium the pressor response following dehydroascorbic acid was partly reduced. Total blocking never occurred and this corresponds with observations by Patterson and Mastin (1951). In order to ascertain whether the failure of hexamethonium to abolish the pressor response to dehydroascorbic acid was due to an incomplete ganglion block, tetraethylammonium which has a potent ability to interrupt transmission in sympathetic ganglia (Moe and Freyburger 1950) was also used. After treatment with this drug a complete inhibition of the blood pressure response of dehydroascorbic acid was observed. These results show that the pressor effect of dehydroascorbic acid is not evoked from sites below ganglionic level (compare Chapter I Part b).

The pressor effect of dehydroascorbic acid was blocked by premedication with guanethidine and bretylium. Both substances are known to block adrener-

gic transmission (for references see Cass and Spriggs 1961 Chang *et al.* 1967) It is thought that they exert their adrenergic neuron blocking activity by means of different mechanisms of action and perhaps at different sites (Boura and Green 1962 Brodie *et al.* 1965) It is presumed that they prevent the liberation of adrenergic transmitter from nerve endings (Maxwell *et al.* 1959 1960 a, b Boura and Green 1959) As the pressor response to injected dehydroascorbic acid was blocked by guanethidine and bretylium it may be suggested that dehydroascorbic acid causes activation of adrenergic nerves, similar to electrical nerve stimulation (Kurpekar *et al.* 1969)

It is evident that the pressor response observed after dehydroascorbic acid injection is the result of an activation of adrenergic α-receptors since the hypertensive response is abolished by the blocking of these receptors. This is *in agreement with observations by Patterson and Martin (1951) and* Wegman (1958) After an α-receptor blockade dehydroascorbic acid injection resulted in a hypotensive response in some of the tested animals. Similar response of dehydroascorbic acid injection was observed in rats by Patterson and Martin (1951) In the present investigation it was found that this depressor response was strongly reduced by premedication with propranolol, a β-receptor blocking agent. This suggests that the adrenergic β-receptors also are activated following dehydroascorbic acid administration.

In the reserpine treated rabbits the pressor effect of dehydroascorbic acid was abolished. Reserpine treatment leads to a strongly impaired adrenergic transmission (Carlsson *et al.* 1957) At the same time there is a marked depletion of noradrenaline. Reserpine causes a blockade of the uptake and storage mechanism in the amine storage granules (Hillarp and Malmfors 1964 Lindmar and Muscholl 1964 Malmfors 1965 Carlsson and Waldeck 1965) The membrane pump mechanism is not affected by reserpine (Malmfors 1965)

The abolished pressor response to dehydroascorbic acid in the reserpinized animals is interpreted as being a consequence of decreased amounts of adrenergic transmitter available for release. In this respect the blood pressure response to dehydroascorbic acid injection is very similar to electrical or chemical stimulation of sympathetic nerves, this response to stimulation being blocked by prior reserpine treatment (Bertler *et al* 1956 Muscholl and Vogt 1958)

A complete inhibition of the pressor response to injected dehydroascorbic acid was observed after treatment with some so called membrane pump blocking agents, Imipramine, desmethylimipramine and cocaine. These substances are supposed to block the cellular uptake of noradrenaline (Whitby *et al* 1960 Dengler *et al.* 1961 Muscholl 1961 Titus *et al.* 1966) It is believed that certain drugs which release intraneuronal noradrenaline are

transported by the membrane pump to reach their site of action e.g. tyramine (for references see Ross 1967). The results from the present experiments suggest that the pressor effect of dehydroascorbic acid is brought about by its being taken up into the adrenergic nerve cells by means of the imipramine-desmethylimipramine- and cocaine-sensitive transport mechanism, and that dehydroascorbic acid thus behaves like an indirectly acting sympathomimetic compound. The probable site and mechanism of action of dehydroascorbic acid will be further discussed and put in relation to other experimental data obtained (see General Discussion).

Earlier it was shown that intravenous injection of high doses of dehydroascorbic acid facilitated neuromuscular transmission in the cat (Chapter I Part 4). In the present experiments it was found that the depressor response to acetylcholine was enhanced after treatment with dehydroascorbic acid in cats and rabbits. It is supposed that there is a common mechanism of action of dehydroascorbic acid in the two different investigations mentioned. In both cases the effect of dehydroascorbic acid is plausibly related to its cholinesterase-inhibiting action (Chapter V). As early as 5 minutes after the injection, dehydroascorbic acid had obviously lost its capacity to potentiate the depressor response caused by acetylcholine. This may be explained by the fact that dehydroascorbic acid is rapidly reduced in the organism forming ascorbic acid (Patterson and Mastin 1951, Hammarström 1966) which is devoid of cholinesterase-inhibiting action (Chapter V). It may also be pointed out that the cholinesterase-inhibiting action of dehydroascorbic acid is weak and consequently fairly high doses are required to produce a demonstrable effect in the organism.

CHAPTER III

Effects of Various Compounds on the Accumulation of ^{14}C-Dehydroascorbic Acid and ^{14}C-Ascorbic Acid in Brain and Heart Tissues of Mice *in vivo*

The pressor effect observed after injection of dehydroascorbic acid was shown to be inhibited by prior treatment with so called membrane pump blocking agents (Chapter II) This inhibition was interpreted as being due to the blocking of dehydroascorbic acid uptake at the intraneuronal transmitter sites. The pressor effect was also found to be inhibited by prior reserpine treatment (Chapter II) It was thus suggested that dehydroascorbic acid (or metabolically formed ascorbic acid) releases noradrenaline from reserpine-sensitive amine storage granules. In order to affect the noradrenaline content of these granules dehydroascorbic acid (or metabolically formed ascorbic acid) has to pass the adrenergic cell membrane and reach the storage sites at the granular membrane.

The aim of the present experiments was to study the influence of certain membrane pump blocking agents and reserpine on the tissue uptake of labelled dehydroascorbic acid and ascorbic acid. The uptake was studied in brain and heart tissues of mice. The brain was chosen because the pressor effect of dehydroascorbic acid is mainly of central origin (Chapter I Part 6) and the effect is probably due to an interference with the intraneuronal transmitter in this tissue. The heart was chosen for studying the uptake in a peripheral tissue with known adrenergic activity

Materials and methods

Radioactive compounds

Ascorbic acid-1 ^{14}C with a specific activity of 3.36 millicuries per millimole was obtained from New England Nuclear Corporation, Boston, Mass., USA. The radiochemical purity was tested by chromatography (N.E.N.) and was found to be 99 % (see below) The radioactive ascorbic acid was dissolved in redistilled water to give a concentration of 0.26 mg/ml (corresponding to 5 μCi/ml)

For preparation of labelled dehydroascorbic acid 2.6 mg (50 μCi) of C-ascorbic acid was dissolved in 10 ml redistilled water Benzoquinone (1.6 mg) dissolved in 10 ml ether was added and the mixture was shaken for 15

minutes. The ether phase was removed and the water phase was washed 3 times with ether. The final dehydroascorbic acid concentration was estimated to be 0.26 mg/ml (corresponding to 5 μci/ml).

Chromatography

The purity of the prepared solutions of ^{14}C-dehydroascorbic acid and ^{14}C-ascorbic acid was checked by thin-layer chromatography and by subsequent autoradiography (Hammarström 1966).

One to 5 μl of each solution was spotted under a stream of nitrogen on 250 μ thick silica gel thin-layer plates for chromatography. The plates were prepared as described by Stahl (1967). Silica gel H and 3 % metaphosphoric acid were used for the preparation. The plates were activated by heating at 120° C for 1 hour.

The solvent system for thin-layer chromatography used was benzene-methanol-acetone-acetic acid (70 : 20 : 5 : 5). The development of the chromatogram was performed under nitrogen. The front was allowed to run approximately 14 cm.

Non-radioactive ascorbic acid and dehydroascorbic acid were always run on each plate as reference substances. After development of the chromatograms these were located by spraying the plates with 2,6-dichlorophenol indophenol in 96 % ethanol (w/v) and 0.1 % 2,4-dinitrophenylhydrazine in 96 % ethanol (w/v) to which had been added 1 ml concentrated HCl.

The radioactive compounds were located by autoradiography. The plates were pressed against Kodirex (Kodak) or Structurix (Gevaert) X-ray film. The exposure time was 1—2 weeks. Then the radioactive spots were scraped off with a razor blade into liquid scintillation vials and counted in a Packard Tri-Carb liquid scintillation spectrometer as a suspension in 15 ml of 0.5 % 2,5-diphenyloxazole + 0.03 % 1,4-bis-2(4-methyl-5-phenyloxazolyl)benzene + 4 % silica gel (Aerosil®) in toluene (Snyder and Stephens 1962). Correction for quenching was made by the external radiation source of the spectrometer.

When the ^{14}C-ascorbic acid solution was run 3 radioactive spots were detected. The major spot had the same R_f value as non-radioactive ascorbic acid, one spot corresponded to dehydroascorbic acid (about 10 % of the total radioactivity) and one spot remained at the starting point (5—10 %). Moreover when ^{14}C-dehydroascorbic acid solution was chromatographed 3 spots were observed. The major spot had the same R_f value as non-radioactive dehydroascorbic acid, one spot corresponded to ascorbic acid (5—10 %) and one spot remained at the starting point (5—10 %).

Animals

Seventy-two female mice of NMRI strain with an average body weight of 20 g were used. The mice were fed a complete pelleted diet and water *ad lib* (see Chapter I Part 1)

Experimental procedure

The mice were divided into 12 groups with 6 animals in each group. In some groups the animals were premedicated with various compounds (see Premedication) C-ascorbic acid (1 μci/animal) was given intravenously into a tail vein to each animal in 6 of the groups and ^{14}C-dehydroascorbic acid (1 μci/animal) to the other 6 groups. The mice were sacrificed by stretching the spine 30 minutes after injection of the labelled substance.

The brain stem and the heart were used for analyses. The two tissues from each animal were weighed and placed separately in vials containing 1 ml sample solubilizer (Soluene™ 100 Packard Instrument Company Ill., USA) The samples were heated to +40 C and within 3 hours the tissues were dissolved. To each sample 15 ml toluene-PPO (5 g diphenyloxazole in 1 l toluene) was added. Radioactivity assay was then performed by liquid scintillation of the whole sample in a Packard Tri-Carb liquid scintillation spectrometer

The body weights of the animals varied between 18 and 22 g and no dose correction was made.

No attempts were made to separate C-dehydroascorbic acid and C-ascorbic acid or labelled metabolites of these substances in the tissues.

Premedication

The animals in the 12 experimental groups were premedicated with 4 different compounds, desmethylimipramine (Pertofrin® Geigy 10 mg/kg) cocaine (cocaine chloride 10 mg/kg) protriptyline (protriptyline hydrochloride 10 mg/kg) and reserpine (Serpasil® Ciba 5 mg/kg) These substances were given intraperitoneally 30 minutes prior to intravenous injection of either of the labelled substances. The animals in the 2 control groups were given physiological saline solution intraperitoneally in equivalent volume to the substances mentioned above (0.1 ml/10 g body weight)

Statistical evaluation

Tests of significance were conducted using Student's t test. The level of significance used was $P < 0.05$

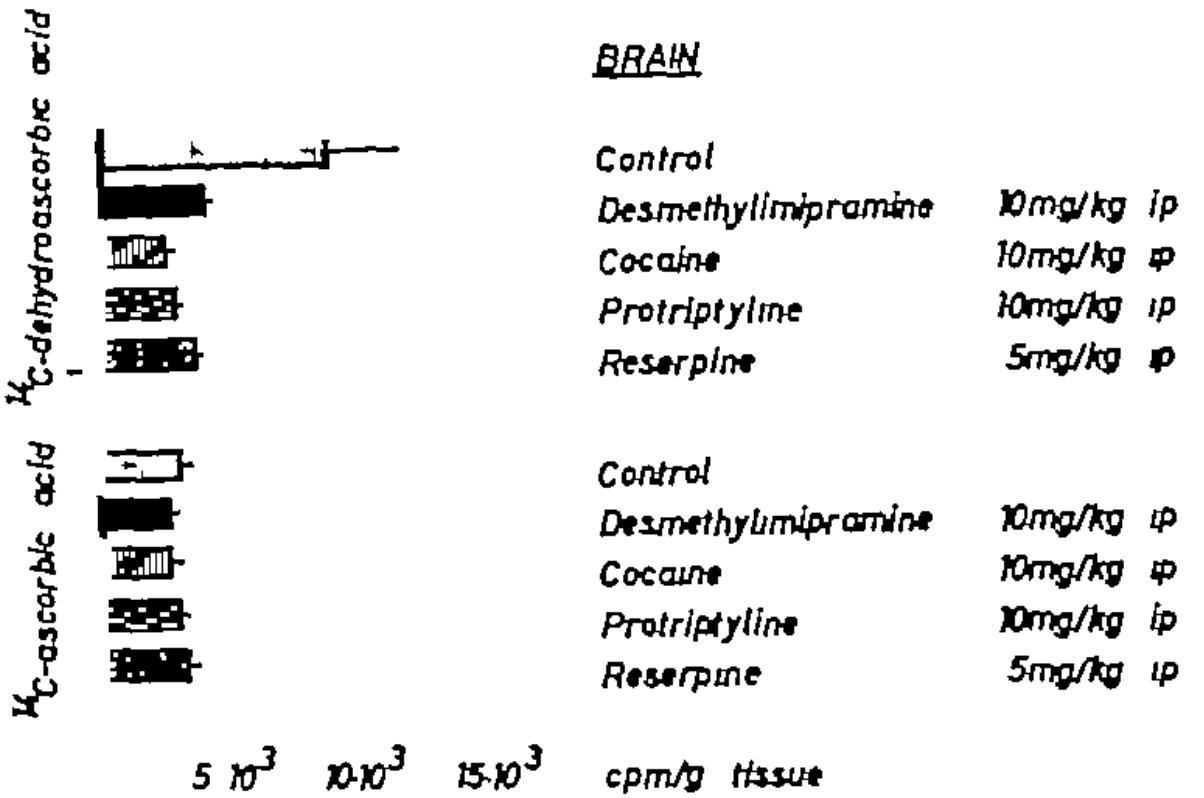


Fig. 33 The total amount of radioactivity per gram tissue in brains of mice 30 minutes after intravenous injection of 1 μci ^{1} C-dehydroascorbic or ^{14}C-ascorbic acid. Each value represents the mean of 6 determinations (standard error of the mean horizontal bars) Some groups of animals were premedicated with various compounds (see text) 30 minutes prior to injection of the labelled substance.

Results

Figs 33 and 34 show the total amount of radioactivity per gram tissue (wet weight) in brains and hearts of mice injected with labelled dehydroascorbic acid or ascorbic acid. The recovered amounts of radioactivity 30 minutes after injection in these 2 tissues were considerably larger after administration of ^{14}C-dehydroascorbic acid than after injection of ^{1} C-ascorbic acid.

After premedication with desmethylimipramme, cocaine protriptyline and reserpine, significantly smaller amounts ($P < 0.05$) of radioactivity were recovered in both tissues following injection of labelled dehydroascorbic acid. In brains and hearts of animals injected with labelled ascorbic acid, on the other hand, no significant differences were observed.

The concentration of radioacti ity in both tissues of premedicated animals injected with labelled dehydroascorbic acid was almost equal to that noted in the tissues of the control animals injected with ^{1}C-ascorbic acid.

The differences in recovered amounts of radioactivity between the premedicated groups given ^{14}C-dehydroascorbic acid were not statistically significant.

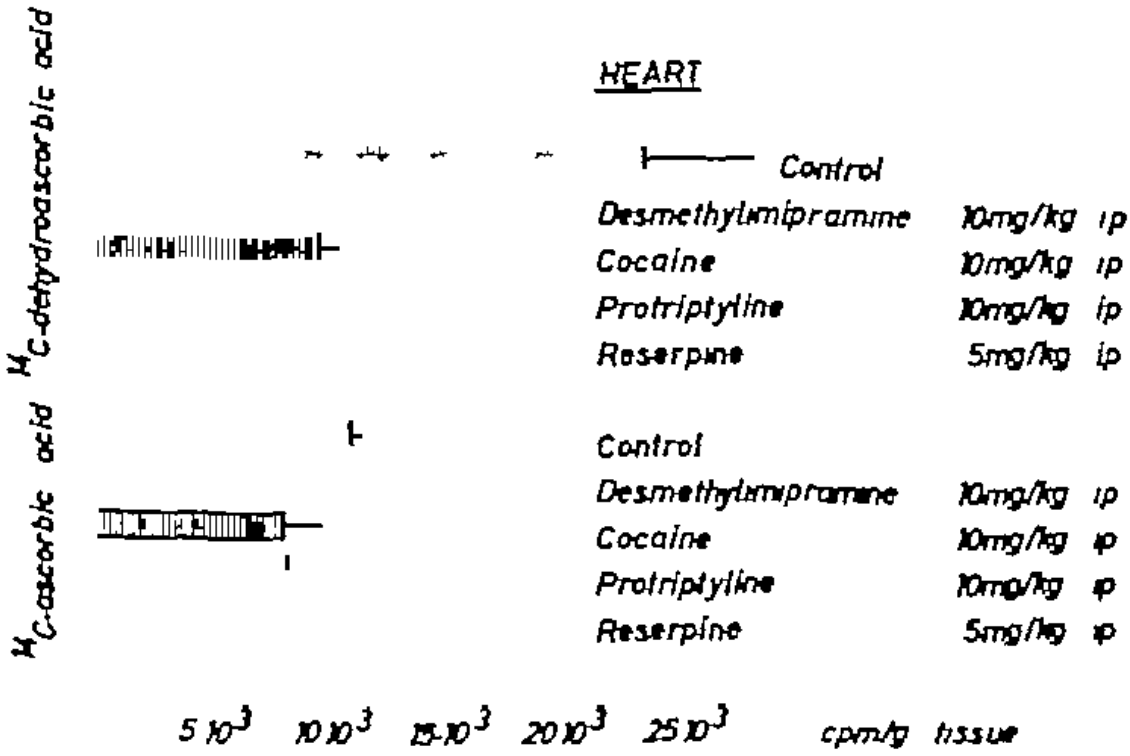


Fig. 34 The total amount of radioactivity per gram tissue in hearts of mice 30 minutes after injection of ^{14}C-dehydroascorbic acid or ^{14}C-ascorbic acid. For further details see Fig. 33 and text.

Discussion

The decreased amount of radioactivity after injection of ^{14}C-dehydroascorbic acid recovered from brain and heart tissues of animals pretreated with desmethylimipramine, cocaine and protriptyline compared to that found in corresponding tissues of control animals is interpreted as a decreased uptake of radioactivity. Desmethylimipramine, cocaine and protriptyline are potent inhibitors of amine uptake through the adrenergic nerve cell membrane, the so called membrane pump (Hillarp and Malmfors 1964 Lindmar and Muscholl 1964 Carlsson and Waldeck 1965 Carlsson *et al.* 1966). In the present experiments it was noticed that all 3 blocking compounds inhibited the uptake of ^{14}C-dehydroascorbic acid to the same extent.

From the present results it may be suggested that the membrane pump blocking agents inhibit the transfer of C-dehydroascorbic acid across the adrenergic cell membrane. This is compatible with the observation that the pressor response of injected non-labelled dehydroascorbic acid is inhibited by prior treatment with membrane pump blocking agents (Chapter II). The uptake is equally inhibited in both brain and heart tissues which suggests that ^{1} C-dehydroascorbic acid is taken up by the same mechanism in both tissues.

Presumably the inhibition was complete, since the amount of radioactivity recovered after premedication was almost identical with that found in animals injected with ^{14}C-ascorbic acid. The latter compound is not supposed to be taken up by the same mechanism (see below)

After premedication with reserpine a smaller amount of radioactivity was recovered from both tissues than from those of the controls following injection of ^{14}C-dehydroascorbic acid. It is well established that reserpine blocks the transmitter uptake and storage mechanisms in amine granules (Hillarp and Malmfors 1964 Lindmar and Muscholl 1964 Malmfors 1965 Carlsson and Waldeck 1965) From the present results it is assumed that these mechanisms must be intact in order to fully concentrate radioactivity in brain and heart tissues of animals injected with ^{1} C-dehydroascorbic acid.

Heath and Fiddich (1966) studied the uptake of labelled dehydroascorbic acid in the rat retina *in vitro* and found that 75 % of the dehydroascorbic acid which entered the cells was reduced to ascorbic acid intracellularly Autoradiographic investigations with labelled dehydroascorbic acid have shown that the injected substance is rapidly reduced to ascorbic acid in the tissues and then retained for several days in the nervous tissue (Hammarström 1966) Nichelmann *et al.* (1966) showed that ascorbic acid is bound to a protein in the nervous tissue. It has been suggested that ascorbic acid is required for dopamine β-hydroxylation (Kaufman and Friedman 1965) The hydroxylating enzyme has been shown to be attached to the amine storing granules *in vitro* (Stjärne and Lishajko 1967) and it is possible that the injected ^{14}C-dehydroascorbic acid may be bound to the granular membrane most probably in the form of ascorbic acid. In the present experiments this binding mechanism may be inactivated by the reserpine treatment which might explain the differences in recovered radioactivity in the tissues between reserpine premedicated and control animals after injection of ^{14}C-dehydroascorbic acid.

The amount of radioactivity recovered from brain and heart tissues of animals *pretreated with any of the compounds after injection of* ^{14}C-*ascorbic* acid was almost the same as that found in the control animals. Based on this observation it is concluded that ^{14}C-ascorbic acid is not taken up in the cells by *the same mechanism as* ^{14}C-*dehydroascorbic acid. The amount of radio*activity observed in these tissues is most probably derived from extracellularly located radioactivity or from ^{14}C-ascorbic acid taken up by some other mechanism.

CHAPTER IV

Effects of Dehydroascorbic Acid and Ascorbic Acid on Levels of Endogenous Noradrenaline and Dopamine in the Brain and of Noradrenaline in the Heart of Mice

It has been shown that injection of dehydroascorbic acid to various species is followed by responses indicating that adrenergic mechanisms are involved, preferentially in the brain (Patterson and Martin 1951 Wegman 1958, Chapters I and II) The rise in the blood pressure caused by injected dehydroascorbic acid has been shown to be blocked by drugs interfering with the uptake of dehydroascorbic acid into brain and heart tissues (Chapters II and III)

The aim of the present experiments was to determine whether dehydroascorbic acid and/or ascorbic acid were able to release noradrenaline from brain and heart tissues and if they caused any changes of the normal levels of noradrenaline and dopamine. The dopamine content in the heart was too small to be detected by the method used and therefore only the amount of dopamine in the brain has been estimated.

Materials and methods

Animals

Female mice of NMRI strain were used. The average body weight was 20 g The mice were fed a complete pelleted diet (AB Ewos) and water *ad lib*

Experimental procedure

The experiments were performed on 87 groups of mice (6 animals in each group) The mice were injected intravenously in a tail vein with dehydroascorbic acid (obtained by ascorbic acid oxidation) or ascorbic acid. These solutions were prepared as described in Chapter I Part 1 and diluted with redistilled water Each animal was given 0.1 ml of either solution. Control animals were injected intravenously with the corresponding volume of redistilled water

The mice were sacrificed by stretching the spine and the heart and brain were rapidly removed from each animal. Six organs (1 group) were pooled in each sample, weighed and homogenized in 0.4 N perchloric acid (10 and 1 ml for the heart and brain samples respectively) by an Ultra-Turrax™ homo-

genizer After centrifugation and neutralization (K_2CO_3) cation exchange chromatography was performed (Dowex 50 W—X 4) Noradrenaline and dopamine were eluted and determined in a spectrophotofluorometer (Aminco —Bowman) One determination per group. The recovery of noradrenaline and dopamine after elution was calculated to be 90 % and 80 % respectively The conversion of noradrenaline and dopamine to fluorescent compounds was calculated to be 100 % for both substances. The methods of analysis are described in detail by Bertler *et al* (1958) (noradrenaline) and Carlsson and Waldeck (1958) *modified by Carlsson and Lindquist* (1967) *(dopamine)*

In a first series of experiments varying doses of dehydroascorbic acid (10 50 100 and 125 mg/kg) or ascorbic acid (50 100 150 and ?00 mg/kg) were injected into 32 groups of mice. The survival time was 60 minutes. Eight groups served as controls.

In a second series of experiments, 100 mg/kg dehydroascorbic acid was injected intravenously into 16 groups of mice which were sacrificed at different time intervals (5 30 60 and 120 minutes) after the administration. Four groups served as controls.

Each value in the results from these two series of experiments is the mean of 4 different determinations ± standard error of the mean.

In a third series of experiments, ?7 groups of mice were premedicated with 500 mg/kg of a methyl ester of α methyltyrosine (H 44/68) given by intra peritoneal injection 3 hours prior to intravenous injection of 100 mg/kg dehydroascorbic acid or ascorbic acid. H 44/68 is a potent inhibitor of cate cholamine synthesis at the tyrosine-dopa step (Corrodi and Malmfors 1966) The survival time was 30 minutes after the last injection. Three of the groups were controls (Table 7)

Statistical evaluation

Tests of significance were conducted using Student s t test. The level of significance used was $P < 0.05$

Results

Table 4 illustrates the recovered amounts of endogenous noradrenaline in the brain and heart 60 minutes after injection of various doses of dehydroascorbic acid and ascorbic acid. It was noticed that injection of ascorbic acid caused no changes at all in the noradrenaline content In either of the tissues. In the hearts of animals gi ven dehydroascorbic acid a slight increase with the dose up to 100 mg/kg was observed. However the differences in the noradrenaline concentration between the treated and control groups were not found to be statistically significant.

TABLE 4 Endogenous levels of noradrenaline (NA) (μg/g tissue) in hearts and brains of mice 60 minutes after intravenous injection of different doses of dehydroascorbic acid or ascorbic acid. The values are means of 4 determinations ± S.E.M.

Dehydroascorbic acid			Ascorbic acid	
Dose mg/kg	Heart NA	Brain NA	Heart NA	Brain NA
Control	0.72 ± 0.06	0.42 ± 0.02	0.85 ± 0.03	0.43 ± 0.01
10	0.74 ± 0.05	0.44 ± 0.02		
50	0.81 ± 0.05	0.48 ± 0.05	0.85 ± 0.07	0.46 ± 0.02
100	0.82 ± 0.08	0.38 ± 0.03	0.84 ± 0.05	0.47 ± 0.03
125	0.72 ± 0.09	0.39 ± 0.04		
150			0.88 ± 0.05	0.42 ± 0.02
200			0.89 ± 0.05	0.40 ± 0.01

The noradrenaline level in the heart tended to increase slightly with increase in survival time after administration of dehydroascorbic acid (Table 5) The amount of noradrenaline recovered from the brain, however was not found to be changed.

The dopamine content in the brains of animals given ascorbic acid was found to be almost identical with that of the controls (Table 6) In the dehydroascorbic acid treated animals on the other hand the dopamine content tended to increase slightly with the dose and also with increase in survival time (Tables 5 and 6)

TABLE 5. Endogenous levels of noradrenaline (NA) (μg/g tissue) in brains and hearts of mice and of dopamine (DA) (μg/g tissue) in brains of mice after injection of 100 mg/kg dehydroascorbic acid to mice sacrificed t different survival times after the injection. The values are means of 4 determinations ± S.E.M.

Survival time (min)	Brain NA	Heart NA	Brain DA
Control	0.39 ± 0.02	0.61 ± 0.03	0.59 ± 0.04
5	0.38 ± 0.03	0.60 ± 0.06	0.55 ± 0.03
30	0.45 ± 0.03	0.67 ± 0.06	0.61 ± 0.03
60	0.38 ± 0.02	0.70 ± 0.05	0.77 ± 0.03[a]
120	0.41 ± 0.01	0.76 ± 0.06[b]	0.73 ± 0.07

[a] $P < 0.10$
[b] $P < 0.05$

TABLE 6. Endogenous levels of dopamine (DA) (μg/g tissue) in brains of mice 60 minutes after intravenous injection of different doses of dehydroascorbic acid or ascorbic acid. The values are means of 4 determinations ± S.E.M.

Dose mg/kg	Dehydroascorbic acid	Ascorbic acid
Control	0.73 ± 0.05	0.75 ± 0.02
10	0.75 ± 0.07	
50	0.80 ± 0.07	0.72 ± 0.03
100	0.84 ± 0.08	0.73 ± 0.03
125	0.88 ± 0.10	
150		0.73 ± 0.07
200		0.77 ± 0.06

In control animals treated with H 44/68 3 ½ hours before sacrifice, the contents of noradrenaline and dopamine in the brain were noticeably reduced and the noradrenaline level in the heart was slightly decreased compared to non-treated control animals. This indicates a blocking of the catecholamine synthesis in the two organs.

After premedication with H 44/68, injection of 100 mg/kg of dehydroascorbic acid caused a marked decrease of the amount of endogenous noradrenaline in both brain and heart tissues (Table 7). The dopamine level was the same in the treated animals as in the control animals. Administration of the same dose of ascorbic acid did not change the amounts of noradrenaline and dopamine in either of the two tissues.

TABLE 7. Endogenous levels of noradrenaline (NA) (μg/g tissue) in brains and hearts and of dopamine (DA) (μg/g tissue) in brains 30 minutes after an intravenous injection of 100 mg/kg dehydroascorbic acid or ascorbic acid to mice. All the animals were premedicated with H 44/68 (500 mg/kg i.p.) 3 ½ hours before sacrifice. The values are means ± S.E.M. The number of determinations is shown in brackets.

	Brain NA	Heart NA	Brain DA
Control	0.21 ± 0.02 (3)	0.61 ± 0.05 (3)	0.23 ± 0.01 (3)
Dehydroascorbic acid	0.16 ± 0.01[1] (1)	0.51 ± 0.01[1] (12)	0.23 ± 0.01 (12)
Control	0.17 ± 0.01 (3)	0.50 ± 0.03 (3)	0.26 ± 0.02 (3)
Ascorbic acid	0.18 ± 0.01 (11)	0.51 ± 0.02 (12)	0.26 ± 0.02 (11)

[1] $P < 0.05$

Discussion

In the present investigation it was observed that the recovered amount of endogenous noradrenaline in the brain of animals injected with either dehydroascorbic acid or ascorbic acid was almost identical with that of the control animals. When the catecholamine synthesis was blocked with H 44/68 injection of dehydroascorbic acid resulted in a pronounced decrease in the level of endogenous noradrenaline. Administration of ascorbic acid on the other hand did not appear to influence the noradrenaline level. These results indicate that injection of dehydroascorbic acid is followed by a release of brain noradrenaline. It is assumed that a similar noradrenaline release occurs in other species and may explain the central adrenergic effects of injected dehydroascorbic acid described earlier (Chapter I, Parts 2, 5 and 6)

The fact that the noradrenaline level was unchanged when the catecholamine synthesis was intact is most certainly due to the circumstance that the release caused by injected dehydroascorbic acid is compensated by new formation of noradrenaline, indicating an increased turnover of noradrenaline. It has been considered that increased activity in adrenergic nerves produced by e.g. electrical stimulation has very little or no effect on their noradrenaline level (Euler and Hellner Björkman 1955) due to effective mechanisms for keeping a constant level of transmitter concentration.

The noradrenaline levels in the hearts of normal and premedicated animals were affected in a similar manner as observed in the brains after injection of dehydroascorbic acid and ascorbic acid. This shows that injection of dehydroascorbic acid is also followed by a release of noradrenaline in the heart. This observation and the fact that the noradrenaline level in the heart was unchanged after injection of dehydroascorbic acid into animals with intact catecholamine synthesis, indicate that dehydroascorbic acid causes an increase in noradrenaline turnover in the heart. Injected ascorbic acid, however did not seem to possess any releasing action on the heart noradrenaline.

In Chapter III it was suggested that dehydroascorbic acid was taken up into adrenergic nerve cells in the brain and heart tissues of the mouse and that the uptake of ascorbic acid on the other hand was more doubtful. The present results support this suggestion, since injected dehydroascorbic acid, by contrast to injected ascorbic acid, clearly interferes with the noradrenaline content in both tissues.

CHAPTER V

Effects of Dehydroascorbic Acid and Ascorbic Acid on Cholinesterases *in vitro*

Some of the effects of injected dehydroascorbic acid, e.g. facilitation of neuromuscular transmission (Chapter I Part 4) were thought to be due to an interaction with cholinergic mechanisms. This facilitatory effect was only seen when high doses of dehydroascorbic acid were given intravenously showing an action similar to cholinesterase inhibitors (see review by Karczmar 1967). Administration of ascorbic acid on the other hand did not appear to cause facilitation of neuromuscular transmission.

It has also been observed that injection of high doses of dehydroascorbic acid potentiates the depressor response to injected acetylcholine in cats and rabbits (Chapter II) suggesting an inhibition of cholinesterases by dehydroascorbic acid (or metabolically formed ascorbic acid).

The aim of the present investigation was to determine whether dehydroascorbic and/or ascorbic acid were able to inhibit cholinesterases. The investigation was performed on blood cholinesterases in man, cat and rabbit by using an *in vitro* method.

Materials and methods

The presumed inhibitory effect of dehydroascorbic acid (obtained by ascorbic acid oxidation) on cholinesterases was determined by an electrometric routine method (Tammelin 1953) using erythrocytes and plasma from man, cat and rabbit. Ascorbic acid was tested only on human cholinesterases in erythrocytes and plasma. For preparation of dehydroascorbic acid and ascorbic acid see Chapter I Part 1.

Erythrocytes and plasma from human, cat and rabbit blood were used as sources of acetylcholinesterase and butyrylcholinesterase respectively and were prepared from heparinized blood by centrifugation. The red blood cells were washed 3 times with physiological saline solution and suspended in distilled water in the proportion 1 + (v/v). The plasma was also diluted with distilled water in the proportion 1 + (v/v). One ml of the erythrocyte or plasma solution was used for each determination. The cat erythrocytes, however had a low normal cholinesterase activity.

The enzyme preparations were incubated at 25 C for about 10 minutes in

test tubes with different concentrations of either dehydroascorbic acid (10^{-3}—10^{-5} M) or ascorbic acid (up to 2×10^{-3} M) To the incubation tubes were also added 3 ml of a veronal buffer (pH 8.2) (Michel 1949) and distilled water to a final volume of 6 ml.

Acetylcholine iodide was used as a substrate for the cholinesterases. Of a standard solution of acetylcholine iodide (2.2 g/100 ml) 0 6 ml was added to the incubation solution after 30 minutes incubation of the enzyme with the inhibitor The acetylcholine concentration was 7.3 mM.

One of the incubation solutions of each run (comprising 6 determinations) served as a control and therefore no inhibitor" was added.

The hydrolysis of acetylcholine by the cholinesterases was recorded as the pH changes in the reaction vessels, caused by the liberated acetic acid. Spontaneous hydrolysis was corrected for The inhibitory effect was expressed as the remaining cholinesterase activity in per cent of the control activity in the presence of different concentrations of inhibitor The inhibitory power was expressed as pI_{50} (the negative logarithm of the inhibitor concentration giving 50 % inhibition)

Each value presented in the results is the mean of 2 determinations.

Results and comments

Determinations of the inhibitory effect of dehydroascorbic acid and ascorbic acid on cholinesterases showed that ascorbic acid had no effect on either acetylcholinesterase (erythrocytes) or butyrylcholinesterase (plasma) from human blood. The highest concentration of ascorbic acid tested was 2×10^{-3} M. Dehydroascorbic acid on the other hand showed slight inhibitory action on both kinds of cholinesterases in human and rabbit blood. The cholinesterase inhibiting effect, expressed as pI_{50} of dehydroascorbic acid in erythrocytes and plasma in the different species are presented in Table 8. Cholinesterase in rabbit erythrocytes was inhibited somewhat more than in the plasma. The lowest inhibitory action was found in plasma from cat blood.

It may be pointed out that the observed anticholinesterase properties of dehydroascorbic acid are very weak compared to conventional cholinesterase inhibitors which should inhibit a cholinesterase in concentrations below 10^{-8} M (for comparison see Augustinsson 1963)

It is known that dehydroascorbic acid easily decomposes at room temperature and at neutral pH values (see Chapter I Part 1) This probably means that the dehydroascorbic acid added to the incubation tubes gradually decomposed and consequently the cholinesterase-inhibiting effect observed was lower than it would have been if the dehydroascorbic acid had been protected

TABLE 8. Cholinesterase-inhibiting effect, expressed as pI_{50} of dehydroascorbic acid in erythrocytes and plasma in different species.

Species	Erythrocytes	Plasma
Man	2.8	2.6
Rabbit	3.2	2.3
Cat	~ 3.5	1.7

against decomposition. Erythrocytes also contain a factor which reduces dehydroascorbic acid to ascorbic acid (Panteleeva 1952 Christine *et al.* 1956 Hendry *et al* 1964) thus causing at least a partial decomposition of added dehydroascorbic acid in the erythrocyte preparations. However as mentioned at the beginning of this chapter high doses of dehydroascorbic acid had to be injected *in vivo* in order to visualize interference with cholinergic mechanisms. Consequently the amount of circulating dehydroascorbic acid is fairly high and may inhibit cholinesterases not only in the blood but also at cholinergic transmitter sites, e g the neuromuscular junction in skeletal muscles. This may be a possible explanation of the observed *in vivo* effects after injection of dehydroascorbic acid. It may be noted that ascorbic acid, which appears to be devoid of cholinesterase-inhibiting action was found not to interfere with neuromuscular transmission (Chapter I Part 4)

General Discussion

Earlier investigations (Patterson and Mastin 1951 Wegman 1958) and the present study show that injection of dehydroascorbic acid is followed by some marked pharmacological responses which may be referred to as activation of certain mechanisms in the central and peripheral nervous systems. In the present study it has been observed that injected dehydroascorbic acid interferes with both cholinergic and adrenergic mechanisms in cats and rabbits and the results are compatible with those obtained in other species (Patterson and Mastin 1951 Wegman 1958)

Injected ascorbic acid, on the other hand, did not reveal any conspicuous pharmacological activity on the various parameters studied. Earlier investigations have shown that injected ascorbic acid causes hypertension (Reuter 1938) or hypotension (Kreitmair 1934 Wegman 1958) Wegman (1958) showed that intravenous injection of a strongly acidic solution (pH 2.2) of ascorbic acid resulted in a fall of the blood pressure, but if the solution was buffered to pH 4—6.5 it did not cause any alteration at all in the blood pressure when injected. The transient depressor response observed in the present experiments may be ascribed to the low pH of the injected solution (pH 2.2) or possibly the rather large volume injected (1—3 ml/kg)

The most prominent responses observed after injection of dehydroascorbic acid into both conscious (rabbits and mice) and anaesthetized (rabbits and cats) animals were the pronounced increase in salivation and lacrimation. It was observed that these effects were inhibited by premedication with atropine (Chapter I Part 2) decerebration and spinalization (Chapter I Part 6) These results suggest a central site of action and most probably an interaction with cholinergic mechanisms. The present results are compatible with observations in conscious rats by Patterson and Mastin (1951)

Similar site and mechanism of action of injected dehydroascorbic acid may also be assumed when causing an increase in lacrimation.

In the anaesthetized cats, ear twitching was frequently observed following injection of large doses of dehydroascorbic acid. This phenomenon has been noticed after administration of compounds known to interfere with cholinergic mechanisms such as cholinesterase inhibitors (see Karczmar 1967) and nicotine (Armitage *et al.* 1966 Hall and Rent 1966) Since this action of nicotine is referred to as an activation of central cholinergic mechanisms, it is believed that injected dehydroascorbic acid activates these mechanisms, most probably

by means of cholinesterase inhibition, analogous to the interpretation of some peripheral effects of high doses of injected dehydroascorbic acid.

Facilitation of neuromuscular transmission and increased depressor response to injected acetylcholine were observed following intravenous administration of high doses of dehydroascorbic acid. Similar effects on neuromuscular transmission were also obtained following close intraarterial injection of small doses of dehydroascorbic acid. Both these effects are interpreted as being due to a cholinesterase-inhibiting action of dehydroascorbic acid (Chapter V)

Ascorbic acid, on the other hand, did not affect neuromuscular transmission. Neither does ascorbic acid seem to possess cholinesterase-inhibiting properties, judging from experiments on human blood cholinesterases (Chapter V)

Another pronounced response observed following injection of dehydroascorbic acid was increased motor activity (Chapter I Part 2) This effect was noticed in conscious mice and rabbits as well as in anaesthetized rabbits and cats. Similar response of injected dehydroascorbic acid has earlier been reported in other species such as conscious rats (Patterson and Mastin 1951) and guinea-pigs (Lyhs and Tegeler 1963) The arousal effect in the anaesthetized animals was shown to be inhibited after decerebration or spinal cord section (Chapter I Part 6) demonstrating a central site of action. As mentioned in Chapter I Part the effects in both conscious and anaesthetized animals are thought to be expressions of the same mechanism(s) Facilitation of neuromuscular transmission caused by dehydroascorbic acid is presumably a contributory factor to the observed increase in motor activity

The behavioural pattern (rage and arousal symptoms) observed in the conscious and anaesthetized animals after injection of dehydroascorbic acid is very similar to that observed after chemical stimulation of certain areas in the brain e.g after administration of centrally active cholinesterase inhibitors. The behavioural changes caused by these substances are controlled by atropine (Langley and Kato 1915) The increased motor activity caused by injected dehydroascorbic acid, however was found to be unchanged by atropine treatment, suggesting that dehydroascorbic acid influences other central mechanisms than cholinergic when causing this response.

It has been reported that activation of certain sympathetic centres in the brain evokes similar behavioural patterns (reviewed by Gellhorn 1943) to those observed after administration of dehydroascorbic acid. Electrical stimulation of these areas produces so called sham rage which is characterized by peripheral signs of sympathetic hyperactivity and aggressiveness. Thus stimulation of the amygdaloid nucleus results in facial twitching, alertness, mydriasis, mastication salivation and vasoconstriction (Gunne and Reis 1963) These effects were associated with a lowering of the brain noradrenaline content. Central excita

tion is also observed following morphine treatment, which has been associated with the noradrenaline releasing properties of morphine (Vogt 1954) Since dehydroascorbic acid causes similar responses and since it evidently causes an increased release of noradrenaline in the brain (Chapter IV) a stimulatory effect on central sympathetic centres is suggested as the trigger mechanism. Autoradiographic investigations with labelled dehydroascorbic acid have shown that the substance easily passes the blood-brain barrier and that the gray matter of the brain accumulates more of the labelled substance than the white matter but that the distribution is relatively diffuse (Hammarström 1966)

Histochemical and autoradiographical studies have also shown that ascorbic acid and dehydroascorbic acid are accumulated in peripheral nervous tissue (Clara 1954 Hammarström 1966) Furthermore there appears to be a correlation between the localization of these substances and the sites of catechol amine synthesis (Hammarström 1966) It has also been shown that injection of dehydroascorbic acid causes a rise in blood pressure in a similar manner to indirectly acting sympathomimetic amines (Chapter II) Wegman (1958) presented evidence indicating a slight peripheral sympathomimetic effect on the heart of the cat by injected dehydroascorbic acid. In order to check the possible peripheral action of dehydroascorbic acid and ascorbic acid the influence of these substances on the nictitating membrane was investigated.

When tested on the non stimulated nictitating membrane injected dehydroascorbic acid caused two different types of response, relaxation and constriction. The type of response appeared to be dependent on the anaesthetic used.

The relaxing effect of injected dehydroascorbic acid on the nictitating membrane in pentobarbitone anaesthetized cats was inhibited by atropine and even turned into a constriction. These results may suggest an influence on cholinergic mechanisms by dehydroascorbic acid. Whether the activation is evoked from central or peripheral sites is not known.

After injection of ascorbic acid, on the other hand, no convincing pharmacological effect was observed.

In the chloralose-urethane anaesthetized cats, injection of dehydroascorbic acid caused constriction of the nictitating membrane. This response was greatly reduced after treatment with adrenergic α- and β-receptor blocking compounds, indicating an involvement of adrenergic mechanisms. It was further shown that the constrictor response was inhibited by preganglionic sympathectomy thus indicating a central activation of the sympathetic nerves running to the nictitating membrane.

The observed differences between the response in the pentobarbitone and the chloralose-urethane anaesthetized animals are most probably due to different modifying properties of the anaesthetic on the central nervous system.

Ascorbic acid on the other hand, did not produce sympathommetic effects on the blood pressure when injected (Chapter I Part 5) Neither did it influence the levels of endogenous noradrenaline (Chapter IV) It was also observed that very little of this substance was taken up in brain and heart tissues as compared to dehydroascorbic acid (Chapter III) which is in agreement with earlier observations (Hammarström 1966) The differences in uptake between the two substances may be attributed to different physico-chemical properties, dehydroascorbic acid being more lipoid soluble than ascorbic acid (Grab 1965) It has also been suggested that dehydroascorbic acid is the form for transport of ascorbic acid across cellular barriers (Patterson and Martin 1951 Martin 1961 Martin and Mecca 1961)

From the present study it seems as if dehydroascorbic acid but not ascorbic acid is taken up into adrenergic nerve terminals. Autoradiographic studies have revealed that both substances are distributed fairly equally in the body A significan uptake of both substances has been observed in sympathetic ganglia (Hammarstrom 1966) where the cell bodies of the postganglionic fibres are located and here the amine granules are thought to be synthetised (review by Andén *et al.* 1969) If ascorbic acid really acts as a co-factor at dopamine β-hydroxylation *in vivo* (cf Kaufman and Friedman 1965) it would enter its site of action in the cell bodies. Then it should be transported down the axon together with amine granules (see Andén *et al.* 1969) Laduron and Belpaire (1968) showed that dopamine-β-hydroxylase accompanies the amine granules on their way down the axon. After reaching the nerve terminals the enzyme still functions probably without losing any vital co-factor such as ascorbic acid. When ascorbic acid is utilized it is oxidized to semidehydroascorbic acid (an unstable product with only one of the hydroxyl radicals of ascorbic acid oxidized) or dehydroascorbic acid which is rapidly reduced again, regenerating ascorbic acid (review by Schneider and Staudinger 1964) It would appear improbable that ascorbic acid is lost during participation in this reaction. Thus it may be assumed that there is no need for additional extracellular ascorbic acid for dopamine β-hydroxylation. Consequently there would appear to be no need for dehydroascorbic acid to function as a form of transport for ascorbic acid across adrenergic cell membranes at nerve terminals as has previously been suggested.

Injected dehydroascorbic acid has been shown to be fairly rapidly reduced to ascorbic acid in the tissues (Patterson and Martin 1951 Hammarstrom 1966, Heath and Fiddick 1966) Patterson and Martin postulated that central nervous system stimulation following injection of dehydroascorbic acid is produced by metabolically formed ascorbic acid. However their explanation is rather doubtful for several reasons.

As shown by distribution studies, the metabolically formed ascorbic acid from injected dehydroascorbic acid is retained in nervous tissue for several days (Hammarström 1966) and if due to formed ascorbic acid the pharmacological effects of injected dehydroascorbic acid would be more long lasting. The latent period would also be more prolonged. In the present experiments all the pharmacological responses were transient, lasting for about 2—3 minutes.

Ascorbic acid is thought to participate in catecholamine synthesis and has been shown to function as a co-factor in dopamine β-hydroxylation *in vitro* (reviewed by Kaufman and Friedman 1965). Experimental data have been presented suggesting that ascorbic acid is also involved in this enzymatic reaction *in vivo*. Izquierdo *et al.* (1968) reported that ascorbic acid injected intraperitoneally into rats caused an increase in the endogenous noradrenaline level and a decrease in the dopamine level in various parts of the brain which was associated with an increase in dopamine-β-hydroxylase activity. In the present study injection of dehydroascorbic acid was shown to cause an increased release of noradrenaline in the brain (Chapter IV).

There is evidence suggesting that ascorbic acid is implicated in the storage mechanism of noradrenaline. Thoa *et al.* (1966) observed a reduced ability of the heart of scorbutic guinea pigs to take up and bind injected labelled noradrenaline. They also noticed a reduction of the level of endogenous noradrenaline in these hearts. Recently Sears (1969) found that endogenous noradrenaline concentration in the iris of the scorbutic guinea pig is decreased to about 50 % of the amount of noradrenaline present in the iris of normal animals. In the same investigation it was found that the turnover time of noradrenaline was much shorter in the scorbutic iris than in the control and it was suggested that the scorbutic iris has a reduced storage capacity for noradrenaline. The same conclusion was earlier drawn by Bhagat *et al.* (1966) making the same observations in isolated atria from scorbutic guinea pigs. In the present study it was noticed that injection of dehydroascorbic acid was also followed by an increased turnover of noradrenaline.

The relationship between the pharmacological effects of injected dehydroascorbic acid and the suggested mechanisms of action of ascorbic acid therefore appears to be small. It appears more likely that dehydroascorbic acid enters adrenergic cells at nerve terminals, reaches storage sites for noradrenaline and interferes with the storage mechanism in a different manner to ascorbic acid.

In many respects the action of dehydroascorbic acid is very similar to that of sulphydryl inhibitors. Mercury derivatives, e.g. parachloromercuribenzoate, exert a pronounced releasing activity on granular catecholamines *in vitro*

(D Iorio 1957) Keswani *et al.* (1969) presented strong evidence that the uptake of SH inhibitors by storage granules is a reversible exchange process involving protein-sulphydryl groups. They suggested that SH groups arising from the granule protein serve as anchoring sites for catecholamine complexes (involving ATP and Mg^{++}) The SH inhibitors are believed to engage the sulphydryl sites within the granule, thus reducing the possibility of formation of an ATP—Mg^{++}—catecholamine complex.

It has been reported that dehydroascorbic acid and SH compounds (glutathione, cystein) react to form a complex or addition compound *in vitro* (Drake *et al.* 1942) It is also known that sulphydryl compounds are essential for the reduction of dehydroascorbic acid in the tissues (Hughes 1964 Grimble and Hughes 1967) In red blood cells glutathione is involved in the conversion of dehydroascorbic acid into ascorbic acid, the latter being retained within the corpuscles (Borsook *et al.* 1937 Panteleeva 1952, Hughes 1964) Dehydroascorbic acid is also taken up in leucocytes and reduced, this being inhibited by SH inhibitors (Hendry *et al.* 1964)

It may be assumed that dehydroascorbic acid, like genuine SH inhibitors, occupies granular SH groups thus inhibiting the binding mechanism of noradrenaline. This impairment of the amine storage sites would result in an increased amount of non-bound noradrenaline available for release and consequently leading to an increased response when stimulated. This may be an explanation for the observed sympathomimetic effects (increased sympathetic tone) of injected dehydroascorbic acid.

There seems to be a relationship between sites of high rate of noradrenaline turnover and sites where injected dehydroascorbic acid produces sympathomimetic responses.

The turnover of noradrenaline is much faster in the central than in the peripheral neurons (see Andén *et al.* 1969) Injection of dehydroascorbic acid is followed by a pronounced central activation of efferent adrenergic nerves causing a rise in the blood pressure and constriction of the nictitating membrane. Data are presented suggesting that the pressor effect of injected dehydroascorbic acid is evoked from *adrenergic sites in the central nervous system*. Peripheral adrenergic effects were found to be very small.

From these conclusions the following summary may be made regarding a possible mechanism of the sympathomimetic action of dehydroascorbic acid. It is suggested that injected dehydroascorbic acid is taken up into adrenergic cells and reaches amine storage granules. Here it interferes with the binding mechanism of noradrenaline by causing an impairment of the amine storage sites. Dehydroascorbic acid probably occupies binding sites for noradrenaline resulting in an increased amount of free amine in the granules. When these

granules are activated (physiologically) a larger amount of noradrenaline will be released, resulting in an increased response. Dehydroascorbic acid was found to increase the noradrenaline turnover in both brain and heart tissues, indicating an action at both central and peripheral adrenergic sites. However the pharmacological action of injected dehydroascorbic acid was far more pronounced at central sites. This may be explained by the fact that the normal noradrenaline turnover is much faster in the brain than in the peripheral tissues and therefore more sensitive to changes. It may be possible that dehydroascorbic acid and ascorbic acid play a physiological role in the binding (storage) mechanism of noradrenaline in granules.

Summary

In the present study dehydroascorbic acid and ascorbic acid were injected into various species of laboratory animals (conscious and anaesthetized). The pharmacological responses to both substances were observed in normal and premedicated animals. General pharmacological tests were performed and the parameters studied were motor activity, salivation, lacrimation, tone of the nictitating membrane, sympathetic ganglionic transmission, neuromuscular transmission and blood pressure (Chapter I). The response to injected dehydroascorbic acid following decerebration, spinal cord section and bilateral adrenalectomy was observed (Chapter I). From the results it became evident that ascorbic acid did not cause any conspicuous pharmacological responses. On the other hand, when dehydroascorbic acid was injected, marked pharmacological effects were recorded. Some of these were considered as being due to interaction with cholinergic mechanisms and others with adrenergic mechanisms. Most of the responses following injection of dehydroascorbic acid were found to originate from the central nervous system.

In conscious mice and rabbits administration of dehydroascorbic acid was followed by increased motor activity, salivation and lacrimation. In anaesthetized cats and rabbits an arousal effect, increased salivation and lacrimation were observed. These effects were shown to be evoked in the central nervous system. The increased salivation and lacrimation were inhibited by atropine, indicating involvement of cholinergic mechanisms.

When dehydroascorbic acid was tested on the non-stimulated nictitating membrane two kinds of response were obtained depending on the anaesthetic used. In pentobarbitone anaesthetized cats, injected dehydroascorbic acid caused a relaxation which was converted to a constriction after atropine treatment, suggesting an activation of cholinergic mechanisms. In chloralose-urethane anaesthetized cats, dehydroascorbic acid caused a constriction of the nictitating membrane. This response was inhibited by premedication with adrenergic α- and β-receptor blocking compounds indicating an activation of adrenergic mechanisms. The constrictor response was also shown to be evoked in the central nervous system. Injection of ascorbic acid, on the other hand, caused only an occasional, small constriction of the nictitating membrane.

Injected dehydroascorbic acid seemed to affect the response of the stimulated nictitating membrane in a similar manner to catecholamines. Ascorbic acid, however, did not influence the response to stimulation at all.

Facilitation of neuromuscular transmission was observed after administration of dehydroascorbic acid but not ascorbic acid.

In both cats and rabbits injection of dehydroascorbic acid was followed by a transient rise in the blood pressure. This response was abolished after spinal cord section but did not change after decerebration. Premedication with ganglion blocking compounds inhibited the pressor response to injected dehydroascorbic acid (Chapter II). The results indicated that the hypertensive effect was mainly of central origin most probably evoked in the vasomotor centre in the medulla oblongata.

After injection of dehydroascorbic acid into spinalized cats and rabbits, an increase of the systolic blood pressure was observed. This response was believed to be due to a peripheral action of dehydroascorbic acid at adrenergic sites.

The pressor response was strongly inhibited following treatment with various agents interfering with adrenergic mechanisms reserpine guanethidine, bretylium, desmethylimipramine, imipramine, cocaine, propranolol and dihydroergotamine (Chapter II). The results suggested that dehydroascorbic acid interferes with adrenergic mechanisms causing an increase of the tone maintaining the blood pressure.

The accumulation of ^{14}C-labelled dehydroascorbic acid and ascorbic acid in brain and heart tissues of mice was studied (Chapter III). The labelled substances were injected intravenously and the total amount of radioactivity in each tissue 30 minutes after the injection of either substance was estimated.

Some groups of animals were premedicated with compounds known to inhibit the uptake of sympathomimetic amines desmethylimipramine protriptyline cocaine and reserpine. It was observed that the accumulation of labelled dehydroascorbic acid in both tissues was strongly inhibited by premedication with any of the inhibitory compounds. The recovered radioactivity in organs from animals given labelled ascorbic acid did not change after treatment with any of the compounds. Based on these observations and those showing that the pressor response to injected dehydroascorbic acid was inhibited by premedication with the inhibitory compounds. It was suggested that dehydroascorbic acid enters its site of action (most probably adrenergic amine granules) by a mechanism which may be blocked by the compounds known to inhibit cellular uptake of some sympathomimetic amines.

The influence of dehydroascorbic acid and ascorbic acid on the levels of endogenous noradrenaline and dopamine in brain tissue and of noradrenaline in heart tissue of mice has been studied (Chapter IV). Mice were injected intravenously with dehydroascorbic acid or ascorbic acid and the catecholamine content in both tissues was estimated. It was observed that neither dehydroascorbic acid nor ascorbic acid changed the levels of endogenous cate-

cholamines in either tissue. However injection of dehydroascorbic acid after inhibition of catecholamine synthesis was followed by a significantly increased depletion of noradrenaline in both brain and heart tissues. Ascorbic acid, on the other hand, did not change the catecholamine levels after synthesis-blocking

The inhibitory effect of dehydroascorbic acid on cholinesterases was determined *in vitro* by an electrometric method using erythrocytes and plasma from man, cat and rabbit (Chapter V) Ascorbic acid was tested only on human cholinesterases in erythrocytes and plasma. It was observed that ascorbic acid had no effect on the cholinesterases in either erythrocytes or plasma. Dehydroascorbic acid on the other hand showed a slight inhibitory action of both kinds of cholinesterases.

From the various results in the present study it became evident that injected dehydroascorbic acid activates cholinergic mechanisms. It was suggested that the cholinesterase-inhibiting action of dehydroascorbic acid plays a significant role.

Injected ascorbic acid did not seem to interfere with cholinergic mechanisms and was also found to be devoid of cholinesterase-inhibiting activity

The symptomatic action of dehydroascorbic acid was shown to be mainly of central origin and evidences are presented suggesting that dehydroascorbic acid mediated the recorded responses rather than metabolically formed ascorbic acid as suggested by Patterson and Mastin (1951) A probable mechanism of action is discussed.

After injection of ascorbic acid no signs of interference with adrenergic mechanisms were observed

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SYMPOSIUM ON AXOPLASMIC FLOW IN ADRENERGIC NEURONS

Göteborg, June 5-6, 1970

ABSTRACTS

ACTA PHYSIOLOGICA SCANDINAVICA
SUPPLEMENTUM 357

Nordic Society for Cell Biology European Cell Biology Organization

AXOPLASMIC FLOW IN ADRENERGIC NEURONS

Symposium
held at
Institute of Neurobiology
University of Gothenburg
June 5–6 1970

Organized for the Societies by Annica Dahlström

A B S T R A C T S

NSCB The Biological Institute of the Carlsberg Foundation 16 Tagensvej 2200 Copenhagen N Denmark

ECBO Laboratorium voor Celbiologie en Histologie der Rijksuniversiteit te Leiden Rijnsburgerweg 10 Leiden Netherlands

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PROGRAMME

(Figures refer to abstract numbers)

Axoplasmic transport of neuronal components

Transport and life-span of amine storage granules studied by histochemical and biochemical method A Dahlström & J Häggendal (4)

Electronmicroscopical and biochemical studies on the transport of noradrenaline-containing granules D Mayor & P Banks (18)

Movement of mitochondria within constricted noradrenergic nerve P Banks & D Mayor (2)

The accumulation of DOPA-decarboxylase and MAO activity in rat sciatic nerves after constriction. J Jonsson (10)

Axonal flow of enzymes involved in the biosynthesis of catecholamines P Laduron (16)

Axonal transport in central and peripheral axons J Sjöstrand & J-O Karlsson (22)

A possible role of lysosomal accumulation in constricted in constricted sciatic nerves P Laduron (15)

Transport of Herpes Simplex virus in peripheral nerves K Kristensson E Lycke & J Sjostrand (14)

Axoplasmic transport and nerve function

Immunochemical evidence for the transport of neurophysin in neurosecretory neurons of the dog R Alvarez-Buylla D G Livett L. G Uttenthal B H Milton & D B Hope (1)

Axonal transport of neurohypophyseal proteins in rats under some experimental conditions A Norström & J Sjöstrand (20)

Nerve function after reserpine and axonal flow of amine granules J Häggendal & A Dahlström (8)

Two populations of noradrenaline-particles in the dog spleen W De Potter I W Chubb & A F Schaepdryver (5)

Chromogranins in the catecholamine-storing granules of the adrenal medulla and the splenic nerve trunk. K B Hall (7)

Immunologic l technique for studying the distribution transport and release of chromogranins B G Livett L. B Geffen & R A Rush (17)

Release of chromogranin A and dopamine-β-hydroxylase from adrenergic nerves during nerve stimulation W De Potter A F Schaepdryver & A D Smith (6)

Axoplasmic transport and regeneration

The effect of cut ligation or crush on the synthesis and transport of amine storage granules in rat sciatic nerve L. Karlsson & A Dahlström (12)

Modes of regeneration of adrenergic nerves L. Olson (21)

Mechanisms of axoplasmal transport

Effects of mitosi inhibitors on the transport of amine storage granules in monoaminergi neurons in the rat A Dahlström (3)

Electronmicroscopical observations on the distribution and transport of noradrenaline storage parti l after local treatment with mitosi inhibitors T Hökfelt & A Dahlström (9)

Effect of intravenous vinblastine on intraneuronal transport of noradrenaline in the rat F Kasa & A Livingston (13)

Transport of neurotubular protein and the effect of colchicine on axonal transport J -O Karlsson & J Sjöstrand (11)

Morphology and properties of treatlike lements of the neuron J Matusala (19)

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1. IMMUNOHISTOCHEMICAL EVIDENCE FOR THE TRANSPORT OF NEUROPHYSI IN NEUROSECRETORY NEURONS OF THE DOG R Alvarez Buylla E G Livett L O Uttenthal E H Milton and D B Hope Departments of Pharmacology Human Anatomy and the Nuffield Department of Experimental Surgery University of Oxford England

The neurosecr tory nerve endings in the posterior pituitary rise from the supra-optic and paraventricular nuclei in the hypothalamu These neurons re chara terized by the presence of Gomori-positive neurosecretory material and, at the ultrastructural lev l by large dense-cored vesicles (1300 Å diam) which store the peptide hormones oxytocin and vasopressin The hormones nd their binding proteins (neurophysins) are probably synthesized in the hypothalamic nucl i and transported down the neuro-hypophyseal tract by oplasmic flow

Two major neurophysin (I and II) have recently been isolated on a large scale from ac tone-dried powder of pig posterior lobes (Uttenthal and Hope Biochem. J. 1970 116 899) and we have prepa ed antibodies to porcine neurophysin-II for use in the indirect or "sandwich method of immunofluorescence histochemistry (Nairn Fluo escent Protein Tracing 1969 Livingstone) to localise this protein in the hypothalamus neuro-hypophyseal tract and posterior lobe of the pig (Livett Uttenthal and Hope Proc int endocrin soc. Bristol, April 1970 in press; Proc roy, soc B in press)

More recently we have made u of an antiserum which cro reacts with dog neurophysin to provide evidence for axoplasmic flow of neurosecretory ve icles in the neurohypophyseal system of the dog The neurohypophyseal stalk of a dog wa exposed at operation by removal of the zygomati arch parietal bone and squamou part of the temporal bone The stalk wa constricted at point with fine forceps and following operation the dog wa allowed to recover consciou ne s After 20 hour the dog wa sacrificed by decapitation under nembutal nd para sagittal nd sagittal sections of the hypothalamo-neurohypophyseal system processed for immunofluorescence Marked ccumulation of fluoresceno ttributabl to neurophysin proximal to the constri tion nd its absence from the portion immedi tely distal to the constriction provides evidence for oplasmic flow of neurophysin from the cell bodi in the hypothalamus to the nerve terminals in the po terior lobe of the pituitary

2. MOVEMENT OF MITOCHONDRIA WITHIN CONSTRICTED MONADRENERGIC AXONS P Banks and D Mayor Depts of Biochemistry and Human Biology & Anatomy University of Sheffi ld England

Following th ligation of cat hypoga tri nerves in vivo mitochondria cha cterized by their electron microscopic morphology and by their cytochrome oxidas otivity ccumulate within the ons on both ids of the constriction and particularly in close proximity to it (Kapeller and Mayor Proc roy soc. B 1969 172 39 and 51; Bank Mangnall and Mayor J. Physiol. 1969 200 745) Thi ccumulation in the 3 mm immediately proximal to a single ligature i always lightly greater than that of th 3 mm immediately di tal to the ligature Within a 1 cm segment of nerve isol ted by two lig tures the total cytochrome oxidase activity does not change significantly with time but is redistributed so that the regions immediately adjacent to the two constrictions contain the highe t enzyme tivities and the greatest number of mitochondrial profiles This ob ervation indicates that the axonal mitochondria are able to redi tribute themselves independently of the cell body by moving in both proximo-di tal and disto-proximal directions This is in trong contrast to the uni-directional movement of dense-cored

vesicles It seems probable therefore that mitochondria and den e-cored ve icles move by different mechanisms

In addition to a redistribution of existing mitochondria it seems probable that the cell bodies contribute to the accumulation of mitochondria on the proximal side of a ingle constriction ince the total cytochrome oxidase of the nerve from ganglion to ligature rises above the control level In contrast it falls below the control level a few mm distal to the constriction

Mitochondria very close to a constriction (within 0 1 mm) tend to be very swollen electron lucent and are probably degenerating Slightly further from the constriction they are still somewhat swollen but not obviously degenerated About 0 5 - 1 mm from the constriction the majority have the elongated and electron dense appearances of normal axonal mitochondria

It has recently been established that mitochondria distribute themselves in constricted axons in vitro in the same manner as in vivo

The factors involved in inducing and sustaining mitochondrial movement in axons are not yet understood

3. EFFECT OF MITOSIS INHIBITORS ON THE TRANSPORT OF AMINE STORAGE GRANULES IN MONOAMINERGIC NEURONS IN THE RAT

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The proximo-distal transport of amine granules is of the fast type and as such suggested to involve microtubules (cf Schmitt Proc nat Acad Sci 1968 60 1092) Colchicine (COL) and vinbla tine (VIN) both disrupting microtubules were applied locally to adrenergic neurons in the PNS and in the CNS (spinal cord) Effects were obtained with 0 035 and 0 001 M concentrations of COL and VIN respectively

Soaking lumbar sympathetic ganglia with COL (Dahlström, Eur J pharm 1968 5 111) or VIN for 10 min caused a medium increase of noradrenaline (NA) in cell bodies and intraganglionic axons already after 3 h and a marked increase by 24 h Reserpine abolished the NA fluorescence totally indicating that all NA was stored in granules Sciatic nerve ligation 1 h before death 24 h after treatment caused very little or no NA accumulations Controls had normal accumulations The results indicate an arrest of the transport of amine granules from ganglia to axons

Unilateral soaking of cervical superior ganglia with VIN caused in addition to the above mentioned accumulation of NA in cells andintraganglionic axons a disappearance of almost all nerve terminals in the iris of the treated side while the contralateral iris (saline soaked) was normal Unilateral pto is (occuring 18 24 h after operation) wa always observed Administration of α-methyl-NA did not increase the number of nerve terminals observed indicating nerve terminal degeneration Five - 10 d after treatment growth cones were observed in branches of the few remaining nerve terminals Local VIN treatment thu probably cause interruption of not only transport of amine granules but also of substances needed to maintain the nerve terminal membrane

Local application of VIN or COL on the spinal cord caused accumulations of NA and 5-hydroxytryptamine (5 HT) in descending axons of NA and 5-HT containing bulbo-spinal neurons respectively

The results indicate that COL and VIN inhibit the transport of amine storage granules in both central and peripheral monoamine containing neurons Since both substance depolymerize microtubules the results may support the view that microtubules are involved in fast transport of amine storage granules

4. TRANSPORT AND LIFE SPAN OF AMINE STORAGE GRANULES STUDIED BY HISTOCHEMICAL AND BIOCHEMICAL METHODS A Dahlström and Jan Häggendal Institute of Neurobiology and Department of Pharmacology University of Göteborg Göteborg Sweden

A proximo-distal transport of noradrenalin (NA) containing granules has been demonstrated to take place in peripheral and central aminergic neurons (for ref see Dahlström and Häggendal in: Biochemistry of Simple Neuronal Models eds Costa and Giacobini Raven Press 1970 65) Ligation of adrenergic nerves results in an accumulation of NA proximal to the ligation The NA increases approximately linearly up to about 48 h indicating a rate of transport of about 5 mm/h in rat sciatic nerve (Dahlström and Häggendal Acta Physiol. Scand 1966 67, 278) Between simultaneous double ligations a proximo-distal shift of NA was observed but no clear net change in NA in the separated nerve part (Dahlström Acta physiol. scand. 1967 69, 158 Bank Mangnall and Mayor J. Physiol. (Lond.) 1969 200, 745) Based on a) the amount of NA accumulating in the sciatic nerve during 12 h b) the NA amount in the nerve terminals of the adrenergic axons in the sciatic nerve and c) the assumption that amine granules are equally loaded with NA in all parts of the neuron a life-span of about 5 week was earlier calculated for amine granules in adrenergic nerv terminals of the rat

Recent result indicate however that amine granules may be less loaded with NA in the axons than in the nerve terminals Three consecutive 1 cm parts of unligated sciatic nerve were examined for NA content A gr dient wa observed the lowest part of nerve having somewhat higher NA content than the more proximal parts This may indicate either an increa e number of granules in the distal parts of the nerve or an increased loading of NA in the granules occurring during their passage down the axon In support of the last mentioned interpreration delayed double ligations (a low ligation followed 6 h later by a second ligation 1 cm proximal to the first one) resulted in a NA increase of about >0% 9 h after the second ligation Reserpine could depl te the NA in the separated nerve part indicating that the NA increase probably occurred in the amine granules accumulated in thi nerve part during the 6 h after the first low ligation (Dahlström Häggendal Larsson and Magnusson in prep) These results have been u ed for a re-estimation of the rate of transport and life-span of amine torage granules in rat adrenergic neurons The rate of transport is then calculated to about 3-4 mm/h whereas the averag life-span of the granules regarding their capacity to tore endogenou NA ha been calculated to about 3-4 weeks

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5. TWO POPULATIONS OF NORADRENALINE-PARTICLES IN THE DOG SPLEEN W P De Potter I W Chubb A F De Schaepdryver Heymans Institute of Pharmacology University of Ghent Belgium

The presence of noradrenaline (NA) storage particles in sympathetic nerves a hown by biochemical and morphological tudi s is now well proven and generally accepted The ite of synthe i of these parti les is thought to be in the cell body of the neuron and that they are supplied to the periphery by a process of axoplasmic flow

Recent morphological evidence ha shown that for the cat spleen there ar two ires of vesicles in the terminals only one of which is present in the splenic nerv axons (Geffen and Ostberg J. Physiol., 1969 204, 583) Biochemically evidence ha been presented for a dual di tribution of NA in the rat heart (Roth, Stjärne Bloom and Olarman J Pharmacol exptl Therap 1968 162, 203) and

in the dog spleen (De Potter (1968) Thesis University of Ghent)

More recent work in this laboratory using denervated spleens has shown that both populations are intraneural and that the addition of KCl Mg^{++} or buffers to sucrose does not alter the NA distribution on gradients The densities of the two particles are (means ± S D) 1 128 ± 0 005 (11 spleens) and 1 178 ± 0 011 (12 spleens)

Dopamine-β Hydroxylase activities shown to coincide with NA particles from splenic nerve (Smith De Potter De Schaepdryver Arch, int Pharmacodyn ther, 1969 179 495) was measured on gradients from the spleen The enzyme reflected the NA distribution of the more dense particle but not of the other This finding plus the evidence of a single di tribution of NA from the splenic nerve axons indicates that while one particle may be synthesized and transported from the neuronal cell body the less dense particle must be derived from some other site

6. RELEASE OF CHROMOGRANIN A AND DOPAMINE β-HYDROXYLASE FROM ADRENERGIC NERVES DURING NERVE STIMULATION W P De Potter A F De Schaepdryver nd A D Smith Heymans Institute of Pharmacology University of Ghent Belgium and Department of Pharmacology University of Oxford England

Evidence has accumulated over the pa t few years for an exocyto is type release of catecholamine from the adrenal medulla Experiments were carried out in this laboratory to determine whether such a mechanism was possible for sympathetic nerves One approach to this problem i to determine whether the intravesicular contents are released concomitantly with noradrenaline on nerve timulation

Biochemical evidence has shown that dopamine-β-hydroxylase and chromogranin A the mayor protein constituents of the adrenal chromaffin granule are also present in the noradrenalin-containing vesicles of bovine splenic nerve (Smith De Potter and De Schaepdryver Arch, int Pharmacodyn ther 1969 179 495; Hörtnagl Hörtnagl and Winkler J. Physiol. (Lond) 1969 205 103)

Bovine calf spleens were perfused with Tyrode solution and the splenic nerves were stimulated at 30/sec supramaximally The perfusate from the splenic vein was collected and assayed for noradrenaline dopamine-β-hydroxylase and chromogranin A The results clearly show a imultaneou release of all three The ratio s of both proteins to noradrenaline were much lower than those found in intact plenic nerve vesicles Of the possibilities that could explain these differenc the two most likely are a slow rate of diffusion of the proteins or the presence of a noradrenaline-containing particle lacking either or both proteins The relea s of noradrenalin and proteins from calf pleen was not prevented by the addition of either hexamethonium-bromide (3 mM) or phenoxybenzamine (0 05 mM) to perfusion fluid However omis ion of calcium from the perfusion fluid trongly inhibited the relea e of noradrenaline a well s dopamine-β-hydroxyla e nd chromogranin A

Release of the two vesicle-proteins upon stimulation of the nerve wa not accompanied by release of DOPA-decarboxylase an enzyme present in the soluble xoplasm and so the observ tions are consistent w th the idea that the neurotransmitter is released by exocyto i

7. CHROMOGRANINS IN THE CATECHOLAMINE-STORING GRANULES OF THE ADRENAL MEDULLA AND THE SPLENIC NERVE TRUNK K B Helle Institute of Physiology University of Bergen Norway

Two proteins have so far been found to be characteristic of the catecholamine-storing granule namely chromogranin A and dopamine-β-hydroxylase By immunological techniques both proteins purified from the adrenal medulla have been shown to be identical to proteins present in the noradrenaline-rich particulate fraction of splenic nerve (Bank Helle & Mayor Mol. Pharmacol. 1969 5 210; Geffen Livett & Rush J. Physiol. 1969 204 589)

In the chromaffin granule of the adrenal medulla both chromogranin A (Helle & Serck Hanssen Pharmacol. res. comm. 1969 1 25) and dopamin β-hydroxylase (Laduron & Belpaire Biochem. Pharmacol. 1968 17 1127) occur in a membrane-bound as well as in a soluble form In its bound form chromogranin A has been found to be associated with phospholipids in a complex which can be distinguished from the soluble chromogranin A by its lower mobility in an electric field

In particulate fraction of splenic nerve homogenates chromogranin A has been detected mainly as the water insoluble membrane-bound form and this fraction accounted for about 80% of the total chromogranin detected in the particulate fractions The ratio of CA chromogranin was found to be approximately 1000 nmoles/mg (Bank & Helle Proc roy Soc (Lond) 1970) This value is five-fold less than that obtained for medullary granules and also much less than for granules isolated from nerve terminals (De Potter Proc. roy. Soc. (Lond) 1970) The nerve granules may thus not be fully loaded with transmitter while being transported down the axon

One of the main functional differences between the medullary granules and the nerve granules may well be related to their difference in content and distribution of lipids illustrated by the detection of chromogranin A mainly in the membrane bound form in the nerve granule The water-soluble fraction of the nerve granule apparently accounts for a much smaller proportion of the total granule than is the case for the medullary granule Thus the fraction of protein expected to leave the nerve granule by an exocytotic mechanism of secretion of transmitter must be rather small as indeed experimentally shown (De Potter De Schaepdryver Moerman and Smith J. Physiol. 1969 204 102P)

8. NERVE FUNCTION AFTER RESERPINE AND AXONAL FLOW OF AMINE STORAGE GRANULES J Häggendal and A Dahlström, Depts of Pharmacology and Histology University of Göteborg Göteborg Sweden

The amine storage granules play a central role (e g for the release of transmitter) for functions of the amine containing neurons Several of the functions can be blocked by reserpine Thus after reserpine the granules in e g the sympathetic nerves are emptied of their noradrenaline (NA) content they lose their normal capacity to take up and retain small amounts of labelled NA and no functional response is obtained to stimulation of the nerve fibres

Based on quantitative studies on endogenous NA and ^{3}H-NA retention in tissues with intact or by axotomy interrupted axonal flow the recovery of these functions after reserpine both in the CNS and in the PNS will be discussed mainly being due to axonal transport to the nerve terminals of new granules formed in the cellbody after the reserpine administration The dynamics of transport of newly formed granules and their accumulation in the

terminal appear to be responsible for the recovery of the endogenou NA levels to normal a recovery which requires several weeks to be completed In the nerve terminals the granules appear to have an average life- pan of several weeks with respect to their average capacity to tore endogenou NA

On the other hand the capacity of the nerve terminals to take up and store tracer amounts of labelled NA appears mainly to be dependent on the presance of newly formed granules With respect to this function the amine granules appear to have a much shorter life span in the order of some day( ) The life span of the amine granules may thus differ with respect to different functions (Dahl trön & Häggendal J Pharm, Pharmac 1966 18 Häggendal & Dahlström Subcellular Organization and Function in Endocrine Tissu s Bristol April 1970 in press)

We sugge t that the granules during their life-time may change their morphology (from larger to smaller) their chemical composition (e g content of chromogranin A nd DA β-hydroxylase) and their capacity to take up store and relea e transmitter

The newly formed granules may by suggestion mainly con titute the small pool easily vailable for release whereas the old granule may represent the large stabile pool in which the tr nsmitter appears to be more firmly bound

9. ELECTRON MICROSCOPIC OBSERVATIONS ON THE DISTRIBUTION AND TRANSPORT OF NORADRENALINE STORAGE PARTICLES AFTER LOCAL TREATMENT WITH MITOSIS INHIBITORS Tomas Hökfelt and Annica Dahlström Dept of Histology Karolinska Institut t nd Institute of Neurobiology Univer ity of Göteborg Göteborg Sweden

Colchicine (Sandox) or Vinbla tine (Velbe® Lilly) was applied locally on the lumbar sympathetic ganglia or on the interganglionic connecting nerves of the sympathetic chain Three or 24 h after the treatment these tissu s were fixed for electron microsc py either with 3% potassium permang nate ($KMnO_4$) (Richardson Nature 1966 210 756) or with gluta aldehyde followed by osmium tetroxide (OsO_4)

Principally both mitosi inhibitor produced the same type of effects on the fin tructure of the nervous ti sue tudied Colchicine in higher concentrations than Vinbla tine (0 35 M and 0 01 M respect vely) The effects were con id rably more pronounced after 24 h than fter 3 h A judged from ingle sections the individual neuron seemed to be affected to varying degree

Cell bodies. In number of ell bodies strands of densely packed neurof laments were found running through the cytoplasm Often small clu ter of both small nd larg granular vesicles were found close to these strand The Golg -apparatus seemed to be enlarged often containing rather high numbers of granular vesicles No microtubul were seen Many cell bodi s howed no obvious hange as compared to control In control gangli no strands of neurofilaments were observed but often ingle microtubul could be seen

Axons. The xon profiles could be div ded into several groups to the effect on their fine structure

1 Enlarged xon containing large numbers of electron dens particles probably representing damaged mitochondria

2 Enlarged axons containing abundant tubular and vesicular stru tures some of which contained electron dense cores

3 Enlarged xons containing large amounts of small and large granular ve icl s In these axons the granular vesicles were often lying in the peripher l part of the axon surrounding strand of densely packed neurof laments No neurotubules were visible in these xons

4 Axon w th normal appearance

The present findings re interpreted to indicate that mitosis inhib tors when applied locally arrest the transport of

noradrenaline storage particles from the cell body (Golgi apparatus) to the axon terminals Thi seems to be a sociated with a disappearance of microtubules and n appearance of dense strands of neurofilaments Thi may indicate a fundamental importance of microtubules for intraneuronal transport mechanisms (c f Schmitt Proc nat Acad Sci 1968 60 1092)

10. THE ACCUMULATION OF DOPA-DECARBOXYLASE AND MAO ACTIVITY IN RAT SCIATIC NERVES AFTER CONSTRICTION J Jonason Depart ment of Pharmacology University of Göteborg Sweden

The ctivities of DOPA-decarboxylase and MAO in rat sciatic nerve was determined at different times after constriction of the nerve Both the enzyme activities were found to increase above the ligatur after the con triction However the change in activity of DOPA-decarboxylase differed from those of MAO with respect to time course and magnitude DOPA-decarboxylase tivity in the part proximal to the lesion wa found to increase to about 5 times the normal level during the second day; a reduction from thi high level was observed after 7 day and 14 days fter the operation the values were almost normal An increase in MAO activity in the proximal part of the nerves to a level of bout 2 5 times normal wa observed fter 7 days At three weeks fter the operations the MAO activity was almost in th normal rang

In th parts below the constriction changes in enzyme activities were lso observed DOPA-decarboxylase ctivity wa found to increas to about 2 5 times normal after 4 days wheres MAO activity wa slightly decreased by 2-3 week

Lumbar sympathectomy reduced the activity of DOPA-decarboxy-lase in nonconstri ted nerves to about half of that of normal nerve wheres the MAO ctivity was not significantly changed after thi procedur No increase in DOPA-decarboxylase activity was obtained fter constriction t sympathectomized rats indicating that the increa in DOPA-decarboxyla e ctivity observed in normally inner-vated rats after ligation occured within the sympathetic po tganglioni nerve fibres Furthermo e the real increase in neuronal DOPA decarboxy-las i probably about 10 times that in normal constricted animals ince the a tivity of thi enzyme in unligated nerve of sympathec tomized animals is about half of normal (see above) After lumbar sympathectomy ligation of the sciatic nerve produced an increase of MAO tivity to a level of more than twice the n rmal However this increas reached the maximal level about one week later than in normal constricted rats

It might be concluded that the behaviour of DOPA-decarboxy-lase nd MAO after nerv ligation i quite different from each ther a well from that of nor drenaline (Dahlström & Häggendal Acta physiol scand 1966 67 278) nd dopamine-β hydroxylase (Laduron & Belpair Life sci. 1968 7 1) The pr sent results indicate that n ther DOPA-decarboxylase nor MAO is localized within the amin torag gr nules

11. TRANSPORT OF NEUROTUBULAR PROTEIN AND THE EFFECT OF COLCHICINE ON AXONAL TRANSPORT J -O Karlsson and J Sjöstrand Institute of Neurobiology Univer ity of Göteborg Sweden

The 6 S colchi ine binding protein has been shown to exi t n microtubules (Shelanski nd Taylor J. Cell Biol. 1967 34 549) nd it ha been sugg sted that thi protein is the subunit of neurotubules (Wei enberg et al. Biochemistry 1968 7 4466) In order to study the xonal transport of this protein we injected ^{3}H leu ine into the eye of the rabbit

At various intervals following the injections the retina optic nerves and tracts was dissected out and the colchicine-binding protein was isolated with a batch DEAE Sephadex elution procedure essentially as described by Weisenberg et al. After reduction and alkylation the isolated protein migrated as a single band on 7 5% polyacrylamide gels Incubation of the purified protein with ^{3}H-colchicine showed that it bound 0 24 M colchicine per 120 000 g protein

Determination of the specific radioactivity of the colchicine-binding protein at various interval following injection showed that this protein was synthesized by the retinal ganglion cells and transported along their axons with the slow phase of the axonal transport in this system (1 5 - 2 mm/day)

^{3}H-colchicine (which binds to microtubular protein in vitro) did not seem to be transported in the axons of the retinal ganglion cells in the same manner as the microtubular protein

The role of neurotubules in the different phases of axonal transport will be discussed

12. EFFECT OF CUT LIGATION OR CRUSH ON THE SYNTHESIS AND TRANSPORT OF AMINE STORAGE GRANULES IN RAT SCIATIC NERVE
Lars Karlström and Annica Dahlström Dept of Histology Institute of Neurobiology University of Göteborg Göteborg Sweden

Ligation of peripheral nerves carrying adrenergic axons results in a rapid accumulation of noradrenaline (NA) storing particle The proce appears approximately linear up to about 48 h in rat sciatic nerve Thereafter the amount of NA above the ligation ppeared unchanged up to at least 96 h indicating decrease in accumulating NA Different theories have been put forward to explain this decrease e g cell body reactions resulting in a decreased synthesis and transport of amine storage granules (Dahlström & Häggendal Acta physiol scand. 1966 67 278)

To test this nd to tudy if different kinds of axonal trauma had the same effect the following experiments were done Sprague-Dawley rats (200 g males) were operated upon at a low level of the sciatic nerv which wa cut crushed without ilk ligature or ligated (with remaining ilk-ligature) 1- 23 d before death Controls were shamoperated One or 2 h before death the sciatic nerve was crushed 1 5 - 2 cm above the level of the initial operation Thi high nerve part wa then di sected out and treated for fluorescence microscopy ccording to Falck and Hillarp

The shamoperated r ts always showed strongly green fluo escent fibres with accumulations bove the high crush In the operated rats the amount of accumulated NA was decreased 3 d after operation Between 5 and 13 d no or very little NA accumulated In rats with crushed nerves weakly fluo escent accumulations were again observed at 15 d and around 19 - 21 d the fluorescence intensity nd size of the accumulations appeared normal in the microscope In rats with cut or ligated nerves no recovery of the NA ccumulations above the high crush was seen during the observed period Similar observation have been done in splenic nerve of cat 21 d fter constriction of the nerve (Boyl & Gill spie J. Physiol. 1968 195 27 P) Our results indicate that axotomy cause depre sion in synthe i and transport of amine torage granules in drenerg c nerves A ligation with remaining ligature or a cut appear to cause a more long la ting depression in synthesis and transport of amine tor ge granules than crush or ligation without remaining ligature

13. EFFECT OF INTRAVENOUS VINBLASTINE ON INTRANEURONAL TRANSPORT OF NORADRENALINE IN THE RAT P Keen and A Livingston Department of Pharmacology University of Bristol Bristol England

The transport of noradrenaline storage granules down sympathetic nerves may be interrupted by the local application of colchicine which is known to interact with neurotubules (Dahlström Europ J pharmacol 1968 5 111) Vinblastine also disrupts neurotubular organisation (Wisniewski J cell biol 1968 38 224)

Vinblastine was administered intravenously to rats in an attempt to block intraneuronal transport of noradrenaline Such a chemical ligation might allow a) prolonged inhibition of axonal transport without the peripheral degeneration which follows mechanical ligation and b) study of granular transport in the heart which has no discrete sympathetic nerves which can be ligated

Vinblastine (3 mg/kg iv) reduced the noradrenaline content of the pleen heart and vas deferens to respectively 10% 20% and 50% of that of the controls but had no effect on brain noradrenaline levels Notable features of the effect of vinblastine were a) that noradrenaline levels did not commence to fall until 24 hours after administration of vinblastine b) that the fall was more rapid in the heart and spleen than in the vas deferens possibly a reflection of the lower turnover rate in the latter) that recovery of noradrenaline levels commenced approximately 12 days after the injection of vinblastine; at 21 days heart noradrenaline had risen to 40% of control values Stress increased the rate of depletion of heart noradrenaline following vinblastine suggesting that the decline of noradrenaline levels depends on the normal leaky process Hearts depleted of their noradrenaline by vinblastine were unable to store systemically administered ^{3}H-noradrenaline; experiments with isolated perfused hearts prepared from these animals showed that this was because the neuronal membrane was unable to take up ^{3}H-noradrenaline Electron microscopy of the coeliac ganglion and splenic nerve of vinblastine treated rats did not reveal any abnormality and ligation of the sciatic nerve of these animals showed that noradrenaline transport was proceeding at normal rate suggesting that the effect of intravenously administered vinblastine may be primarily on the nerve endings

14. TRANSPORT OF HERPES SIMPLEX VIRUS IN PERIPHERAL NERVES Kerstin Kristensson Erik Lycke and Johan Sjöstrand Departments of Pathology and Virology and Institute of Neurobiology University of Göteborg Göteborg Sweden

It is well known that herpes simplex virus may reach the CNS along peripheral nerves The problem of in which structural component of the nerve (epi peri or endoneural spaces Schwann cells axons) the infectious agent travels has been discussed in the literature After intradermal inoculation of the virus the lesions were observed to follow the sensory pathways whereas after intramuscular inoculation the motor fibres were primarily affected (Kristensson Acta neuropath (in press)) The findings suggest that there may be a direct transport of the infectious agent within axons To test the existence of a distal-proximal transport in side the axons experiments with different kinds of axonal injuries were carried out

Suckling mice were inoculated with herpes simplex virus intradermally in the sole of the hind foot Signs of paresis developed on the inoculated leg after 4 to 5 days and all animals died one or two days later Titrations for virus were performed from different levels of the sciatic nerve dorsal ganglia and spinal cord Ligatures of the sciatic nerve prevented the spread of the infection if applied within 24 hours after inoculation A similar effect was observed after

freezing of the nerve It ha been reported that colchicine blocks axonal tran port in various nerve (Dahl trön Eur J pharm 1968 5 111 Karlsson and Sjöstrand Brain res. 1969 13 617 Kreutzberg Proc nat Acad Sci 1969 62 722) Soaking of the sciatic nerve with colchicine prevented to a high degree the spread of the infection The ultrastructural changes of the nerve following colchicine treatment were studied and the mechanism for the blocking effect is discu sed It i concluded that in suckling mice a relatively rapid transport in axons from the periphery to the perikaryon most likely responsible for the transmission of herpes simplex viru in the nerve trunks

15 AXONAL FLOW OF ENZYMES INVOLVED IN THE BIOSYNTHESIS OF CATECHOLAMINES P Laduron Department of Biochemical Pharmacology Janssen Pharmaceutica Beerse Belgium

Despite Udenfriend s unitarian theory that the three enzyme of catecholamine biosynthesi (tyrosine hydroxylase DOPA-decarboxylase and dopamine β-hydroxylase) are sociated within the granule containing catecholamines (cf Adrenergic neurotransmission CIBA Foundation 1968) we are now well documented to claim that the two fir t steps of catecholamine biosynthe is occur outside the granules

The rate of accumulation of these three enzymes in the proximal part nd their disappearance in the distal part of constricted nerves provide a suitable tool to study the intracellular localisation of these enzymes

The fact that the rapid accumulation of dopamine-β hydroxy lase above a ligature parallels that of noradrenalin together with some particular aspects of latency and sedimentation indicates its granular belonging On the contrary the low increase or the unchanged activity of DOPA-decarboxylase and tyrosine hydroxylase above a l gature suggests that these enzymes are not linked to a subc llular structure

Ther fore the xonal flow in a way performs a physiolog cal fra tionation of enzymes which can be estimated by the kine tic of accumulation and thus provid s a new pproach to th clas ical methods of ti sue fractionation

However the exact its of synthesi of noradrenaline in the neuron remains questionable If in the neuronal system only the nerve ending re apt to relea e neurotransmitters by exocyto i it eems reasonabl to assume that becau e of the presence of a large amount of noradrenaline along the axon an important part of thi amin is synthe ised in the cell body

16 A POSSIBLE ROLE OF LYSOSOMAL ACCUMULATION IN CONSTRICTED SCIATIC NERVES P Laduron Department of Biochemical Pharmacology Janssen Pharmaceutica Beerse Belgium

In the axoplasmic flow experiments using ligatures of adrenerg c nerves it was generally observed that the gradual cumulation of different components bove the ligature rea hed a maximal level 2 to 4 days after the operation Thereafter a decline period was found especially for the DOPA-decarboxylase

In contra t the ri of lysosomal (cid phosphatase nd acid deoxyribonuclease) and microsomal accumulation (inosine diphosphatase) in the same experimental conditions behaved quite differently in such way that their activities were found to increase gradually on both sides of the constr ction especially from the econd day to reach a maximal level 9 days after the operation A clearcut dissociation between the two kinds of enzymes due to their different intracellular local zation was recently reported (Laduron Arch. int.

Pharmacodyn. 1970 185 200)

A tructure linked latency of acid phosphatase wa demon trated in homogenates in both the proximal and distal egment of nerves which is compatible with previous results showing the presence of variou digestive vacuoles of lysosomal type in cru had sci tic nerves (Holtzman Lysosomes in biology and Pathology 1969)

Furthermore experimental data u ing two ligatures nd various kinetic of coumulation, suggest a possible double origin of these lysosomes During the fir t days after lig ture the accumulation of lysosomal enzymes wa greater in the proximal nd di tal segments than in the segments located between the two ligatures suggesting that at lea t part of the accumulated lysosomes originate from the axons probably by axoplasmic flow However from the second day on a possible extra axonal origin probably in the Schwann cell i suggested by the increase of lysosomal enzymes between two ligatures

Consequently from thes results we put forward working hypothesis according to which the accumulation of granules containing catecholamines nd mitochondria in on tricted nerves disappear within a few days owing to the digestive proces of v rious lysosomal enzymes

This phenomenon mu t be kept n mind to specify the scope of the axoplasmic flow conc pt

17. IMMUNOLOGICAL TECHNIQUES FOR STUDYING THE DISTRIBUTION TRANSPORT AND RELEASE OF CHROMOGRANINS B G Liv tt L B Geffen and R A Rus h Department of Pharmacology University of Oxf rd England, nd Departments of Biochemistry nd Physiology Monash University Clayton Victoria Australia

W have used the opportunity presented by th drenal medulla a relatively homogenous and concentrated source of catecholamin containing vesicles to study by immunological techniques wh ther the soluble vesicl proteins (chromogranin) a also present in sympath ti neurones nd to examine their distribution tran port and participation in the transmi ion proce (Geffen Livett and Rush J. Physiol. 1969 204 58P; J. Physiol. 1969 204 593)

The formaldehyd ind ced fluorescence method for catechol mines by Hillarp and Falck (for methodology see Corrodi nd Jonsson J Histochem Cytochem 1967 15 65) ha been a us ful technique for studying the di tribution torage and transport of the transmitter in drenergic neurones (Dahlström J. Anat. 1965 99 677; Dahlström N S Arch exp Path Pharmak. 1967 257 93) Flu r scence microscopy can also be used to tudy the di tribution of specif c proteins if ntibodie to these proteins a e labelled with fluo escent molecul such a fluorescein isothiocyanate or bodamine B200 The u fulne s of the immunofluorescence hi tochemical technique derives from the fa t that it combine the sensitivity of fluore en e microscopy with the specifi ity of the immune reaction (vide Nairn Fluores ent Protein Tr ing 1969 Livingstone) With thi technique we have shown that chromogr nin-A nd dopamine β-hydroxyla ar cytoplasmic co ti tuents of the cell bodies axons and xon terminal of noradrenergic neurones nd that they ccumulate proximal to a nerve ligation Comps ison of immunofluorescenc hi tology for the two proteins with the f rmaldehyde-indu ed flu r scence method f catecholamines showed that 11 three ha e imilar di tribution

Quantitative microcomplement fixation naly i ha been ed to show that the chromogranin re present in microsomal and soluble fraction of he p vasa deferentia sympathetic ganglia nd plean in the same r lative mounts s noradrenaline nd that chromogranin-A and dopamine β-hydrocylase re relea ed into the pe fusates from isolated sheep pleens pon timulation of th splenic nerves t 10 c p s Th results tog ther with thos of Blank Helle and Mayor (Mol c pharmacol 1969 5 210) and De Potter De

Schaepdryver Moerman and Smith (J. Physiol. 1969 204 102P) suggest that chromogranins are contained within noradrenergic vesicles nd are released together with noradrenaline by exocytosi

18. ELECTRONMICROSCOPICAL AND BIOCHEMICAL STUDIES ON TRANSPORT OF NORADRENALINE-CONTAINING GRANULES D Mayor and P Bank Dept of Human Biology & Anatomy and Biochemi try Sheffield University England

Studies on sympathetic nerves constri ted at one or two points in vivo have shown that noradrenaline and granular vesicles accumulate proximal but not distal to the constriction The amount of noradrenaline above the distal of a pair of constrictions was pproximately equal to the normal noradrenaline content of the piece of nerve isolated between the two lesion Above the proximal constric tion there was a progres ive increase in the number of granular ve- sicles and in the amount of noradrenaline accumulating This reached a peak by 4 days after operation The association between granular ve icles and noradrenalin in these nerves was further confirmed by using reserpin and mono-amin oxidase inhibitors (Banks Mangnall and Mayor J. Physiol. 1969 200 745; Banks Kapeller and Mayor Brit J Pharmac 1969 37 10)

Similar results have recently been obtained using an in vitro preparation of con tricted cat hypoga tric and colonic nerve attached to the inferior mesent ric ganglion Colchicine added to the incubation medium caused a marked reduction in the number of granular vesi l s and in the amount of noradrenaline accumulating above a constriction

The ultrastructural details of cat granular v sicles varied slightly depending on the fixative used However in general most of the granular vesicle accumulating above constriction were lightly smaller than those found in r lation to the Golgi apparatu in the cell body but they were larger than the majority of the granular vesicles in drenergic terminals

Thu granular ve icle containing noradrenalin only move proximo-distally in ligated cat ympathetic nerves both in vivo and in vitro Since this movement occurs in isolated pieces of the nerv it s not entirely dependent on the cell body of the neuron which is however essential for the production of the granular ve icle Furthermor thi movement can take pla e in the absence of preganglionic timulation The precise mechanism by which granular vesicl s mov along sympathetic axon t present uncertain but th inhibition produced by colchicine suggests that the xonal microtubule may be involved

19 MORPHOLOGY AND PROPERTIES OF THREADLIKE ELEMENTS OF THE NEURONE J Matuzals Institut of Neurobiology Faculty of Medicin Univer ity of Göteborg Göteborg Sweden

Since e ghty years there ha been a succession of theorie of cytoplasmic streaming scribing the production of the motive force to gel sol transformations among the different regions of the cyto- plasm In th neuron such transformation can be elated to the conformat onal changes of the filamentous cidic protein isolated from the axoplasm of the squid giant n rve fiber whereby Ca^{++} i a major fa tor in determining the behaviour of the protein (Matuzal J Cell Biol 1969 45 Katchalsky Biophys. J. 1964 4)

Based on differential interference microscopy electron microscopy nd physico-chemical observations of the squid giant nerve fiber and of vertebrate neurones model of the motive force of the neuroplasmic movements i pr sented (Matuzals & I zard, J Cell. Biol 1969 45); Matuzal IIIrd Europ reg conf electr micr ráfu

1964 According to this model gel sol transformations occur either: (1) between the outer or ectoplasmic and inner or endoplasmic portions of the axon and of the cell body of the neurone; (2) along the long xis f the axon causing transverse banding pattern of the axoplasm; in myelinated fibers gel - sol transformations occur between the node (gel) and the paranodal (sol) axoplasm Coiling decoiling and f lding unfolding of the protein molecules caused through inter actions with Ca^{++} would be the primary proce s at the molecular level

20. AXONAL TRANSPORT OF NEUROHYPOPHYSEAL PROTEINS IN RATS AT SOME EXPERIMENTAL CONDITIONS A Norstrőm nd J Sjöstrand Department of Histology Institute of Neurobiology Uni ver ity of Göteborg Göteborg Sweden

Axonal transport of proteins in the hypothalamo-neurohypophyseal system of the male rat was studied after a local injection of (^{35}S) cysteine in the region of the supraoptic nucleus The migration of labelled proteins was followed by measuring the specific radioactivity of the proteins in various parts of the hypothalamo-neurohypophyseal tract Between two and four hours after the isotope injection there was a sharp increase in the protein-bound radioactivity of the posterior pituitary lobe demonstrating that a transport of (^{35}S) labelled proteins had occurred from the supraoptic nucleus to th neurohypophysis The rate of the transport wa 2 to 3 mm/h During the first 24 h after the injection a continuou ccumulation of labelled material occurred in the neural lobe Considerable radioactivity could till be recovered 6 days after the isotope injection

Fractionation of the neurohypophyseal proteins by polyacrylamide gel electrophoresis revealed that pproximately 90% of the radioactivity of the soluble proteins was recovered in a single protein fraction Labelling of thi fraction was not observed until 2 h fter the isotope injection The radioactivity increased markedly up to 4 h It is sugge ted that this protein component is involved in the neurohypophyseal response to osmotic trass ince the protein disappeared from the posterior lobe upon dehydration of the rat

Colchicine injected into the subarachnoidal pace before the local injection of (^{35}S)cysteine inhib ted the transport of r diolabelled material

The transport of neurohypophyseal prot ins in female rats was tudied under various phy iological conditions The implications of these findings will be discussed

21. MODE OF REGENERATION OF ADRENERGIC NERVES L. Olson Department of Histology Karolinska Institutet Stockholm Sweden

The different aspects of axonal growth of the sympathetic adrenergic neurons were studied with the Falck Hillarp fluorescence technique for hi tochemical localization of catecholamines in combination with ^{3}H-NA uptake measurements Three principally different types of growth occur 1 Outgrowth of axons and terminal systems during ontogenesis 2 Regeneration after axotomy 3 Collateral prouting when an intact drenergic neuron increases its field of innervation in esponse to various tinuli

The sympaticoblas ts eveal NA fluorescence from an early stage (13 day ecbryo) and the outgrowing axons and terminal nerve plexuses can be followed during development

Regeneration rates of non-terminal NA-axons were determined in the adult rat fter sciatic nerve compressions The main bulk of axons advanced about 1 5 mm/day the first week and 3 mm/day the second week Thin sprouting fibres showed initial growth rates a high 9

mm/day (Olson Hi tochem. 1969 17 349)

When a piece of peripheral tissue is transplanted to the anterior chamber of the rat eye the intrinsic adrenergic nerves of the transplant degenerates A rapid reinnervation will then take place by collateral sprouting from the adrenergic ground plexus in the iris of the host eye The time course of adrenergic reinnervation was followed in detail with histochemical nd bi chemical methods using autologous contralateral iri tran plants Reinnervation of irides was found to be a rapid process continuing until the normal amount of adrenergic nerve was restored after one month

A series of similar experiments using different types of tran plants and pharmacological and physiological techniques permitted the following conclusion Regeneration i specific in the sense that new nerves only grow into denervated tissues where adrenergic nerves normally exists and unspecific in the sense that any part of the adrenergic neuron system can reinnervate any adrenergic receptor tissue The newly formed nerves have an essentially normal transmitter pharmacology and are able to restitute neuromuscular transmission Neither chronic treatment with various drugs affecting the transmitter mechanisms nor chronic treatment with testosterone or cortisone influences the outgrowth Impulse flow is not necessary for regene ration Slight infections will often cause a pronounced hyperinnerva- tion of host irides even if no transplant is placed in the anterior chamber (Olson Malmfors Acta physs scand 1970 suppl 348)

22. AXONAL TRANSPORT IN CENTRAL AND PERIPHERAL AXONS J Sjöstrand and J -O Karlsson Institute of Neurobiology University of Göteborg Göteborg Sweden

The axonal transport in the retinal ganglion cells of the rabbit was studied after intraocular injection of labelled leucine by measuring the arrival and ccumulation of labelled proteins in the axon terminals of the lateral geniculate body Four different phases of axonal transport was found with a transport velocity of 110-150 40 7 and 1 5 2mm/day respectively The subc llular distribution and the life-span of the labelled proteins in these phase will be discussed

Mo t of the soluble proteins of the axons wa transported with the low (1 5 2 mm/day) phase whereas only some of the soluble proteins was transported with the rapid (110-150 mm/day) phase Triton X 100 extraction of the microsomal fraction (containing a considerable part of the rapidly transported proteins) solubilised highly labelled proteins belonging to the rapid phase

The xonal transport of labelled protains was studied in vagus and hypoglossal nerves of rabbits fter n intramedullary in je tion of (^{3}H) leucine Two migrating protein components were described Th rapid component moved at rate of about 400 mm/day and 300 mm/day nd the slow component at an average r te of 26 mm/day and 5 mm/day in the vagu and hypoglo sal nerve respectively

Cell fractionation tudies of the nerves showed that the rapidly migrating component wa larg ly associated with the particulate fraction with the highest pecific radioactivity in the microsomal fr ction In the low component the highe t specific activity wa found in the soluble protein fraction The effect of Triton X 100 on the subcellular di tribution will be discussed

When the axons had been crushed by a ligature the enzyme accumulated proximal to the ligature The transport velocity s calculated from the rate of accumulation was highest in the vagus nerve both for AChE and ChAc Approximately the same rates were observed for both enzymes i e 15 mm/24 h in the vagus nerve and 5 mm/24 h in the hypoglossal nerve These rates were of the same order as thos observed for the slow axoplasmic transport of radioactively labelled proteins in unligated nerve

AUTHOR INDEX

The f gures refer to the abstract numbers

Alva sa Boylia R 1

Bank P 2 18

Chubb I W 5

Dahlström A 3 4 8 9 12

De Potter W P 5 6

De Scheepdryver A F 5 6

Geffen L B 17

Hall K B 7

Hope D B 1

Högqendal J 4 8

Hökf lt T 9

Jonsson J 10

Ka lsson J -O 11 22

Ka lström L 12

Keen P 13

Kristensson K 14

Laduron P 15 16

Living ton A 13

Liv tt B G 1 17

Lyck E 14

Mayor D 2 18

Meturals J 19

Milton S H 1

M rström, A 20

Olson L 21

Rush R A 17

Sjö trand J 11 14 20 22

Smith A D 6

Uttenthal L O 1

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LACTATE AND PHOSPHAGEN CONCENTRATIONS IN WORKING MUSCLE OF MAN

with special reference to oxygen deficit at the onset of work.

BY

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From the Department of Physiology Gymnastik och idrottshögskolan, Stockholm, the Department of Pharmacology Karolinska institutet, Stockholm and the Department of Zoophysiology Uppsala Universitet, Uppsala.

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CONTENTS

Chapter I

Introduction 7

Chapter II

General laboratory methods

Abbreviations 13
Definitions 14
Statistics 15
Subjects 15
Procedure 15
Methods 18

Chapter III

Determination of organic muscle metabolites

Enzymatic fluorometric analysis of muscle metabolites 19
Freezing and weighing of muscle biopsy specimens and extraction of metabolites 23
Concentration differences for lactate in a single biopsy specimen and in the contracting muscle 25

Composition of muscle tissue

Water content 30
Sodium and potassium concentrations 31
Nitrogen and protein content 32

General comments on the methods to study muscle metabolites 32

Chapter IV

Results

Lactate accumulation and phosphagen depletion with submaximal and maximal exercise 35
Lactate accumulation and phosphagen depletion in relation to the oxygen deficit during exhaustive exercise 40
Pyruvate and lactate concentrations in muscle and blood and the effect of exercise 45
LDH activity in skeletal muscle after prolonged exercise 47
The effect of a preceding prolonged heavy exercise on the lactate formation during exercise 48

Chapter V

General discussion
The significance of lactate formation 52
Relation between muscle lactate concentration and the perception of exhaustion 55
Muscle lactate with standardized work after prolonged exercise 56
Relationship between muscle lactate and blood lactate concentration 57
Glycogen depletion and anaerobic glycolysis 59
Possible factors regulating glycolytic activity 61

Chapter VI

Summary 63

Acknowledgements 65

References 66

The present report is based on investigations carried out at the Department of Physiology Gymnastik och idrottshögskolan, Stockholm, and the Department of Pharmacology Karolinska institutet, Stockholm, from 1966—1970 Except for some hitherto unpublished results, it summarizes the following papers, which will be referred to by their Roman numerals.

I. Diamant, B., J Karlsson and B. Saltin. Muscle tissue lactate after maximal exercise in man. *Acta physiol. scand.* 1968. *72* 383—384

II. Karlsson, J., B. Diamant and B. Saltin. Muscle metabolites during submaximal and maximal exercise in man. *Scand. J clin. Lab Invest.* 1970 *26* 385—394

III Karlsson, J and B. Saltin. Lactate, ATP and CP in working muscles during exhaustive exercise in man. *J appl. Physiol.* 1970. *29* 598—602.

IV Karlsson, J and B. Saltin. Oxygen deficit and muscle metabolites in intermittent exercise. *Acta physiol. scand* 1971 *Preprint*

V Karlsson, J Pyruvate and lactate ratios in muscle tissue and blood during exercise in man. *Acta physiol scand* 1971 *Preprint.*
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VI. Karlsson, J., B. Diamant and B. Saltin. Lactate dehydrogenase activity in muscle after prolonged severe exercise in man. *J appl. Physiol.* 1968. *25* 88—91

VII Karlsson, J Lactate in working muscle after prolonged exercise at standardized work loads. *Acta physiol. scand.* 1971 *Preprint*

CHAPTER I

INTRODUCTION

According to du Bois—Reymond (1877) Berzelius wrote to Lehmann in 1841 stating that he had found lactic acid in the muscles of exhausted game, and du Bois-Reymond explained the acidic reaction of tetanized muscles with this observation. According to the same reference, Berzelius has stated dass ein Muskel desto mehr Milchsäure enthält, je mehr er vorher an gestrengt worden ist." Berzelius did not continue these experiments on chemical events in muscle tissue, and an explanation of lactate formation could *not be given at that time.*

Araki was one of the first physiologists to conduct further studies into the *conditions of lactate formation in intact animals* (Araki 1891 a and b) In 1891 he demonstrated an increase in lactate concentration in urine and blood when dogs, rabbits or hens were subjected to hypoxia. Thus, he confirmed von Liebig's hypothesis (1847) that lactic acid accumulated in blood when the O supply was restricted. Several investigators concomitantly found lactic acid formation in other experimental situations (for ref see Bang 1935) e.g. when the liver was extirpated (Minkowski 1885)

No observations were made until the beginning of this century that defined in greater detail the conditions necessary for lactate accumulation in cells. Fletcher and Hopkins (1907) examined the relationship between lactic acid formation and the effect of an oxygen deficient milieu on contractions. Furthermore, using a frog muscle specimen, they demonstrated the removal of lactate in an oxygen rich environment. Similar results were obtained by Hastings (1921) when examining blood *lactate concentration in intact dogs* after brief intense exercise, indicating that the oxygen supply was insufficient during heavy exercise and that the circulatory system might be a limiting factor in the energy output of working muscle. With moderate work intensities, however very low blood lactate concentrations were obtained.

The intrinsic role of the circulatory system in the body's lactate formation was further illustrated by Groag and Schwarz in 1927 who showed in man that subjects suffering from cardiac failure had higher blood *lactate values* at a given submaximal work load than healthy subjects.

During the early 1920's Meyerhof and Hill advanced their concept of an oxygen debt. Meyerhof (1920 a, b and c) demonstrated on isolated stimulated frog muscle a decrease in glycogen content at the same time as lactate was formed. Removal of lactate was associated with a resynthesis of glycogen during the animal's recovery from exhaustion. Hill and his associates had shown that blood lactate concentration rose in the initial phase of work. When work ceased, lactate concentration disappeared at a rate similar to the decrement in the oxygen uptake (Furusawa *et al.* 1924 Hill, Long and Lupton 1924 a and b)

Both Hill and Meyerhof deduced that lactate formation was an indicator of O_2 lack during exercise, that the removal of lactate was directly responsible for postexercise excess O_2 uptake (oxygen debt) and that there was quantitative agreement between O deficit and O debt as originally concluded by Krogh and Lindhard (1920) It was subsequently demonstrated, however *that* O_2 *deficit was less than the oxygen debt* (*Schneider and Clarke* 1925 Asmussen 1946, Christensen and Högberg 1950)

Collazo and Lewicki had published results in 1925 which failed to fit the theory of oxygen lack as the significant cause of lactate formation in the body They gave normal subjects 100 g of sucrose orally and found elevated lactic acid concentrations in blood similar results were obtained by other investigators using a carbohydrate-rich diet. These results were confirmed in *animal experiments by Cori and Cori in 1929 who demonstrated lactic acid* formation when giving rats intravenous glucose infusions.

In the work by Ørskov (1931) the conditions necessary for lactic acid formation in the working muscle were studied, considering both local blood supply exercise and carbohydrate-rich diet. Many of his results are still relevant but are usually overlooked by later authors in the field of muscle biochemistry and physiology He showed that certain amounts of lactate (2—8 *mmoles* × kg^{-} *wet weight) were present in the muscles of the resting animal,* supposedly well-supplied with oxygen. The concentrations rose strikingly during exercise, and values about 20—30 mmoles × kg^{-} wet weight were obtained in the groups of working muscle of intact animals. Moreover he illustrated the effect of different diets on blood lactate concentration and showed an increase in blood lactate concentration to 3—4 mmoles × l following a carbohydrate-rich diet.

Lundsgaard (1930 a and b) was able to show that the Iodoacetate poisoned muscle performed lactic acid-free contractions at the same time as energy rich phosphate compounds were depleted in the muscle. Similar observations were made by Schwartz and Oschmann as early as 1924 but these results were evidently neglected and thus forgotten.

One of these compounds was then described as creatine phosphate (CP) (Eggleton and Eggleton 1927 and Fiske and Subbarow 1927) and the other one was described by Lohmann in 1931 as adenosine triphosphate (ATP) Nachmansohn (1928) demonstrated that CP was resynthetized very effectively by anaerobic glycolysis, and Meyerhof and Lohmann (1928) showed that breakdown of CP was an exothermic reaction. But it was not clearly demonstrated until Lundsgaard's studies that CP was a more immediate source of energy for muscular contraction than any of the intermediate by-products of glycolysis. Lohmann (1934) further defined the problem of how chemically bound energy is transformed into mechanical work and demonstrated that adenosine diphosphate (ADP) was a co-factor required for the breakdown of CP by dialyzed muscle extracts. The resynthesis of ATP (Lohmann 1935) by CP is often referred to as the "Lohmann reaction"

As a consequence of these studies on anaerobic energy output by muscle, the terms *lactacid and alactacid anaerobic energy output* were coined (Lundsgaard 1932) Margaria, Edwards and Dill (1933) and Dill *et al.* (1936) developed on this basis and in experiments performed on man the hypothesis of a lactacid and an alactacid portion of the oxygen debt. Thus, they further developed the concept of oxygen debt introduced by Hill and Meyerhof The energy of the lactacid part was derived from glycolysis ending up with lactate formation (anaerobic glycolysis) and of the alactacid part from the fast splitting of ATP and CP stored in the muscle. These two high energy phosphate compounds are usually referred to as *phosphagen*. On the basis of these concepts, total lactate formation during exercise was estimated in man. It has been postulated by Hill Long and Lupton (1924) that muscle lactate and blood lactate concentration would be the same in steady state conditions. Blood lactate concentration was then assumed by Margaria and co-workers to be representative of the concentration of the total body water they were then able to evaluate the alactacid portion of the O_2 debt using this calculation.

In animal experiments Sacks and Sacks (1937) tried to show that a (diffusion) equilibrium as suggested by Hill *et al.* did exist between muscle and blood in steady state conditions but failed. From their experiments they concluded that a lack of free diffusion of lactic acid existed since the plasma of the venous blood issuing from the muscles contains a significantly lower concentration of lactic acid than does the muscle itself" These authors argued that it was unnecessary to postulate any alactacid" mechanism as Margaria and co-workers had done since it was impossible, on the basis of their experiments, to conclude anything about lactate formation from blood lactate data. Similar results were obtained by Ørskov (1931) and Bang (1935)

As illustrated by the investigators in the 1920's, lactic acid could be formed without tissue hypoxia. Huckabee came to the same conclusion (1958 a and b) when he examined the properties of the enzymatic reaction in which lactic acid is formed pyruvic acid + NADH ⇆ lactic acid + NAD catalyzed by the enzyme lactate dehydrogenase (LDH) and advanced the hypothesis of "excess lactate based on these studies. According to formula and the mass action law lactate formation does not necessarily depend on an oxygen lack which would induce elevated extra mitochondrial concentrations of NADH, as later demonstrated by Chance and associates (for ref see Chance, Schoener of Schindler 1964) Increased pyruvate concentration in the cell would similarly cause lactate formation by the cell. Consequently the significance of blood lactate concentrations might be questionable as an indicator of an anaerobic metabolism. As early as 1941 Friedemann and Barbocka had pointed out the theoretical importance of considering the concentration of pyruvate at the same time as that of lactate.

The excess lactate value calculated from lactate and pyruvate concentrations determined simultaneously would better reflect the lactic acid concentration formed due to true tissue hypoxia. This excess lactate hypothesis has been the subject of intense discussion and its relevance has been questioned (e.g by Knuttgen 1962, Margaria *et al* 1963 Olson 1963 Alpert 1965)

In 1909 Krogh suggested that the diffusion rate of O_2 from blood to the muscle cell might be critical in an adequate O supply to the cell. Chance *et al.* (1962) calculated a critical low intracellular O tension of approximately 1 mm Hg necessary for the maintenance of a normal redox state for the mitochondrial fraction of NAD Stainsby and Otis 1964 demonstrated on dogs that an O tension of 10 mm Hg in venous blood was sufficient to provide an adequate O_2 supply Similar low O_2 tensions have, in fact been reported in venous blood draining the exercising muscles during maximal exercise in man (Saltin *et al.* 1968)

In recent years the different sizes of motoneurons in the central nervous system have been advanced as being specific for the innervation of different types of muscle fibers (Henneman, Somjen and Carpenter 1965 a and b, Henneman and Olson 1965) The motoneurons have individually different rated threshold and are activated in special recruitment patterns which are related to the strength of the electrical stimuli. Moreover Kugelberg and Edström (1968) have shown that the fibers of a motor unit have identical enzyme profiles and that relatively more glycolytic fibers are stimulated at high firing rates. It seems therefore reasonable to assume that mainly red muscle fibers are activated in man at low work intensities, but the greater the intensity the greater the number of white fibers activated. The red muscle

fibers have a pronounced aerobic metabolism while the white ones display a more pronounced glycolytic metabolism. Whether the recruitment pattern is related to O_2 tension in the muscle or not is a question frequently discussed but yet unanswered. Further it can be questioned whether lactate formation in the muscle cell as i function of a deficiency in the O supply to the cell or of the intensity of muscle contractions as well.

As early as 1907 Fletcher and Hopkins empirically found that a high lactate concentration occurred at the same time as muscle fatigue appeared. When a muscle specimen was exposed to an oxygen rich environment the muscle regained its irritability at the same time as the lactate formed was removed, thus indicating the key position of lactic acid in the state of muscular fatigue. However the causal relationship has not been established even if Meyerhof's experiments (1925) further emphasized the role of lactic acid as the cause of muscular fatigue by showing increased performance by a muscle specimen in an alkaline Ringer's solution. In 1936 Bang examined lactic acid concentrations in man at rest and with increasing work loads and found concentration of blood lactic acid increasing as work load increased. He postulated that intracellular muscle lactic acid concentrations must reach even higher levels than in blood. Asmussen *et al.* (1948) were of the same opinion. Moreover Asmussen and his associates suggested that the limiting factor for physical work of high intensity was set at a certain concentration level for lactate in the working muscle groups.

As illustrated by Bang (1936) blood lactate concentration tended to decrease if work were continued for longer than 10 min, even if the initial blood lactate concentration had been 8—10 mmoles $\times$ l^{-1}. Astrand *et al.* made similar observations in 1963 when studying the effects of prolonged heavy exercise consisting of cross-country ski racing lasting 1—8 hrs. Lower blood lactate concentrations were found in subjects given standardized submaximal and maximal work loads after the race than in control experiments after prolonged rest. The duration of work with the maximal test load was also reduced. These authors proposed an impaired ability to obtain energy from anaerobic sources, while Bang suggested an increased utilization of exogenous lactate induced by the prolonged work both the suggestions would result in a decreased blood lactate level.

With the re-introduction of the needle biopsy technique (Bergström 1962) originally described by Duchenne in the middle of the 19th century (for ref. see Natrass 1968) it was possible to determine local changes in the concentrations of certain metabolites such as lactate, ATP and CP in the working muscle. By means of this technique and enzymatic analytic methods, Hultman Bergström and McLennan Anderson 1967 were able to demonstrate a decrease in

the local stores of ATP and CP thus confirming the old contention originally advanced by Margaria, Edwards and Dill that anaerobic energy output was in part derived from a depletion of energy rich phosphate compounds stored in the muscle (phosphagen) Data were not, however presented for local lactate concentration in the working muscle, which is why an explicit picture of anaerobic energy yield by the working muscle could not be presented.

It was felt to be of interest to further develop the analytical methods used on muscle biopsy specimen of man in order to make it also possible to determine certain glycolytic intermediary metabolites such as glycogen, pyruvate and lactate in addition to ATP and CP With these methods it should be possible to estimate the alactacid anaerobic energy yield during exercise and to examine the relationship between muscle lactate and blood lactate concentrations and to clarify the extent to which lactate concentrations in muscle and blood are related to a hypoxic state in the working muscle. Moreover it was of interest to include several different work levels in the present report in order to illustrate phosphagen depletion and lactate formation in greater detail in relation to absolute as well as to relative work load and how work stress affected the subject.

More specifically the aims of the present studies may then be summarized as follows

1 to adapt methods for the determination of carbohydrate metabolites, i.e. chiefly glycogen, pyruvate and lactate, plus ATP and CP in small muscle biopsy specimens of human muscle (5—15 mg wet weight)

2 to examine whether or not lactate concentration gradients are present within the muscle during mild and severe exercise and to evaluate the extent to which a single biopsy specimen of the muscle can be considered as representing the muscle as a whole

3 to illustrate the interrelationship between O_2 deficit, phosphagen depletion and accumulation of lactate in the working muscle and

4 to investigate whether preceeding prolonged heavy exercise of several hours duration may influence LDH activity and lactate accumulation in the working muscles.

CHAPTER II

GENERAL LABORATORY METHODS

Abbreviations

ADP	= adenosine 5-diphosphate
AMP	= adenosine 5-monophosphate
atm	= standard physical atmosphere
ATP	= adenosine 5-triphosphate
a-v difference	= arterio-venous difference
BSA	= bovine serum albumin
CP	= creatine phosphate
F-6-P	= fructose-6-phosphate
F 1 6-P	= fructose-1 6-phosphate
g	= gram
G-6-P	= glucose-6-phosphate
hr	= hour
k_m	= the substrate concentration at which the velocity of a reaction is half the maximal
kpm × min	= kilopondmeter per min (= 0.163 Watt)
l	= liter
LDH	= lactate dehydrogenase
M	= moles × l^{-1} of solvent
max $\dot{V}O_2$	= maximal oxygen uptake
min	= minute
NAD	= nicotinamide-adenine dinucleotide
NADH	= nicotinamide-adenine dinucleotide, reduced form
NADP	= nicotinamide-adenine dinucleotide phosphate
NADPH	= nicotinamide-adenine dinucleotide phosphate, reduced form
P O	= arterial oxygen tension
PEP	= phosphoenolpyruvate
P_i	= inorganic phosphate
SD	= standard deviation
SE	= standard error of the mean
sec	= second
V_{max}	= maximal velocity of a reaction or similar process
$\dot{V}O_2$	= oxygen uptake
V	= volt

Definitions

aerobic processes	= chemical processes in which O_2 is the electron acceptor
anaerobic processes	= chemical processes in which other cellular constituents are electron acceptors
aerobic glycolysis	= glycolysis ending up with pyruvate formation
anaerobic glycolysis	= glycolysis ending up with lactate formation
anoxia	= a state with a complete absence of oxygen
hypoxia	= a state with a limited oxygen supply
lactacid anaerobic energy yield	= energy from anaerobic glycolysis
alactacid anaerobic energy yield	= energy from ATP and CP stores
maximal oxygen uptake ($max\dot{V}O_2$)	= the greatest oxygen uptake per unit of time according to the levelling off criterion (Åstrand and Saltin 1961) during physical work and breathing air at sea level
maximal (or supramaximal) work load	= exhaustive work load corresponding to or exceeding the individual $max\ \dot{V}O_2$ at observed mechanical efficiency
phosphagen concentration	= (ATP + CP) concentration
relative work load	= the per cent of the individual $max\ \dot{V}O_2$ demanded by the work load, assuming a complete aerobic energy yield (at submaximal work load = steady state $\dot{V}O_2$)
submaximal work load	= a work load, less than the work load corresponding to the individual $max\ \dot{V}O_2$
supramaximal work load	= see maximal work load

Statistics

Ordinary statistical methods were used to calculate mean, SD and SE.

The error of the methods employed was estimated from SD of a single experiment as calculated from double determinations. Residual error was estimated, if calculated, by means of the formula

$$SD_t = \sqrt{SD_a^2 + SD_b^2 + \quad + SD_n^2}$$

The statistical significance was tested, when possible, on intraindividual differences (Student's "t" test) and in other cases on differences between means. The level of significance is denoted with p values.

Subjects

Some anthropological and physiological data for the 49 subjects studied are presented in Table I. Fifteen of these subjects were studied immediately after induction into military service. They were sedentary while the remainder were physical education students or the equivalent who regularly participated in physical activities only a few subjects were nationally prominent competitors in endurance events.

Procedure

In preliminary tests several days before the experiment day subjects were "mapped" in respect to max $\dot{V}O_2$, maximal heart rate, maximal blood lactate concentration and the work load exhausting the subjects within 2—6 min. Exercise was performed on an electrically (Krogh's bicycle) (Nielsen and Fridrichsen 1938) or mechanically braked bicycle ergometer (von Döbeln 1954)

On the experiment day subjects reported to the laboratory early in the morning after only a light breakfast. After 45—60 min at rest in a supine position, rest measurements were made. Submaximal work loads, if included in the protocol, were chosen so as to elevate blood lactate concentration significantly over rest values, i.e. work load demanding an oxygen uptake exceeding 50 per cent of max $\dot{V}O_2$.

The submaximal work load was generally interrupted after 6—10 min. Immediately after completion of work, the subject still sitting on the bicycle a biopsy specimen was obtained from the lateral portion of the subject's quadriceps muscle. Repeated blood samples were simultaneously obtained

TABLE I Mean and range of pertinant anthropological and circulatory data of the subjects participating in the different investigations.

Series	n	Paper	Age years	Height cm	Weight kg	Maximal oxygen uptake l × min	ml × kg × min⁻¹
1	13	II	24 (22—26)	182 (172—192)	72 (65—83)	4.4 (3.4—5.3)	61 (52—72)
1 A	5	VII	23 (22—24)	180 (172—189)	68 (65—75)	4.1 (3.4—4.7)	60 (52—64)
1 B	8	VII	25 (25—26)	184 (174—192)	75 (69—83)	4.6 (4.0—5.3)	61 (60—72)
2	15	II	20 (19—22)	178 (172—187)	70 (54—83)	3.3 (2.7—4.2)	48 (41—54)
3	4	I	23 (22—25)	176 (169—181)	73 (68—75)	4.2 (3.8—4.5)	57 (55—60)
4	5	III	26 (22—28)	180 (179—181)	74 (71—76)	4.4 (4.1—4.7)	58 (55—63)
5	3	IV	23 (22—24)	175 (172—180)	69 (65—72)	4.0 (3.8—4.2)	58 (54—62)
6	8	V	23 (21—24)	178 (170—184)	70 (63—76)	3.9 (3.5—4.2)	56 (51—62)
7	6	VI	26 (23—28)	176 (175—178)	73 (69—76)	4.4 (4.2—4.6)	60 (56—66)

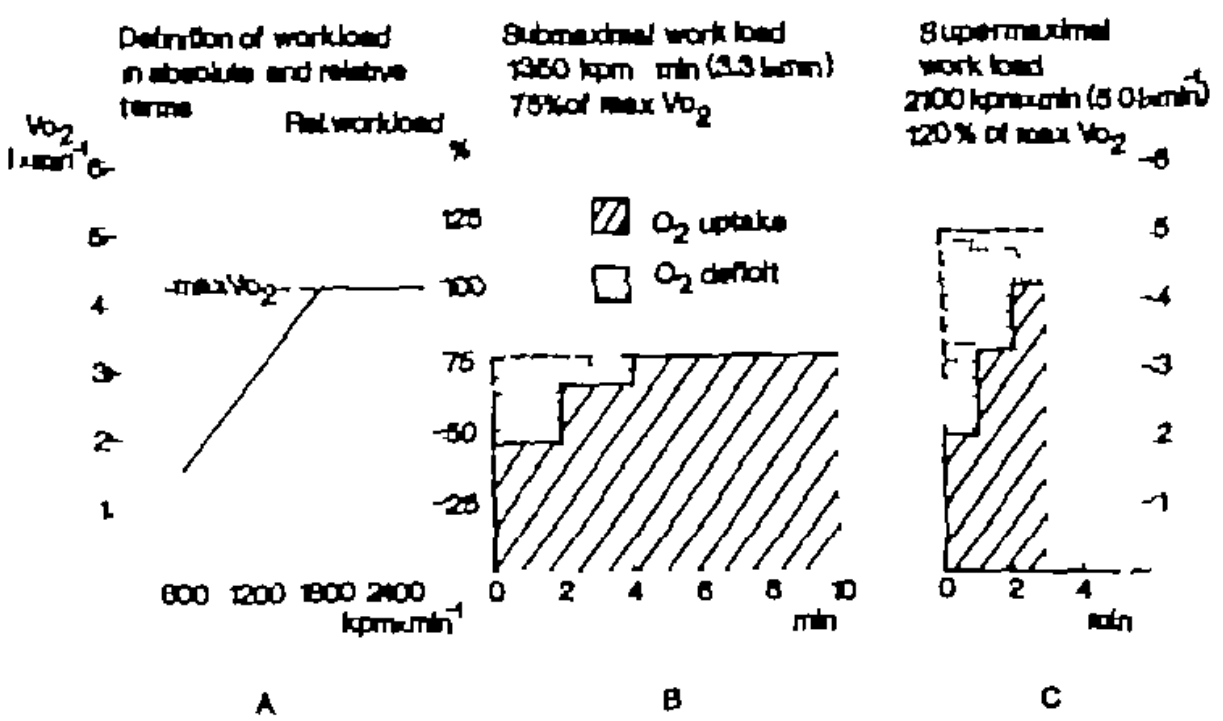


Fig. 1 Principles underlying calculation of relative work load and oxygen deficit. Left $\dot{V}O_2$ is related to work load, max $\dot{V}O_2$ being defined by the levelling off criterion. Centre: oxygen deficit at submaximal load ($\dot{V}O_2$ = 3.1 l × min^{-1} equivalent to 75 % of max $\dot{V}O_2$) Right: oxygen deficit with supramaximal work ($\dot{V}O_2$ = 5.0 l × min^{-1} for complete aerobic energy output, equivalent to 120 % of max $\dot{V}O_2$)

from a prewarmed hand (fingertip blood) The exhaustive work load was performed after 10—20 min of rest and biopsy specimens and blood samples were taken as above.

$\dot{V}O_2$ was determined in all these experiments, either throughout the whole work period or during the final min.

Assuming a mechanical efficiency of 22.5 % with the supramaximal work load relative work load was estimated as described in Fig. 1 a, in which the linear relationship $\dot{V}O_2$ — work load is extrapolated to supramaximal work loads. From this curve it is possible to predict the $\dot{V}O_2$, assuming complete aerobic energy yield even with work loads exceeding max $\dot{V}O_2$.

By definition, the measured $\dot{V}O_2$ with steady state conditions at a certain submaximal work load is equal to the amount of oxygen demanded for complete aerobic energy yield.

O_2 deficit calculations were performed in experiments in which total $\dot{V}O_2$ was determined. This is illustrated in Fig 1 b for a submaximal work load and is based on the assumption of a 22.5 % constant mechanical work efficiency during the initial phase of the work. The O_2 deficit is the difference between

a theoretical oxygen uptake with complete aerobic energy yield and the value actually determined. As shown in Fig 1 c the same calculations have been *made for the exhaustive, maximal and supramaximal work loads.*

Methods

The physical work of the exercise tests was performed on bicycle ergometer Oxygen uptake was determined with the Douglas bag method. The expired gas volume was measured in a Tissot spirometer and gas analyses were made using the *Enghoff Haldane technique.* The individual max $\dot{V}O_2$ was determined according to the levelling off criterion (Åstrand and Saltin 1961)

Blood lactate concentration was determined using an enzymatic method originally described by Scholz *et al.* (1959) on 0 1—0.2 ml of arterial fingertip blood taken from a prewarmed hand. Several blood samples were obtained after exercise.

The LDH activity of biopsy specimens of man was determined from tissue homogenates (in 0.5 ml of ice-cold TRIS-buffer pH 8 0) according to Lowry and Passonneau (1964) The accumulated changes in fluorescence (ΔE) when corrected for the blank values, were used to determine the moles of NADH oxidized according to the formula

$$(NADH) = \Delta E \times \text{fluorometer factor} \times V \times V_h \times V \times W$$

The fluorometer factor = the amount of spectrophotometrically determined NADH, causing a change of 1.0 scale divisions on the fluorometer

V volume of reagent in the fluorometer tube (1.00×10^{-3} l)

V volume of homogenate added to the fluorometer tube (1.58×10 l)

V total volume of the homogenate (0.50×10^{-3} l)

W_w wet weight of the muscle biopsy at zero time (Bergström 1962)

The LDH activity was defined as the initial velocity of the reaction. Two determinations were performed on four muscle specimens, giving values within 0.5 % of the first determination.

CHAPTER III

DETERMINATION OF ORGANIC MUSCLE METABOLITES

Enzymatic fluorometric analysis of muscle metabolites

Chemical analyses of the muscle tissue metabolites were performed mainly in accordance with the St. Louis method introduced by Oliver H. Lowry and co-workers (ref. see Table II)

Information concerning quality concentration in test medium and source of the reagents for the metabolites studied are presented in Table II

Enzymatic reactions were in general carried out in 3 ml prematched test tubes in 1 ml of reagent. The NADPH or NADH concentrations were fluorometrically determined in a Farrand fluorometer Model 24 The increase or decrease in the fluorescence thus obtained was compared in each determination with the corresponding increase or decrease obtained in identical test media with spectrophotometrically predetermined standard solutions. The net changes in concentration of the metabolites were arranged to read in excess of 5—10 % of the scale of the fluorometer

Table II also indicates whether or not reactions were started with the sample or any appropriate auxiliary enzyme. Approximately 20—25 samples were analyzed at a time, and the concentration of auxiliary enzymes was chosen to give a 1—2 min reaction halftime.

With the present methods, it was possible to obtain accurate determinations of metabolites present at concentrations in excess of 0.5 mmoles × kg wet muscle. This corresponds to test tubes concentrations of $1—2 \times 10^{-4}$ mM of NADH or NADPH.

The same analytical procedure was used to check the order of magnitude of the pyruvate concentrations. To obtain more accurate values an indirect method was applied (Lowry Roberts and Kapphahn 1957) using 100 µl of a medium as described in Table II. After a 15 min incubation duplicate samples were treated with 26 µl of 6 M HCl to destroy the remaining NADH. One ml of 6 M HCl was then added and the samples were allowed to remain at 60 C for 15 min before reading in the fluorometer (at room temperature)

The error in double determinations is presented in Table III for the analysis of the same supernatant as well as for separate samples of the same muscle biopsy specimen. Errors in enzymatic assays were 0.4—0.7 mmoles × kg^{-1} wet muscle, except for pyruvate and glycogen for which the errors were 0.006 mmoles × kg^{-1} wet weight and 2.5 mmoles of glucose units per kg, respectively

TABLE II Analytical conditions

Substrate	Reference	Buffers	Enzymes Final concentration	Other reactants Final concentration	Muscle quantity used
Glycogen	Lowry *et al.* 1964	Tris 0.1 M pH 7.5	Yeast Hexokinase[B] 5.0 μg × ml Yeast Glucose-6-P-Dehydrogenase[B] 0.7 μg × ml⁻	$MgCl_2$ 5 mM BSA[B] 0.01 % NADP 0.05 mM	0.05 mg[**)]
G-6-P	Lowry *et al.* 1964	Tris 0.1 M pH 8.0	Yeast Glucose-6-P Dehydrogenase[B] 1.0 μg × ml⁻	$MgCl_2$ 5 mM BSA[B] 0.01 % NADP 0.06 mM	0.2 mg[**)]
Lactate	Lowry *et al.* 1964	Carbonate 0.1 M pH 9.7	*Heart Lactic Dehydrogenase*[W] 5.5 μg × ml	NAD[B] 1.0 mM	0.5 mg[**)]
Pyruvate	Lowry *et al.* 1956 1967	Phosphate 0.1 M pH 7.0	Heart Lactic Dehydrogenase[W] 0.5 μg × ml⁻	NADH 0.0003—0.001 mM, BSA 0.01 %	0.5—0.1 mg[**)]
ATP[*)]	Lowry *et al.* 1964	Tris 0.1 M pH 8.0	See Glucose-6-P-medium + Yeast Hexokinase[B] 2 μg × ml⁻ [**)]	See Glucose-6-P + Glucose 1.0 mM	0.2 mg
CP	Lowry *et al.* 1964	Tris 0.1 M pH 8.0	See ATP-medium + Muscle Creatinekinase[B] 85 μg × ml⁻ [)]	See ATP + ADP[B] 0.03 mM	0.1 mg
ADP	Lowry *et al.* 1964	Phosphate 00.5 M pH 7.0	Heart Lactic Dehydrogenase[W] 9 μg × ml⁻ Muscle Pyruvate-kinase[B] 0.5 μg × ml	$MgCl_2$ 2 mM, BSA[B] 0.01 % ATP[B] 0.02 mM, PEP[B] 0.05 mM, NADH[B] 0.004 mM	0.5 mg[**)]
P	Gatfield *et al.* 1966 Schulz *et al.* 1967	Imidazole 0.01 M pH 7.0	Phosphoglucomutase[B] 3.5 μg × ml⁻ Yeast Glucose-6-P Dehydrogenase[B] 5.5 μg × ml⁻ Phosphorylase-a 75 μg × ml⁻ [**)]	$MgCl_2$ mM BSA[B] 0.02 % NADP[B] 0.04 mM, Glycogen[N] 0.1 % Cleelands 0.5 mM 5-AMP[B] 0.01 mM, EDTA 1.0 mM	0.2 mg

[)] the same sample as for G-6-P

B = Boehringer N = Nutritional.

W = Worthington. S = Sigma.

[**)] denotes whether the reaction is started with the substrate or an auxiliary enzyme.

TABLE III. Methodological error of the studied muscle metabolites expressed as the standard deviation (SD) of a single determination. The residual error (SD) was calculated according to the formula $SD_r = \sqrt{SD_t^2 + SD_a^2}$

Substrate	The error of the total procedure (SD_t)	The error of analysis (SD_a)	Residual error (SD)
Glycogen	4.6	2.3	4.0
(glucose units)	0.62	0.30	0.54
G-6-P*	0.010	0.006	0.016
Pyruvate	0.68	0.48	0.48
Lactate			
Lactate in freeze dried slices**	0.78		
ATP*	0.64	0.47	0.43
CP*	0.81	0.55	0.59
ADP*	0.36	0.16	0.32
P_i	1.13	0.68	0.90

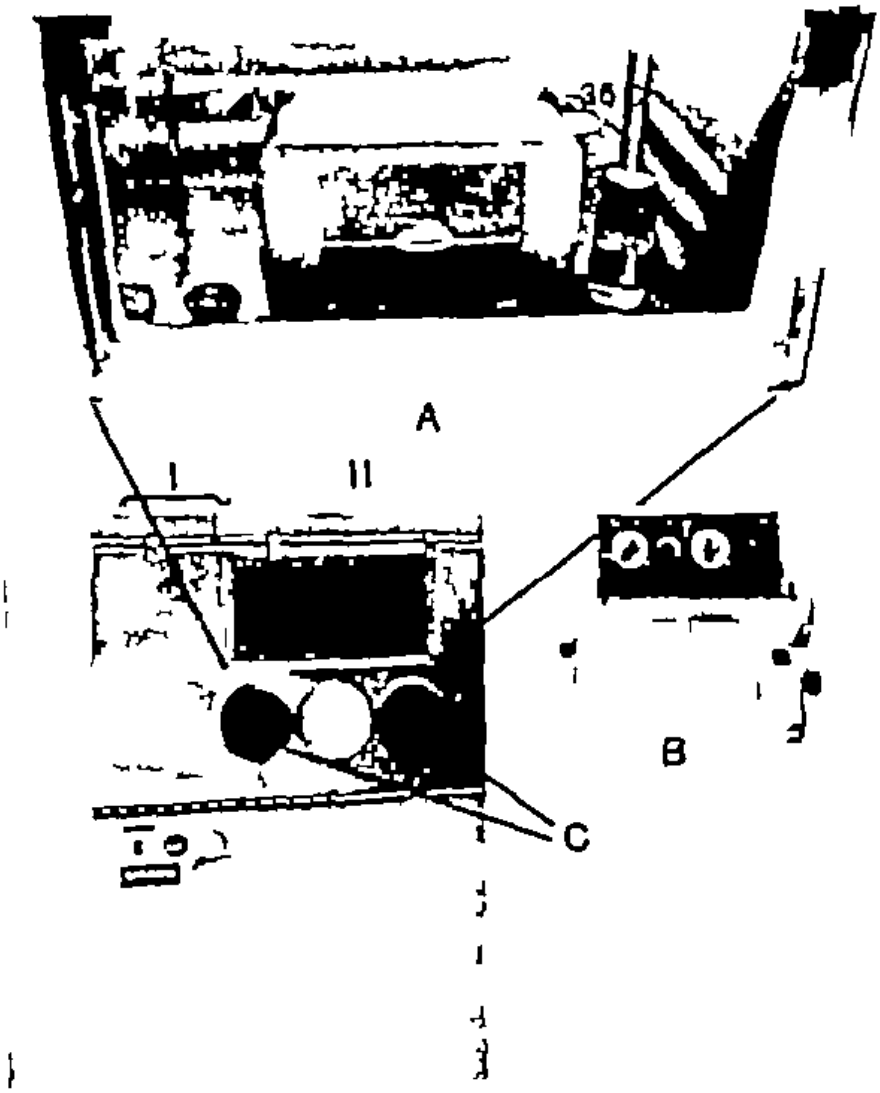
* mmoles × kg⁻ wet muscle
** mmoles × kg⁻ dry muscle

Comments

To evaluate the error of the method for separate analyses, residual error (SD) was calculated in each case from the standard deviation of a single determination of

a) the chemical part of the procedure (SD_a) and

b) the complete procedure, also including weighing and biological variations of duplicate samples (SD_t). The SD was never found to be less than the SD for the metabolites studied. As the weighing error was less than 1 % it is assumed that biological variations were responsible for the main part of the total methodological error. This assumption is supported by other methodological studies in which known amounts of different metabolites were added to the test medium which also included extract of biopsy specimen. Recovery was found to be 98 % (range 95—100)



Ill. 1 Cryostat for weighing frozen muscle biopsy specimen, constructed in collaboration with E. Nilsson Instrument, Solna, Sweden.

The interior of the cryostat — the weighing room (A) supplied with Cahn Electrobalance has temperature of —35 C.

The weight of the sample is tomatically registered outside the cryostat box (B)

The box is constructed with two lids (I and II) the small one (I) is for separat part of the box, divided from the weighing room by a screen This construction makes it possible to quickly put down or remove g biopsy samples from the cryostat without interferring the weighing room. The electrobalance and the interior of the weighing room can be manipulated from the outside (C)

Freezing and weighing of muscle biopsy specimens and extraction of metabolites

Muscle specimens from man were obtained from the lateral portion of quadriceps femoris muscle using a needle biopsy technique. All biopsy specimens were frozen in liquid nitrogen while still in the biopsy needle within 1—2 sec after the needle was withdrawn from the muscle.

The weighing procedure was performed on a Cahn Electro-balance at —20 C, or less either in a freezing room or in a specially constructed cryostat (Ill. 1) The biopsy specimen was dissected to remove blood and connective tissue, constituents easier to observe in a frozen state than fresh, and then powdered in a porcelain mortar After weighing the powder was transferred to frozen 3 M perchloric acid for further treatment.

The biopsy material, freed from frozen blood and connective tissue, powdered and weighed was extracted (deproteinized) in about 150 µl of 3 M $HClO$ at —20 °C. After 2—4 hrs the temperature of samples was raised to + 4 C samples were neutralized with approximately 250 µl of 2 M $KHCO_3$ (to obtain a final pH ranging from 7.8—8.5) and centrifuged. The supernatants were saved for determination of water soluble metabolites. The residues were dissolved and made up to 1.0 ml with 1 M HCl and hydrolyzed for 2 hrs in carefully sealed tubes in a 100 °C water bath for subsequent glycogen determination.

The degree of extraction of the water soluble metabolites was studied by treating various amounts of the same muscle powder with the same volume of $HClO_4$ (see Fig. ?) A muscle tissue specimen with a lactate concentration of 6.9 mmoles × kg wet muscle was divided into several portions with varing weights. The amount extracted (presented as scale readings on the fluorometer of the analysis kit) was plotted against the wet weight of each portion. It may be concluded that lactate was linearly extracted from specimens weighing 2—25 mg of wet muscle, indicating a complete extraction of water soluble metabolites. Re-extraction of the perchlorate precipitate supported the assumption that extraction was complete. In the present study all samples used ranged between 2.8 and 16.4 mg of muscle powder and, consequently were within the linear part of the curve. Similar curves were also obtained for ATP and CP

To test whether or not phosphate compounds were destroyed or were lost by $HClO$ extraction and subsequent neutralization, known amounts of ATP and CP were treated at low temperatures in the same manner as the biopsy specimens. Little or no loss (< 2 per cent) was observed. Lowry *et al.* (1964) previously showed that the procedure employed in deproteinization did not influence endogenous ATP and CP levels.

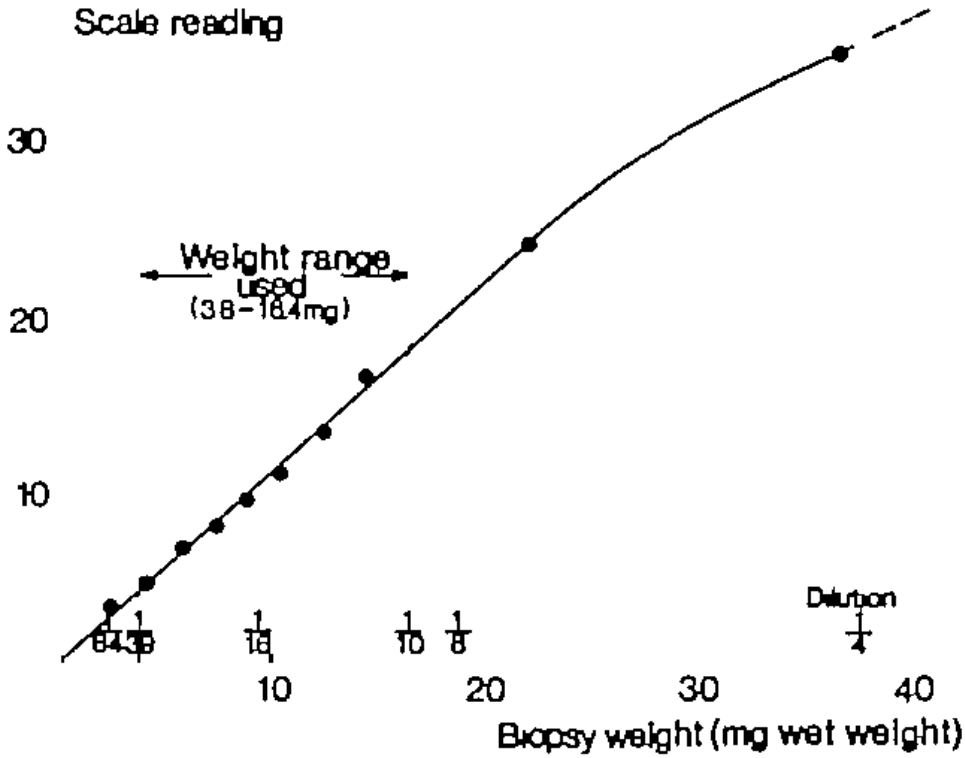


Fig. 2. Extraction of muscle lactate in biopsy specimens ranging between 2.5—38 mg wet weight. Extraction is expressed as the fluorometer scale reading (net) which is proportional to the lactate concentration. The actual weight range used in the present studies is 2.8—16.4 mg, which is within the linear part of the extraction curve.

Comments

The storage of supernatants for longer periods of time at —20° C was observed to depress the concentration of ATP and CP (a 50 % loss was found after six months storage) P_i levels were elevated at the same time, but not stoichiometrically Further measurable amounts of AMP could be detected. In the present study care was taken to check for ATP and CP in the supernatant within a few days after the extraction. None of the other supernatant metabolites was found to change during the time needed for complete analysis.

To evaluate the influence of the freezing procedure s duration of latency on certain muscle metabolites such as ATP CP and lactate, specimens from the canine gracilis muscle were frozen at intervals ranging from zero to 30 min after extirpation (see Fig 3)

Briefly these experiments with delayed freezing produced the following results a very small decrease in CP concentration may occur in the first

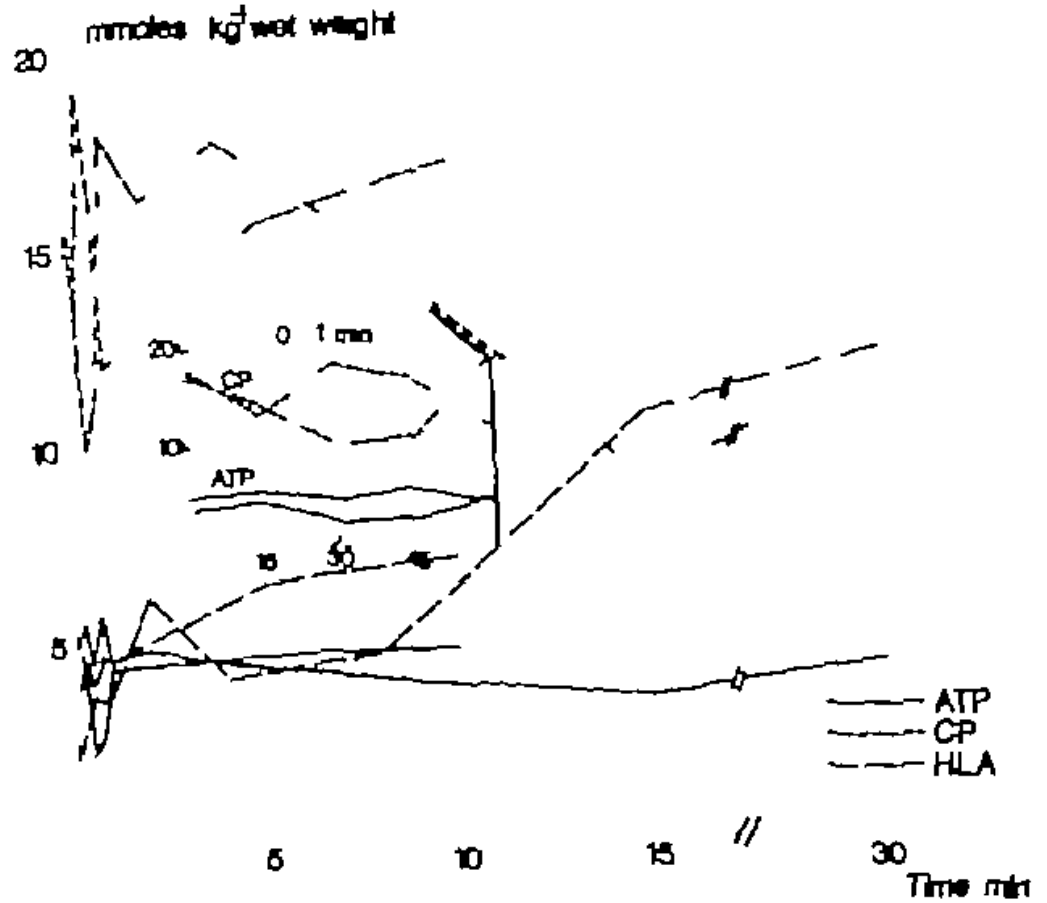


Fig. 3 Changes in ATP CP and lactate concentrations at different time intervals between extirpation of the biopsy specimen and freezing in liquid nitrogen.

3—4 sec before the freezing of the specimen. ATP concentration did not decrease during the time studied, but there was an increase in lactate concentration. This increase was initially (during the first min) rapid but, with time, the rate of increase declined. Thus, lactate values obtained averaged 1.5 mmoles × kg⁻ wet weight at zero time, about 4 mmoles × kg⁻ wet weight after 1 min and 7 mmoles × kg⁻ wet weight after 10 min.

Concentration differences for lactate in a single biopsy specimen and in the contracting muscle

Even if the analytical methods applied were accurate enough for reproducible determinations, the question may arise as to whether or not concentration gradients within contracting muscle in particular influence the extent to which the sample is representative of the muscle as a whole.



Ill 2. A freeze dried slice of biopsy specimen and the definition of different portions analysed for lactate concentration
A fiber tructures
B extra-fiber tructures
C mixed structures

To analyze further the biological significance of lactate determinations, the following items were studied

1 concentration differences within the biopsy specimen
2 concentration differences within a contracting muscle (the studies were performed on canine gracilis muscle stimulated in situ)
3 repeated biopsies from the vastus lateralis of man at rest and after exercise of increasing intensities.

Lactate concentration differences within the biopsy specimens were studied by examining the lactate concentration of separate samples dissected from freeze-dried sections as illustrated in Ill. 2. These sections were obtained with a microtome at —20 C freeze-dried for 36—48 hrs and dissected under light microscope at a magnification of 20 x. After weighing dissected material was put into test tubes for determination of lactate, as described in Table II The error introduced by this modification of the lactate method was 0 78 nmoles × kg⁻ dry weight.

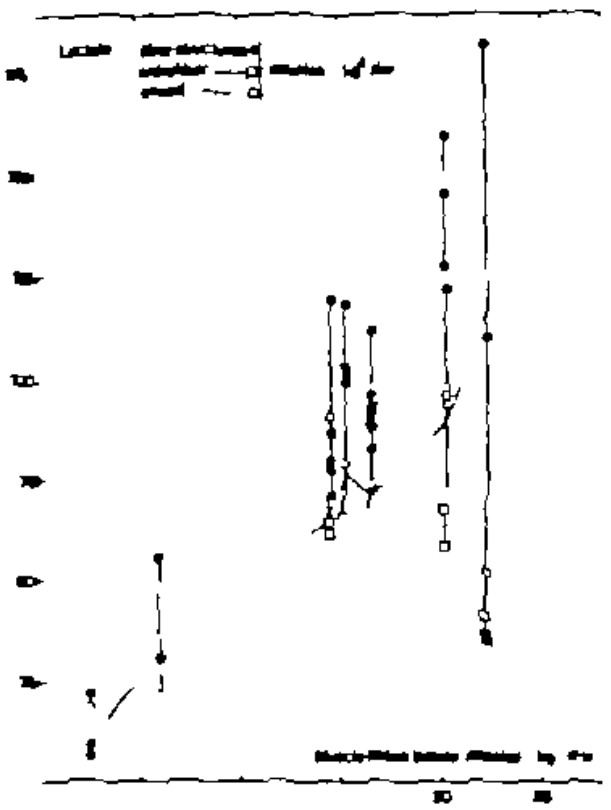


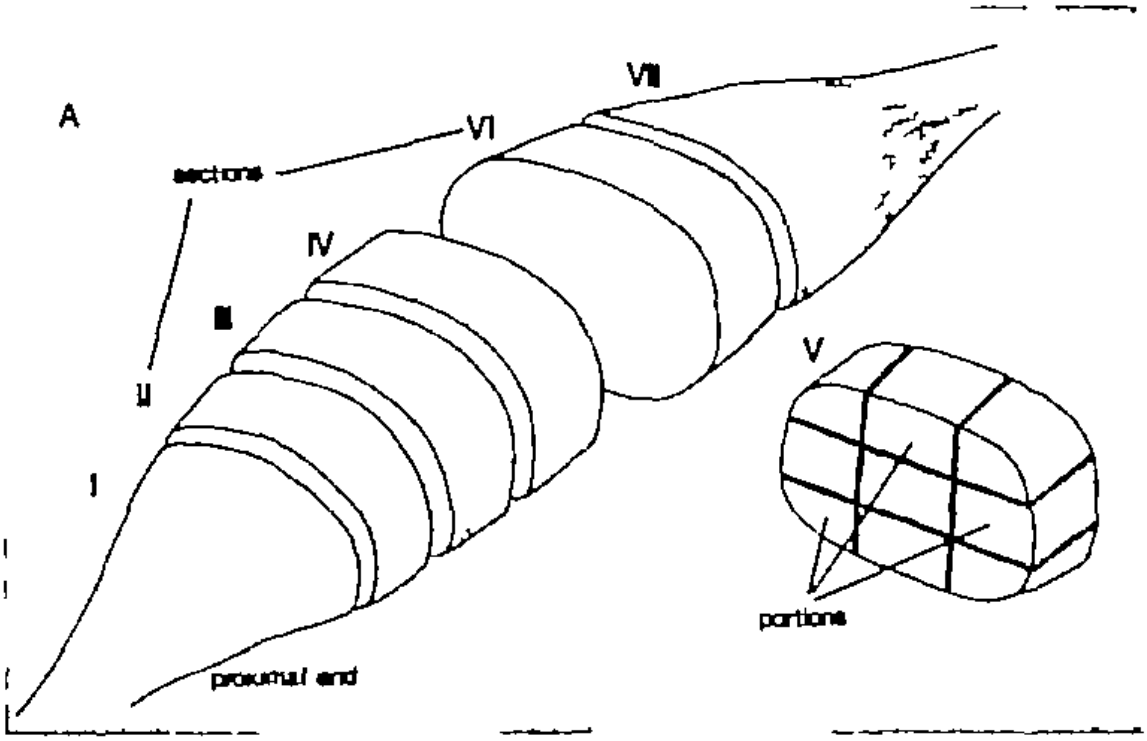
Fig. 4. Lactate concentration in different muscl tissue structures, as defined in Ill. 2, in relation to the muscle lactate concentration of the specimen. The curve connecting the examined specimens denote the lactate concentration analyzed for the whole slice.

In Fig. 4 results are presented expressed in mmoles × kg^{-1} dry weight for the dissected material plotted versus a corresponding sample treated according to the general procedure. Thus, it is possible to compare the lactate content obtained in the biopsy specimen with certain portions of the specimen

The dissected material was classified in three categories according to its appearance in the microscope

a. material consisting of muscle tissue only — fiber structures
b. tissue elements surrounding fiber bundles — extra-fiber structures
c. material containing both these constituents — mixed structures.

In all samples studied the highest lactate concentration value was obtained in the fiber structures and the lowest in all but two samples of mixed or extra fiber structures. This was most evident when the lactate content in the biopsy specimen exceeded 20 mmoles × kg^{-1} wet weight. Thus, in two samples the highest lactate concentrations obtained were 187 and 160 mmoles × kg^{-} dry weight or 37 and 32 mmoles × kg^{-} wet weight respectively while the lowest values obtained were 35 and 39 mmoles × kg^{-} dry weight or 7 and 12 mmoles × kg^{-} wet weight respectively assuming 77 % of the wet weight to be water Lactate concentration in the contracting elements of muscle performing heavy exercise would then correspond to values 3—5 times greater than the lowest concentration observed in the muscle specimen.



B

C

Fig 5 Schematic illustration of the dissection of canine gracilis muscle (A) and muscle lactat concentration in the portions of the section after 15 min timulation to contractions using 4/sec 4V and 0.1 msec (B) and 15/sec, 4V and 0.1 msec (C)

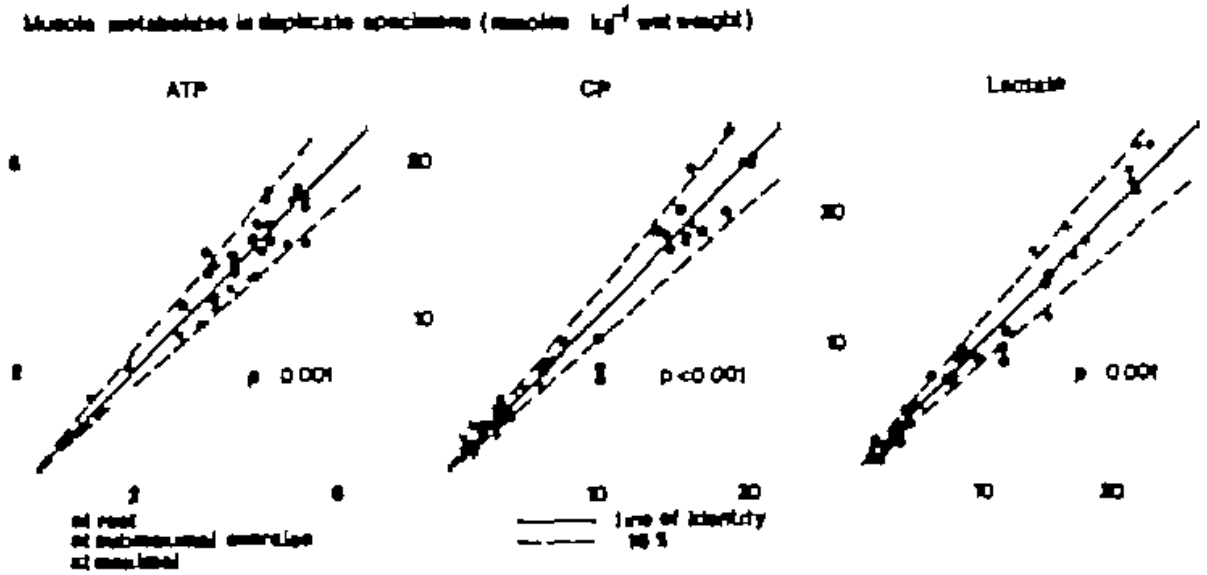


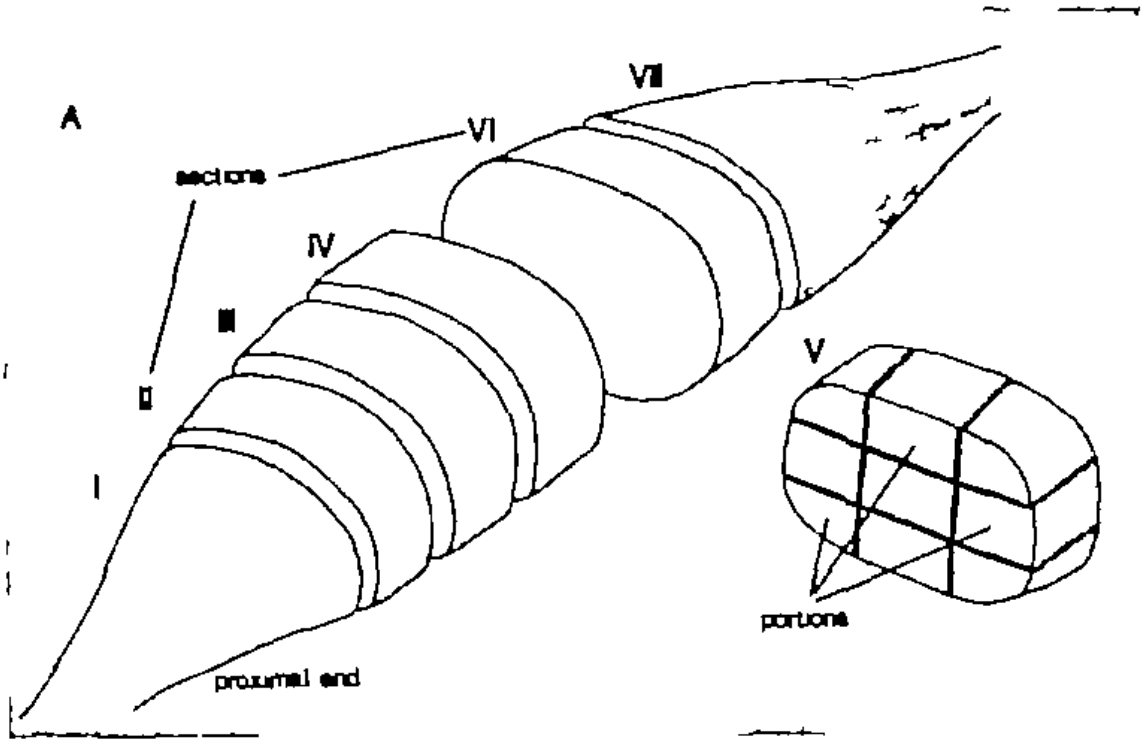
Fig. 6. ATP, CP and lactate concentrations in duplicate muscle biopsy specimens of man obtained from different parts of the quadriceps femoris muscle at rest, after submaximal and brief exhaustive work intensities. The duplicate samples, presented on the x-axis and y-axis respectively were obtained simultaneously at rest and at submaximal work rather than after brief exhaustive work in which the test load was repeated twice with at least 1 hr of rest in between.

SD for a single experiment were for ATP, CP and lactate concentration 0.55, 0.83 and 0.67 mmoles × kg⁻¹ wet muscle respectively.

The question may arise as to whether or not these gradients influence the extent to which the biopsy specimen is representative of the whole muscle examined. To pursue this question further, two canine gracilis muscles were electrically stimulated to contract (4/sec, 4V for 0.1 msec and 15/sec, 4V for 0.1 msec, respectively). After 15 min. stimulation the entire muscle was quickly frozen in liquid nitrogen and then prepared according to Fig. 5 A. The lactate concentration was found to average 7.8 and 22.9 mmoles × kg^{-1} wet weight respectively and SD of a single determination was 0.5 and 0.8 mmoles × kg^{-1} wet weight, respectively within sections II—IV.

Repeated biopsies were obtained from different sites in the thigh at rest and after bicycle work of different submaximal intensities (Fig. 6) in order to investigate whether there was a regional variation in lactate concentration in the vastus lateralis of the quadriceps femoris muscle of man. Repeated work loads were performed so as to obtain similar values after brief maximal exercise and biopsies were included in the protocol. At least one hr of recovery was allowed between each experiment.

Lactate concentration in resting muscle ranged from 1.0—2.7 mmoles × kg^{-1} wet weight in four muscles examined. However, the differences between



B

C

Fig. 3 Schematic illustration of the dissection of canine gracilis muscle (A) and muscle lactate concentration in the portions of the section after 15 min stimulation to contractions using 4/sec 4V and 0.1 msec (B) and 15/sec, 4V and 0.1 msec (C)

TABLE IV Constituents of the muscle biopsy specimens presented as mean ± SE and SD

Solids*)	Protein	N	Na	K
% of wet weight	g × kg^{-1} wet muscle		mEq × kg^{-1} wet muscle	
n = 17	n = 12	n = 12	n = 18	n = 18
23.0 ± 0.4	170 ± 6	28.4 ± 0.6	26 ± 1	84 ± 2
1.7	22	2.0	5	9

*) The biopsy specimens were obtained at rest as well as after exercise. Considering this the corresponding value for subjects at rest, with submaximal and maximal exercise was 22.9 ± 0.6, 23.9 ± 0.9 and 22.3 ± 0.6 respectively

Sodium and potassium concentrations

Muscle concentrations of sodium (Na) and potassium (K) were determined with a flame spectrophotometer on supernatants of muscle specimens homogenized in redistilled water using a Potter Elvehjelm homogenizer with teflon pistills. In specimens assumed to be made only of muscle tissue, sodium concentration averaged 26 mEq × kg^{-1} wet weight and the potassium concentration 84 mEq × kg^{-1} wet weight (Table IV)

Potassium levels lower than 60—65 mEq × kg^{-1} wet muscle were found when a specimen was contaminated with blood (Fig 7)

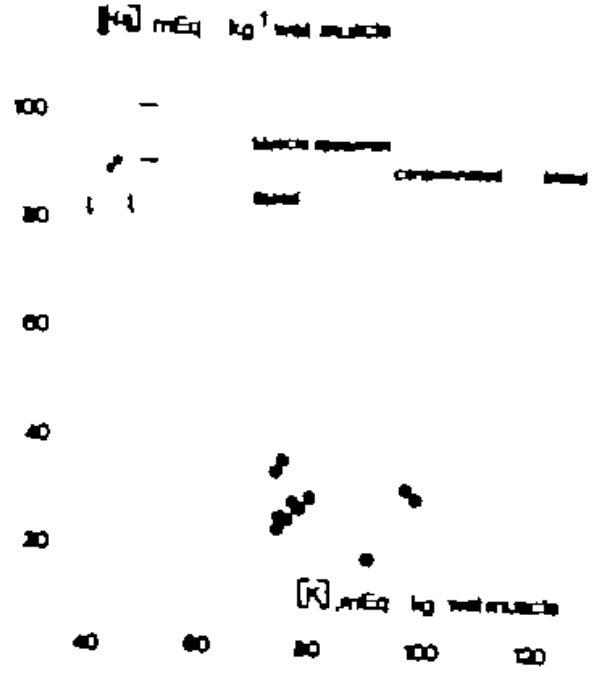


Fig. 7 Sodium (Na) concentration in relation to potassium (K) concentration in the same biopsy specimen of man consisting of muscle tissue or muscle tissue contaminated with blood. The dotted line and the arrows denote the highest potassium and lowest sodium concentrations in blood respectively

Comments

The present values for sodium and potassium agree with those previously reported (summarized by Bergström 1962). The relatively high values found in Bergström's own study calculated at an average at 25 and 99 mEq × kg wet weight respectively were obtained using another technique (activation analysis) which might explain the difference between the present and Bergström's mean potassium values. The activation analysis technique may include potassium present in the muscle cell bound to e.g. proteins. The present methods might be assumed to establish the amount of potassium present in the water phase.

On the basis of these figures intracellular Na—K space can be calculated and was found to range between 76—79 % of tissue water which should be compared to the approximately 75 % obtained by Hollosy and Narahara (1965) (3-methylglucose-^{3}H space) with frog muscle and the 83—91 % (chloride space) found by Bergström, Guarnieri and Hultman in 1971 for man at rest and after 20 min exercise respectively.

Nitrogen and protein content

The specimen's nitrogen content was determined according to the Kjeldahl technique (Jacobs 1965) and the specimen's protein content according to the Folin reaction, modified by Lowry *et al.* (1951) using BSA as a standard.

Nitrogen content averaged 1.0 moles N × kg^{-1} wet muscle, which is equivalent to 28 g N × kg^{-1} muscle. Protein amounted to 170 g × kg^{-1} wet muscle (Table IV).

Comments

In 1962 Bergström reported a N content amounting to approximately 28 g × kg^{-1} wet muscle. A factor of 6.25 for conversion of protein N into protein would provide a protein content corresponding to ca. 178 g × kg^{-1} wet muscle.

General comments on the methods to study muscle metabolites

Assuming approximately the same ratio for water content and solid material in the fiber and extra-fiber structures respectively a marked concentration gradient seemed to exist for lactate as soon as the average concentration of the specimen was elevated compared to resting values. This gradient was

evidently greater with higher tissue levels of lactate. This may indicate that the translocation process for lactate is not fast enough to establish a diffusion equilibrium for lactate between the fiber and extra fiber structures during moderate and heavy exercise of short duration (up to 10 mm)

Kübler *et al.* (1967) demonstrated a $Q_{10} > 2$ for the translocation of lactate from the heart to the venous blood with hypothermic dog heart, indicating that the process was of a nondiffusing type. Similar findings have been reported by Hirche, Langohr and Wacker (1970) and Karlsson, Rosell and Saltin (1971) (Fig. 8) with canine gracilis muscle which, stimulated to contractions, demonstrated a levelling off of venous lactate release related to the increase in muscle lactate concentration. Approximately the same values for V_{max} and K_m were obtained for lactate release in these two studies.

If it is assumed that blood lactate concentration represented extracellular fluid and that conditions were unchanged in respect to total cellular water and the relationship between intra and extracellular water then a saturation relationship must be present in the mechanism for lactate translocation from muscle to blood. Glaviano (1965) Tranquada (1966) and Kübler *et al.* (1967) have even suggested that an active process is involved.

Bergström, Guarnieri and Hultman (1971) demonstrated increased total tissue water content in human biopsy specimens taken from subjects after moderate exercise (1000 kpm × min) and calculated, using chloride space determinations, an increased intracellular water content which was linear to the increased muscle lactate concentration. It must be pointed out, however that the increase is less than 5 % of the wet weight. In the present material, in which the water content of specimens was determined in samples selected at

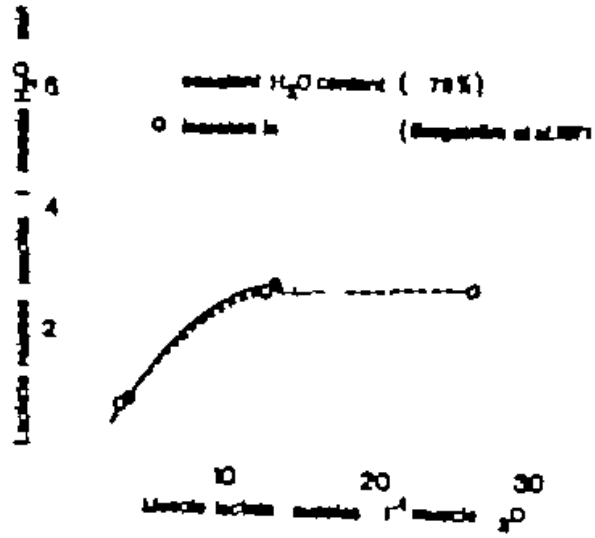


Fig. 8. Lactate release from contracting canine gracilis muscle in relation to muscle lactate concentration. The solid curve denotes the assumption of muscle water content corresponding to 76 % of the wet weight and the dotted curve an increase in muscle water content during contractions according to Bergström, Guarnieri and Hultman (1971) V_{max} of the release is 0.28 mmoles × l⁻ muscle water × min⁻ and the $k_m \sim 6$ mmoles × l⁻ muscle water

random from a number taken from subjects at rest and after exercise, no such tendency was found. Assuming an increase in total tissue water during exercise of the magnitude suggested by Bergström, Guarnieri and Hultman (1971) the data in Fig 8 were recalculated. As illustrated the levelling off in the relationship between venous lactate release to the capillary blood and muscle lactate concentration was more pronounced when considering the increase in muscle water during exercise.

Whether or not arterial finger tip blood obtained in man represents blood concentrations in the legs, especially after brief maximal work can be questioned. The lactate concentration in blood from the brachial artery in man has shown to be of the same order of magnitude as blood from the femoral vein which was simultaneously drawn immediately after submaximal and maximal work (Saltin *et al* 1968) The same experiment also illustrated that lactate concentration in femoral venous blood was only approximately 1 mmole × l⁻ higher than in femoral artery blood after maximal exercise, indicating that there is also a limitation on the translocation of lactate from muscle to blood in man.

Values for the individual muscle metabolites obtained in the lateral portions of quadriceps femoris muscle of man at rest were in good agreement with data previously reported (glycogen, ATP and CP concentration) from other laboratories using different techniques (Hultman 1967) It is concluded that reliable methods have been applied to the muscle biopsy specimen to make quantitative determinations of ATP CP G-6-P pyruvate and lactate in the same supernatant and of glycogen in the residue after perchloric precipitation.

CHAPTER IV

RESULTS

Lactate accumulation and phosphagen depletion with submaximal and maximal exercise

(Papers I, II and IV)

In order to illustrate the breakdown of phosphagen (ATP + CP) and lactate in the working muscle, 28 subjects were examined, 13 trained and 15 untrained.

There was a marked breakdown of the CP stores even with the submaximal work loads in both subject groups, while ATP depletion could only be observed with maximal or near maximal work loads. (Fig 9)

Although there was a marked difference between the two groups of subjects when work loads were expressed in absolute terms (Table V) no difference could be demonstrated between the groups in respect to the degree of CP depletion with the same relative work load (75 % and 100 % of max $\dot{V}O_2$

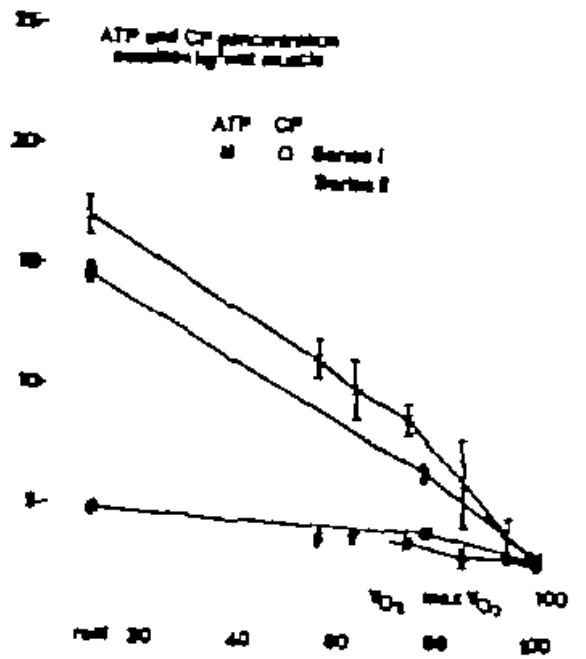


Fig. 9. Mean value ± SE for ATP and CP in relation to the relative work load, i.e. the oxygen uptake expressed as per cent of the individuals' maximal oxygen uptakes. In Series II (untrained subjects) mean values were calculated from the individual results and presented according to the following groupings: 50—59 60—69 70—79 80—89 90—99 and ≥ 100 % of maximal oxygen uptake. There were five or more subjects in each group.

TABLE V Mean values and range for the work loads and oxygen uptakes are given. The muscle metabolite and blood lactate values are presented as means ± SE and SD. The p-values denote the significance level between the observed results at different work loads. These calculations are based on intra-individual values.

	Workload kpm · min	Oxygen uptake l × min	ATP	CP	Glycogen glucose units	Muscle lactate	Blood lactate
			mmoles × kg⁻ wet muscle				mmoles × l
Series I	Rest	—	4.7 ± 0.2 0.6 p < 0.001	14.5 ± 0.4 1.5 p < 0.001	74 ± 2 6 p < 0.001	1.4 ± 0.3 1.2 p < 0.001	1.2 ± 0.2 0.7 p < 0.001
	1500 (1200—1800)	3.4 (2.8—4.4)	3.9 ± 0.2 0.6 p < 0.001	6.4 ± 0.4 1.4 p < 0.001	62 ± 2 8 p < 0.001	10.9 ± 1.1 4.0 p < 0.001	5.3 ± 0.3 1.0 p < 0.001
	2610 (2400—2850)	4.4 (3.4—5.3)	2.8 ± 0.1 0.3	2.9 ± 0.2 0.6	42 ± 3 9	22.7 ± 1.6 5.6	14.6 ± 0.6 2.0
Series II	Rest	—	3.8 ± 0.2 0.5 p < 0.01	16.9 ± 0.8 2.8 p < 0.001	75 ± 4 12 p < 0.001	2.1 ± 0.1 0.4 p < 0.001	1.3 ± 0.2 0.5 p < 0.001
	900	2.2 ± 0.0 0.1	3.5 ± 0.2 0.8 p < 0.05	9.8 ± 0.8 2.8 p < 0.001	57 ± 4 14 p < 0.001	6.6 ± 1.2 4.2 p < 0.001	4.5 ± 0.6 2.2 p < 0.001
	1315 (1140—1500)	3.1 (2.7—3.7)	2.9 ± 0.2 0.6 p > 0.05	4.5 ± 0.9 3.0 p > 0.05	33 ± 3 10 p < 0.001	13.0 ± 1.1 3.7 p < 0.001	10.4 ± 1.2 4.2 p < 0.001
	1595 (1260—1890)	3.3 (2.7—4.2)	2.6 ± 0.2 0.6	3.1 ± 0.4 1.9	22 ± 2 8	16.9 ± 0.7 2.4	12.3 ± 0.7 2.4

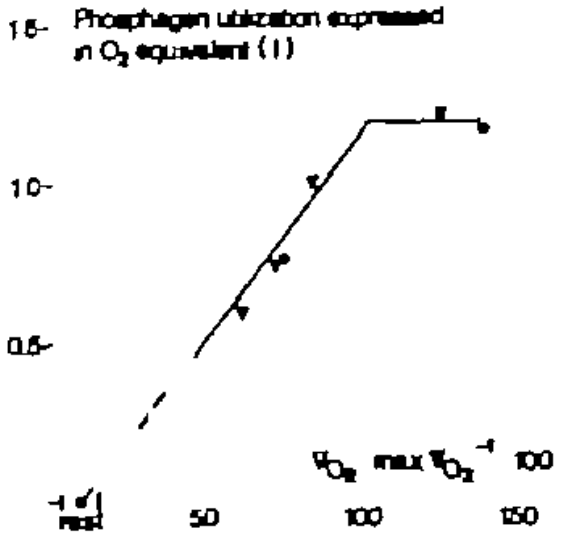


Fig. 10. Total estimated phosphagen depletion expressed in O equivalents in relation to the relative work load. Data are included from Series I (dots) Series II (triangles) and Series IV (data obtained after 2—3 min squares)

respectively) Total phosphagen depletion in the working muscle groups can be expressed in O_2 equivalents, assuming

a) concentration values for ATP and CP are representative of 25 kg of working muscles
b) the formula for complete carbohydrate combustion ($C_6H_{12}O_6 + 6O_2 \rightarrow 6CO_2 + 6H\,O + 38$ ATP) is valid for the calculation of ATP in O_2 equivalents.

The connection between phosphagen depletion and relative work loads (Fig 10) might indicate that there is a linear relationship between alactacid anaerobic energy output and work intensity up to approximately 90—100 % of max $\dot{V}O_2$. With heavier work loads depletion seemed to be limited to approximately 1.3 l of O_2.

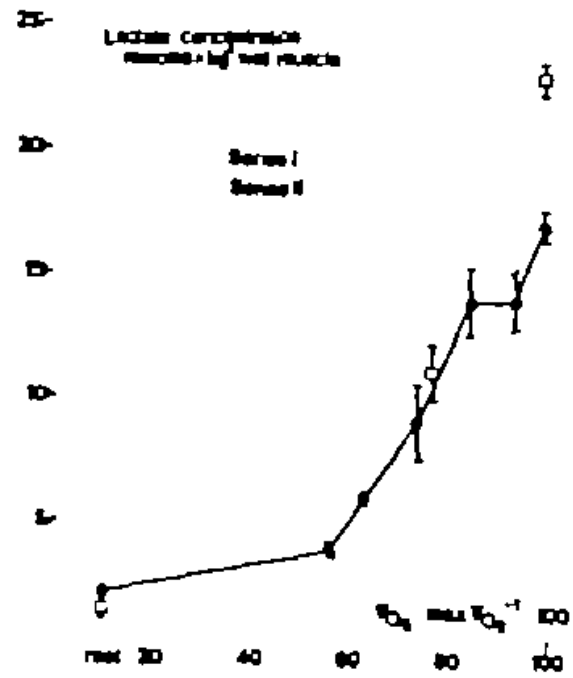


Fig. 11 Mean value ± SE for muscle lactate concentration in relation to the relative work load for Series I (squares) and Series II (dots)

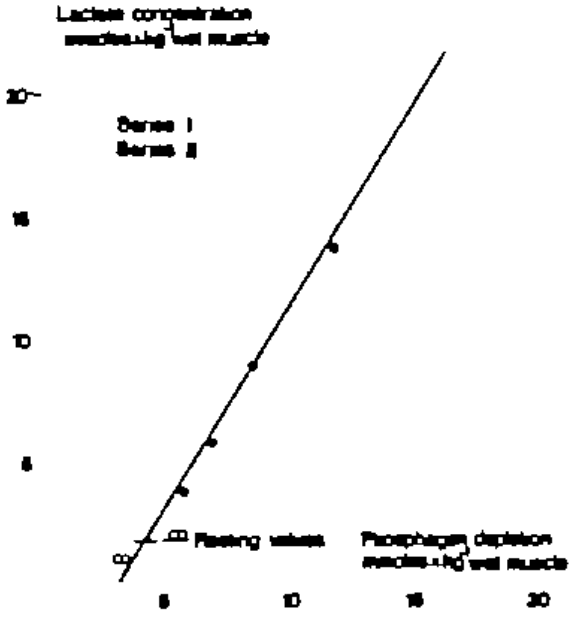


Fig. 12. Muscle lactate concentration in relation to phosphagen depletion for the same subjects as in Fig. 11 The equation for the regression line calculated for the exercise values is $y = 1.6 \times - 5.0$, $r = 0.88$, $p < 0.01$ Open circles, not included in the statistics, denote data obtained by Knuttgen and Saltin (to be published)

A certain lactate concentration was present in the resting muscle, but no marked increase was observed until work loads exceeded 50—60 % of max $\dot{V}O_2$ (Fig. 11) A rapid increase in the accumulation of lactate took place above this work level, reaching maximal values with brief maximal exercise. No difference was demonstrated between trained and untrained subjects in muscle lactate concentration at rest and with a submaximal work load corresponding to 75 % of max $\dot{V}O_2$. There was a significant difference ($p < 0.001$) with the exhaustive work load, however and the mean values obtained were 16.9 and 22.7 mmoles × kg⁻ wet muscle in untrained and trained subjects respectively

With increasing work load phosphagen stores in the muscle display certain depletion before lactate starts to increase beyond values at rest. A regression line can be calculated using the exercise values which intersect the x-axis at a numeric value of 3 1 (Fig. 12)

If muscle lactate concentration is taken into account these results would appear to indicate that phosphagen depletion in the order of 4—5 mmoles × kg⁻ wet muscle must be present before lactate concentration increases beyond rest values.

Repeated maximal or near maximal bursts of activity of 1 min duration led to maximal muscle lactate concentrations as early as at the end of the first exercise period and amounted to 23.2 (range 19 1—26.3) mmoles × kg^{-1} compared to an average value of 22.5 (range 21 1—24.5) mmoles × kg^{-1} wet muscle obtained (individual figures in Fig. 13) after the fifth and last exercise period. These concentrations are comparable to those obtained in the

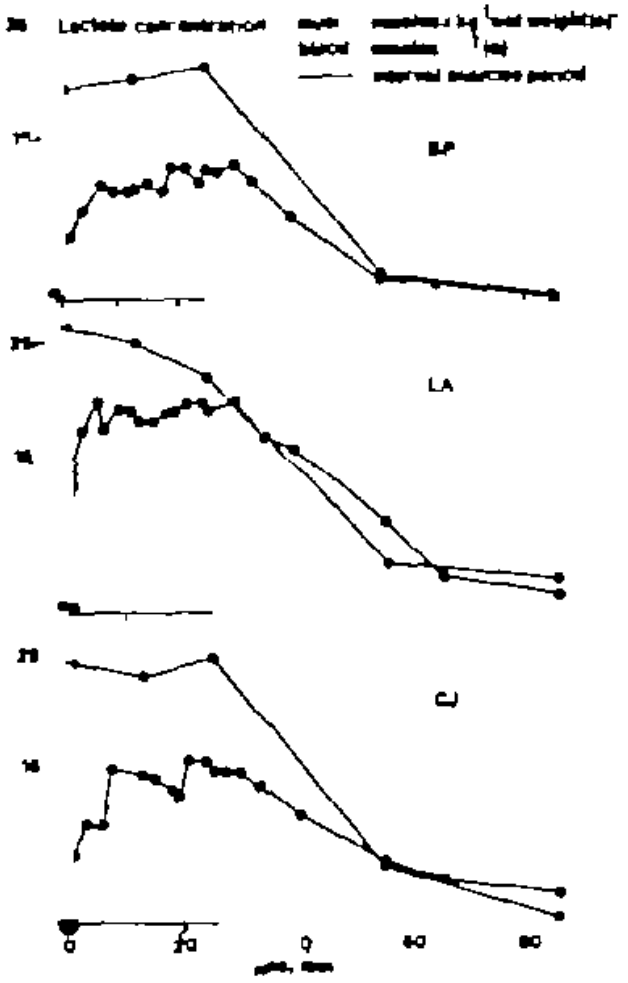


Fig. 13. Lactate concentration in muscle tissue (dots) and blood (circles) for three subjects (Series V) before, during and after intermittent exercise consisting of 5 maximal or near maximal bursts of 1 min duration with 5 min rest between each exercise period.

aforementioned Series II with the brief exhaustive work load (22.7 mmoles × kg^{-1} wet muscle) and those obtained in four other subjects (Series III, 19.1 mmoles × kg^{-1} wet muscle) after brief maximal exercise without any warm-up period.

The fact that a maximal anaerobic requirement was induced by the first burst of activity during the intermittant exercise is further emphasized by ATP and CP concentrations which averaged 2.1 and 2.8 mmoles × kg^{-1} (ATP) and 4.5 and 7.4 mmoles × kg^{-1} (CP) wet muscle respectively after the first and fifth exercise periods respectively.

Comments

The existence of lactate accumulation in the working muscles, despite incomplete phosphagen depletion, suggests that there was energy yield from anaerobic glycolysis at the same time as there was phosphagen depletion. As the breakdown of phosphagen was linearly related to the relative work load (Fig. 10) even with heavy work loads, it seems reasonable to assume that anaerobic glycolysis was relatively less important as an energy

delivering process as long as phosphagen depletion was not maximal. Anaerobic energy yield and the processes by which it is produced may then be summarized as follows

a) by phosphagen depletion with low work intensities (< 50 % of max $\dot{V}O_2$) with a relatively modest anaerobic energy yield
b) partly by phosphagen depletion and partly by anaerobic glycolysis with moderate work intensities (> 50 % but < 90 % of max $\dot{V}O_2$)
c) by maximal phosphagen depletion and anaerobic glycolysis, resulting in very high muscle lactate concentration, with heavy and exhaustive work loads (> 90—100 % of max $\dot{V}O_2$)

It may be concluded that the muscle lactate concentration obtained from muscle after brief exhaustive exercise is the highest attainable, i.e. that the term "maximal muscle lactate concentration" is relevant for use with the value obtained with brief maximal work loads for 1 to ca. 10 min.

Lactate accumulation and phosphagen depletion in relation to the oxygen deficit during exhaustive exercise

(Papers I III and VII)

Muscle lactate concentrations at the end of work were found to be the same, whether exhaustion time was 2—3 min or about 7 min, but were lower when exhaustion time amounted to 10—20 min (Fig 14)

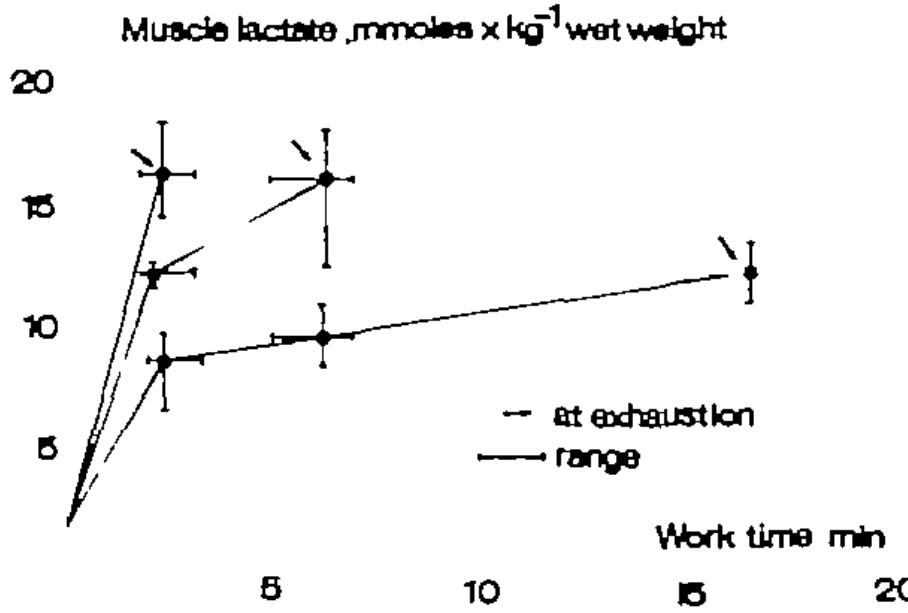


Fig. 14 Mean value and range for muscle lactate concentration with the three different work loads in three subjects (Series IV) in relation to work duration. Not each point in the graph was obtained in separate experiment.

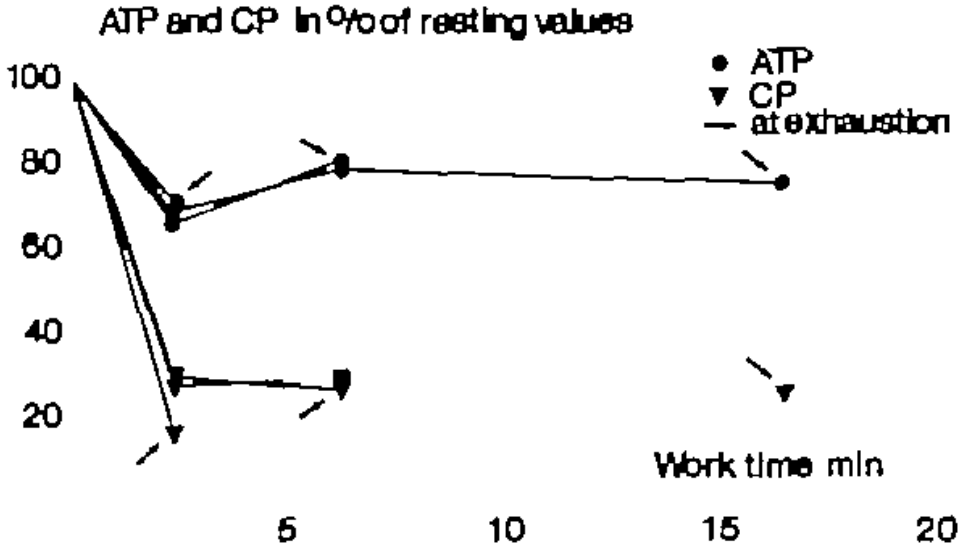


Fig. 15 Mean value and range for ATP and CP concentrations in the exercising muscle during and immediately after exhaustive exercise expressed as per cent of the value at rest. For further information see Fig. 14.

After 2—3 min at the three different test loads muscle lactate concentrations were about 8.5 with the lowest load and about 16.1 mmoles × kg⁻ wet muscle at the highest work load, indicating differences in total anaerobic energy output. However the ATP and CP concentrations obtained simultaneously were evidently reduced to the same extent (Fig 15) pointing to the same alactacid anaerobic energy output, irrespective of work load. ATP and CP concentrations were not depleted further if work continued.

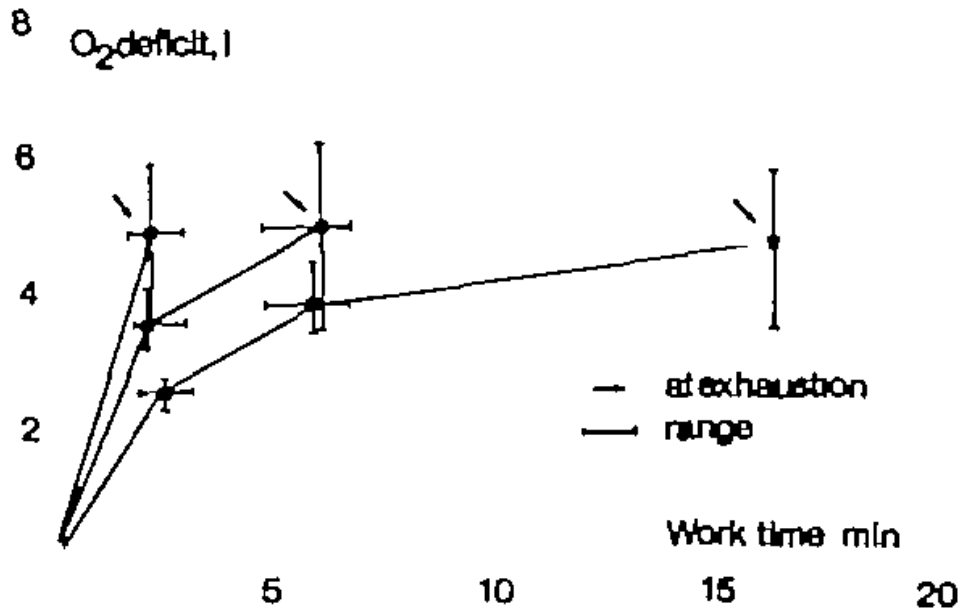


Fig. 16. Mean value and range for O deficit in relation to work time. For further information see Fig. 14

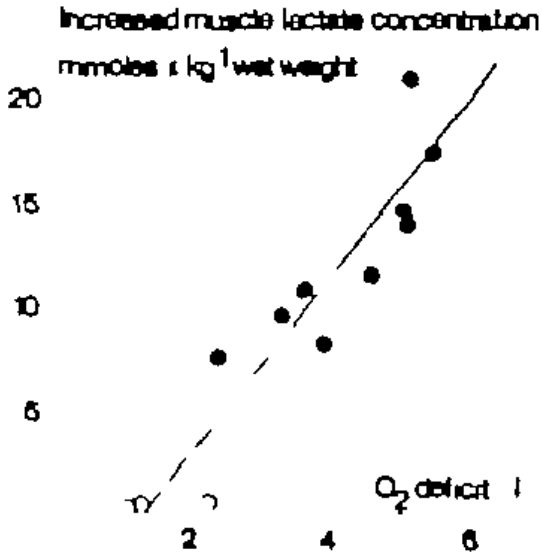


Fig 17 The mean increase in muscle lactate concentration in Series IV in relation to the corresponding oxygen deficit. Data from Series I and from Agnevik *et al.* 1969 are also included. The equation of the regression line is $y = -5.2 + 4.1x$ ($r = 0.88$) The intercept of the x-axis is 1.3 l. This figure may represent the alactacid portion of the oxygen deficit. For explanation of open circles (not included in the statistics) see Fig. 12.

The O deficit values obtained at exhaustion with the three test loads showed an identical total anaerobic energy output (Fig 16)

However after 2 min there was considerable variation in the O_2 deficit which amounted to 2.6, 3 6 and 4.9 l respectively at the three loads.

Since ATP and CP depletion was constant in all six experiments, even if muscle lactate concentration increased, it should be possible to estimate the order of magnitude of the alactacid and lactacid portions of the O_2 defict value by relating the increase in muscle lactate concentration to the corresponding O_2 deficit values. (Fig 17)

Assumng a linear relationship the equation of the regression line was $y = -5.2 + 4.1x$ ($r = 0.88$, $p < 0.01$) the intersection of the x-axis corresponding to 1.3 l. This value would theoretically correspond to total anaerobic energy output without any lactate accumulation in the working muscle, i.e. the total alactacid portion of the oxygen deficit. The excess would then correspond to the lactacid portion. This value agrees with calculated alactacid energy output from maximal phosphagen depletion, which would amount to 1.2—1 3 l of O_2 as presented in the preceding paragraph (Fig 10) Phosphagen depletion in relation to the corresponding O deficit indicated that these stores were not utilized to a maximum until O_2 deficit values were greater than 2—3 l (Fig 18)

Comments

As shown in the preceding paragraph (Fig 10) phosphagen depletion was almost linearly related to work load. The submaximal work loads in that study were interrupted after 6—10 min and it was impossible to establish whether or not ATP and CP concentrations exhibited a gradual decrease. The present data indicate that on work loads exceeding approximately 90—100 %

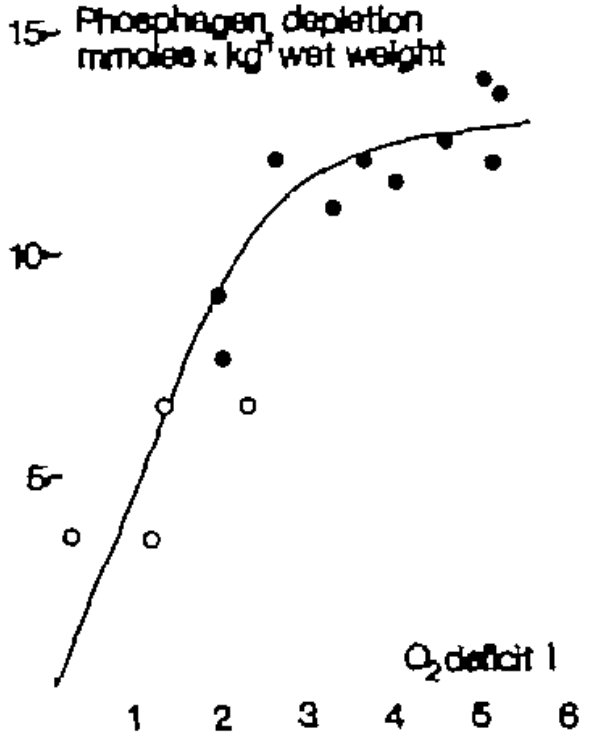


Fig. 18. Phosphagen depletion in relation to the oxygen deficit. Each point represents data from Series I and IV Data from Agnevik *et al.* 1969 Knuttgen and Saltin (open circles, to be published) and Saltin and Karlsson (to be published) are also included.

of max $\dot{V}O_2$, phosphagen stores were depleted to a maximum after only 2 min, whether a subject was exhausted or not. Similar findings have been obtained at lower work loads with less striking CP depletions which stabilized after 4 min and remained constant at that level for 1—2 hrs (Saltin and Karlsson, in preparation)

The portion of the oxygen deficit that could not be accounted for as lactacid anaerobic energy output amounted to a maximum of 1 3 l of oxygen. This figure would then correspond to the alactacid anaerobic energy yield but myoglobin oxygen stores and the decrease in venous oxygen saturation compared to values at rest may modify its magnitude. Human muscle contains myoglobin whose significance has been discussed but it has not been shown that there is any emptying of oxygen stores bound to myoglobin during heavy exercise, even if there are some indications to this effect (Christensen, Hedman and Saltin 1960) The physiological role of myoglobin in muscle tissue is presumably to facilitate the transport of oxygen from capillary blood to the mitochondria (Scholander 1960) and during heavy exercise certain reduction of oxygen stores bound to myoglobin may be expected because of the more pronounced oxygen pressure gradient here (for further information see Wittenberg 1970) However this must be of quantitatively limited importance, amounting to a maximum 0.3 l according to data presented by Björck (1949) Moreover an estimation suggests that approximately 0.3 l of the oxygen deficit may be contributed by a lowering of the oxygen content

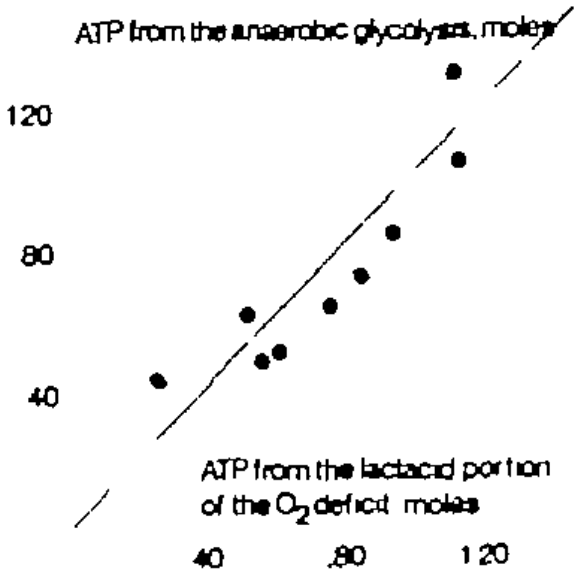


Fig 19 The calculated total mean increase in lactate in the body expressed as ATP formed by concomitant anaerobic glycolysis in relation to the amount of ATP corresponding to the lactacid portion of the oxygen deficit values (actual oxygen deficit — 1 3 l) Data presented in Fig. 17 are used in the calculations.

of venous blood. Thus, maximal O deficit values (4—6 l) would be contributed to 10—15 % of O_2 from myoglobin and venous blood and it seems reasonable to assume that this relative contribution will not increase with submaximal O_2 deficit values. It is therefore concluded that the dominant fraction of the oxygen deficit corresponding to the intercept accounts for alactacid anaerobic energy output. A quantitative relationship was shown to exist between the calculated amount of energy (expressed in oxygen equivalents) which could be derived from phosphagen depletion and the estimated portion of the oxygen deficit contributed by alactacid anaerobic energy yielding processes.

Studies similar to the present one have recently been undertaken in detail in animals (the canine gastrocnemius-plantaris muscle group) by Cerretelli, di Prampero and Piiper (1969) and di Prampero Piiper and Cerretelli (1970) but the results are at variance with the present in several main points. These authors found that lactate formation started only after the complete depletion of phosphagen, which is in accordance with the hypothesis advanced by Margaria (1967) The finding of increased muscle lactate concentrations at an earlier stage was supposedly related to the preparation technique and insufficiencies thus induced.

As an estimation of total lactate production from the present data would be of some interest, the following assumptions have to be made

a) the biopsy is representative of all the muscle groups involved during exercise
b) the involved muscle mass amounts to about 25 kg and the water soluble muscle metabolites would then be diluted in approximately 20 l of water

c) blood lactate concentrations obtained at the same time as the biopsies represent remaining total body water ($\sim$ 20 l)

The total lactate content may then be estimated and the amount of ATP resynthesized by this lactate production (1 lactate molecule = 1.5 ATP molecule) is plotted in Fig 19 in relation to the lactacid portion of the oxygen deficit (oxygen deficit — 1.3 l) expressed in moles of ATP according to the formula on p 37

On the basis of these calculations, there appears to be agreement between the estimated lactacid portion of the oxygen deficit and the amount of lactate present in muscle and blood

Pyruvate and lactate concentrations in muscle and blood and the effect of exercise

(Paper V)

Muscle pyruvate concentration in subjects at rest averaged 0.06 mmoles × kg^{-} wet muscle and increased to 0 13 mmoles × kg^{-} after a brief maximal work load (Table VI) Blood pyruvate concentration started at 0.11 mmoles × l^{-} at rest and ended up at 0.39 mmoles × l^{-} immediately after the maximal load. Thus, a small increase in muscle pyruvate concentration corresponded to a pronounced increase in muscle lactate concentration (Fig. 20)

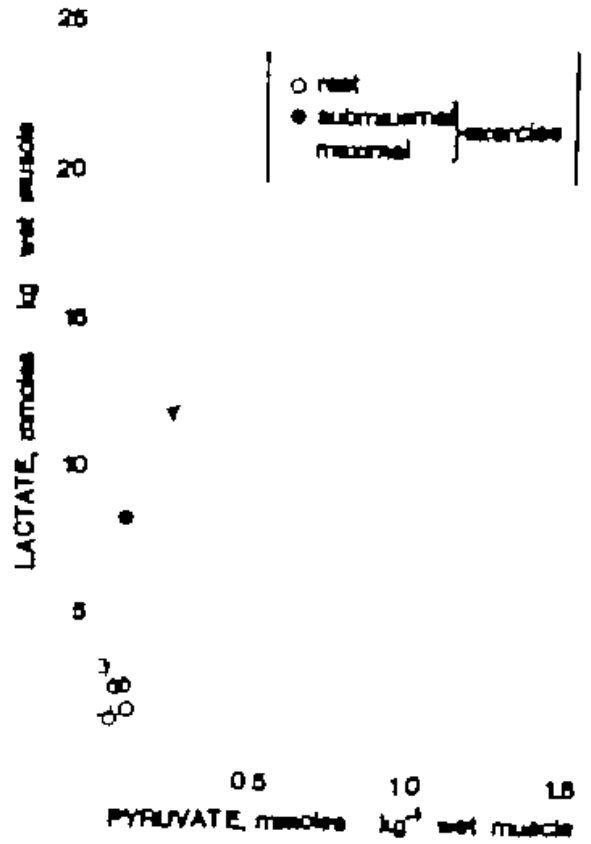


Fig. 20. Muscle lactate concentration in relation to muscle pyruvate concentration at rest and immediately after submaximal and brief maximal exercise in Series VI.

TABLE VI. Mean value ± SE and SD are presented for pyruvate and lactate concentration in muscle and blood in subjects at rest, at submaximal and a maximal work load. The work load and the oxygen uptake are presented as mean value and range

	Work load	Oxygen uptake	Lactate		Pyruvate	
	kpm × min	l × min	muscle [1]	blood [2]	muscle [1]	blood [2]
Rest	—	—	0.06 ± 0.01 0.03	0.11 ± 0.02 0.07	2.3 ± 0.2 0.7	1.6 ± 0.1 0.3
n = 8						
Submaximal work	1290	3.1	0.14 ± 0.02 0.04	0.24 ± 0.03 0.06	10.4 ± 0.6 1.3	7.7 ± 0.5 1.1
n = 5	(1200—1350)	(2.8—3.2)				
Maximal work	2040	3.9	0.13 ± 0.03 0.07	0.39 ± 0.05 0.11	17.1 ± 1.6 3.5	10.0 ± 1.1 2.4
n = 5	(1950—2100)	(3.8—4.1)				

[1] mmoles × kg⁻¹ wet muscle

[2] mmoles × l⁻¹ blood

Comments

Present data would indicate a concentration gradient from blood to muscle for pyruvate on per l of water comparison. Whether or not this would result in an uptake cannot be established since pH differences between the blood and the cell might favour such a gradient (Sjesjö Granholm and Kjällquist 1968)

However *in situ* experiments on the contracting canine gracilis muscle (Karlsson, Rosell and Saltin 1971) disclosed a positive a-v concentration difference for pyruvate at the end of a 50 min stimulation period in five dogs. This immediately changed to a negative a v difference at the beginning of the recovery period, pointing to an uptake of pyruvate during contraction with subsequent release during the recovery period.

Observations at present are too few to make it possible to draw any definite conclusions concerning the relationship between intracellular and extra cellular pyruvate concentrations and their role in the transport of electrons within the intact organism especially when oxygen transport is limited to one organ or organ system. However the increase in muscle pyruvate concentrations observed after exercise is too small to induce a measurable effect on muscle lactate concentration according to the mass action law. It is, therefore, concluded that the excess lactate calculated according to Huckabee (1958) is of no significance in the interpretation of blood and lactate concentrations after muscular exercise.

LDH activity in skeletal muscle after prolonged exercise

(Paper VI)

The mean LDH activity in the vastus lateralis averaged 0 73 (range 0.54—0.88) $\times 10^{-4}$ moles NADH $\times$ g^{-} wet muscle $\times$ min^{-1} which would correspond to a maximal lactate formation rate of 73 mmoles lactate $\times$ kg^{-} wet muscle $\times$ min This value is 3—5 times greater than the rate of formation calculated from the lactate concentrations in muscle and blood after brief maximal work.

LDH activity obtained after endurance work averaged 0.88 $\times 10^{-4}$ moles NADH $\times$ g^{-} wet muscle $\times$ min indicating an increase of the enzyme activity corresponding to 21 % ($p < 0.001$) as shown for two of the subjects in Fig 21

Comments

Studies were not performed to evaluate whether LDH activity depended on a true increase in the amount of enzyme protein or on some change in the

TABLE VI. Mean value ± SD and SD are presented for pyruvate and lactate concentration i muscle and blood in subjects at est, t rubmaximal and maximal work load. The work load and the oxygen ptake are presented as mean value and range.

	Work load	Oxygen uptake	Lactate		Pyruvat	
	kpm × min	l × min	muscle)	blood**)	muscle)	blood**)
Rest	—	—	0.06 ± 0.01	0.11 ± 0.02	2.3 ± 0.2	1.6 ± 0.1
			0.03	0.07	0.7	0.3
n = 8						
Submaximal work	1290	3 1	0.14 ± 0.02	0.24 ± 0.03	10.4 ± 0.6	7 7 ± 0.5
			0.04	0.06	1.3	1 1
n = 5	(1200—1350)	(2 8—3.2)				
Maximal work	2040	3.9	0.13 ± 0.03	0.39 ± 0.05	17 1 ± 1 6	10.0 ± 1 1
			0.07	0.11	5.5	2.4
n = 5	(1950—2100)	(5.8—4 1)				

) mmoles × kg⁻ wet muscle

) mmoles × l ¹ blood

TABLE VII. Mean values ± SE and SD for ATP and CP in subjects at rest and after two standardized work loads for the control study (Series A + Series B) and for Series A and Series B respectively after prolonged heavy exercise. The level of significance denotes the differences based on intraindividual comparisons between the control experiments and those performed after the preceding endurance exercise.

Metabolite		Values obtained in the control study n = 13	Values obtained after —4 hrs' exercise n = 5 (Series A)		Values obtained after 5—7 hrs' exercise n = 8 (Series B)	
Adenosinetriphosphate (ATP) (mmoles × kg⁻¹ wet muscle)	Rest	4.7 ± 0.2 0.6	4.8 ± 0.2 0.5	$p > 0.5$	4.1 ± 0.2 0.4	$p > 0.5$
	75 %	3.9 ± 0.2 0.6	3.9 ± 0.3 0.6	$p > 0.5$	4.0 ± 0.2 0.5	$p > 0.5$
	Max	2.8 ± 0.1 0.5	2.6 ± 0.3 0.7	$p > 0.5$	2.2 ± 0.4 1.1	$p > 0.5$
Creatinephosphate (CP) (mmoles × kg⁻¹ wet muscle)	Rest	14.5 ± 0.4 1.5	13.0 ± 1.2 2.7	$p > 0.5$	13.8 ± 1.1 3.2	$p > 0.5$
	75 %	8.4 ± 0.4 1.4	5.9 ± 0.4 0.8	$p > 0.5$	6.. ± 0.9 2.6	$p > 0.5$
	Max	2.9 ± 0.2 0.6	3.3 ± 0.4 0.9	$p > 0.5$	4.0 ± 0.9 2.7	$p > 0.5$

(5—7 hrs not determined after 2—4 hrs work) rose from 3.3 l in the control study to 4.7 l due to a slower rate of acceleration for the O_2 transport system, indicating increased anaerobic energy output. A slower rate of acceleration was observed even at the maximal test loads, resulting in a faster development of O_2 deficit. After moderately prolonged exercise, the O_2 deficit was similar to that in the control study. Thus, there was no tendency for anaerobic energy output to decrease. On the other hand, after the extremely prolonged heavy exercise O_2 deficit was reduced from 5.8 l in the control study to 4.7 l.

Prolonged heavy exercise did not influence phosphagen concentrations before and after the standardized tests performed after the prolonged heavy exercise periods pointing to the same alactacid anaerobic energy output as in

the *control experiments* (Table VII) Muscle tissue lactate concentration at rest and at the standardized work loads was of the same order of magnitude as in the control experiments (Table VII) with the exception of the maximal test load performed after extremely prolonged exercise (5—7 hrs) (13.3 mmoles compared to 22.0 mmoles × kg^{-1} wet muscle in the control study) Highest concentrations of blood lactate were significantly lower in the standardized tests, except at the submaximal load after moderately prolonged exercise (2—4 hrs) at which a tendency to a decrease was present, however (Table VIII) Blood lactate data consequently confirmed observations reported by Astrand *et al.* (1963)

Comments

Blood lactate concentration decreased at the standardized work loads performed after a preceding prolonged work. The most reasonable explanation for this finding is that prolonged heavy exercise caused an increase in the metabolism of extracellular lactate as earlier suggested by Rämmal and Ström (1949) Increased utilization of blood borne lactate, resulting in a lower blood lactate concentration may facilitate the translocation of lactate from the muscle. *Thus, it seems reasonable to assume an enhanced lactate flux with* standardized work loads after prolonged heavy exercise.

Muscle lactate concentration at the standardized work loads was, with one exception, unchanged by the preceding prolonged heavy exercise period. According to the findings above this should be indicative of at least the same lactate formation as in the control experiments. The decrease in muscle lactate concentration with the brief maximal load after extremely prolonged exercise *was greater than expected from the decrease in the O deficit (for comparison* see Fig 17) The hypothesis of an increased utilization of blood-borne lactate and a subsequent increased release of lactate from the muscle would contribute to an explanation of the lowered muscle lactate concentration with the brief maximal load after extremely prolonged exercise, but a definite explanation cannot be given. The reason why the decrease in muscle lactate concentration was not present with the brief maximal load after moderately prolonged exercise can be explained by the shorter period of work (1.2 compared to 1 7 min) which would minimize the effect of an increased lactate flux.

The short period of work in the present studies is the most probable reason for the higher blood lactate concentration after maximal work preceded by prolonged exercise as compared to the results reported by Astrand *et al.* (1963) In these experiments maximal work of about 5 min duration was used.

Since preceding prolonged heavy exercise did not influence ATP and CP depletion, indicating an intact alactacid anaerobic energy output as compared to that in the control experiments, changes in O_2 deficit may reflect altered

TABLE VIII. Mean values ± SE and SD for muscle lactate, blood lactate and oxygen deficit for Series A and Series B in subjects at rest and after the two standardized work loads in the control study and after prolonged heavy exercise respectively. The level of significance denotes as in Table VII, the difference based on intraindividual comparisons between the control study and the studies performed after prolonged exercise respectively.

		Series n = 5 (Series A)		Series n = 8 (Series B)	
		before	after	before	after
Muscle lactate (mmoles × kg⁻¹ wet muscle)	Rest	1.5 ± 0.8 1.9	1.6 ± 0.2 0.5 p > 0.05	1.4 ± 0.2 0.5	1.7 ± 0.3 0.8 p > 0.05
	75 % of max $\dot{V}O_2$	11.4 ± 2.0 4.4	13.5 ± 2.5 5.5 p > 0.05	10.6 ± 1.4 4.0	10.7 ± 1.4 4.0 p > 0.05
	Max	23.9 ± 3.5 7.8	22.0 ± 1.5 3.4 p > 0.05	22.0 ± 1.7 4.9	15.3 ± 1.3 3.7 p < 0.01
Blood lactate (mmoles × l⁻¹)	Rest	1.0 ± 0.3 0.6	1.3 ± 0.1 0.2 p > 0.05	1.4 ± 0.2 0.7	1.9 ± 0.1 0.1 p > 0.05
	75 % of max $\dot{V}O_2$	5.7 ± 0.2 0.5	4.2 ± 0.8 1.8 p > 0.05	5.1 ± 0.4 1.2	3.9 ± 0.5 1.4 p < 0.01
	Max	14. ± 1.1 2.4	10.1 ± 0.9 2.1 p < 0.01	14.9 ± 0.7 1.9	9.4 ± 1.2 3.4 p < 0.001
O_2 deficit (l)	75 % of max $\dot{V}O_2$	–	—	4.6	3.2 p < 0.01
	Max	3.9	3.6	3.8	4.7 p < 0.01

lactacid anaerobic energy output. The observed increase in O_2 deficit at the submaximal work load after extremely prolonged heavy exercise would then be reflected by a higher muscle lactate concentration. This is not the case even if a tendency was present at the submaximal test load after moderately prolonged exercise. In view of the decreased blood lactate concentration an increased flux would be the most reasonable explanation.

CHAPTER V

GENERAL DISCUSSION

The significance of lactate formation

When man starts to perform dynamic physical work, chemically bound energy is transformed into mechanical work by the muscles. As first demonstrated by Cain and Davies (1962) ATP stored in the muscle cell is the immediate energy source for muscular contraction. The dominant ATP fraction is formed by resynthesis of ATP from ADP in the mitochondria, which however need O_2 to capture electrons from the oxidative processes. As indicated by Chance and his associates (for ref see Jöbsis 1964) the electron transfer systems of the muscle cell are reduced at the onset of a muscular contraction as an indication of increased mitochondrial breakdown of substrates to meet the demand for ATP This demand may vary many hundredfold over a range of isotonic contraction tensions (Davies 1967)

ATP and CP stores in the resting muscle of man are very limited and can only provide energy briefly during intense contractions. Energy in the form of ATP quanta must, therefore, be delivered by other cellular processes, as man can perform extremely heavy exercise for a period longer than fractions of a min. An increased rate of mitochondrial ATP resynthesis will take place since an elevated O supply to the muscle is induced by the work, but the resynthesis of ATP by glycolysis also is of significance in this context. Anaerobic energy yielding processes can supply more than 50 % of the total energy yield at high loads resulting in exhaustion within 2—3 min (Paper III) A considerable amount of energy in ATP equivalents must be generated by glycolysis in such exercise. The present data indicate that breakdown of ATP and CP on the one hand, and anaerobic glycolysis, on the other hand provide amounts of energy equivalent to approximately 1 l and 3—5 l of O_2 respectively This should be comparable to a max $\dot{V}O_2$ in the order of 3—6 l × min the lowest value representative for sedentary subjects and the highest value for athletes (Saltin and Åstrand 1967)

The significance of lactate formation is two-fold

1 it is the end product of anaerobic glycolysis, which is one of the ATP yielding processes

? it is a constituent of the coupled redox processes between pyruvate and lactate and extra mitochondrial NAD and NADH, which are necessary in the maintenance of glycolysis under anaerobic conditions.

The factors involved in the regulation of lactate production have been frequently discussed but are still not well understood. The following factors may be mentioned as being of importance

a. The enzyme LDH is present in mammalian tissue in 5 forms with different affinities for pyruvate and lactate respectively (for ref. see Sund 1968) The M form favours lactate formation under physiological conditions, while the H form favours the formation of pyruvate from lactate and is the most frequent form in heart muscle. Present data on the total LDH activity in the skeletal muscle of man (lateral portions of the quadriceps femoris) indicate that enzyme activity may not be a limiting factor in lactate formation in the working muscle, since this enzyme activity exceeded the highest observed rate of lactate formation by a factor of 3—5

b Pyruvate concentration in the muscle may reach very high levels and influence lactate formation. This was earlier thought to be a mass action effect as suggested by Huckabee (1958) However the present experiments produced an increase of 0 06 to only 0 14 mmoles × kg⁻ wet muscle. Thus, the increased pyruvate concentration may be ignored as a significant cause of lactate formation.

c. Extra mitochondrial concentration of NADH increases and this has been shown in most tissues to depend on the fact that the rate of oxidation by the mitochondria is insufficient when oxygen supply is inadequate (for ref. see Jöbsis 1964) Of necessity this fraction of NADH in the muscle cell is oxidized by pyruvate.

During exercise of relatively brief duration (< 30 min.) no change in muscle LDH activity may occur (Gollnick, Struck and Bogyo 1967) Moreover the increase in NADH concentration in the cell seems to be the most probable cause of increased lactate formation during exercise since the increase in muscle pyruvate concentration is negligible. However the redox system or systems involved seem to be complex in nature, since working muscle producing lactate is also able to oxidize blood-borne labelled lactate in significant amounts (Jorfeldt 1969) In the present studies it seems reasonable to conclude that at start of exercise in man conditions are such as to cause lactate formation in the muscle cell with subsequent accumulation and release. Moreover lactate formation occurs when the total energy yield exceeds that which can be derived from aerobic processes operating at a maximum. In this situation reduction of pyruvate is the only way to oxidize extra-mitochondrial NADH.

The correlation found between O deficit and lactate concentration in working muscles suggests that lactate formation during exercise is caused by a lack in oxygen transport in mainly two situations

a. at the start of exercise and until a steady state level is obtained

b. with supramaximal work loads.

This is in agreement with Lundin and Ström (1947) and their findings when studying the effect of nitrogen-oxygen mixtures on blood lactate concentrations with exercise.

Recently studies have been published which are at variance with this generally accepted view concerning the limiting factors of oxygen supply to the working muscle

Kaijser (1970) reported elevated O_2 tensions in the deep veins of man during rhytmic contractions of the forearm muscle in a hyperbaric oxygen (3 atm) atmosphere (P O 1840—1900 mm Hg compared to 96—100 mm Hg in the control experiments at 0 21 atm PO) However blood lactate concentrations were only moderately reduced in deep venous blood with subjects breathing oxygen at 3 atm as compared to the results of control experiments. The author concluded that the links in the O transport system must be saturated with O_2 and the main limiting factor in the rate of utilization of O would consequently be found at the mitochondrial level. Disregarding the consequence of the aforementioned findings concerning the role of circulatory functions as limiting factors, these results failed to establish whether or not hypoxia was still present in the contracting muscle fibers and whether or not high O_2 levels had a toxic influence on muscle enzymes. In contrast to the results presented by Kaijser Wyndham and associates recently reported an increase in max $\dot{V}O_2$ amounting to 17 % by descending to 3,000 m below surface in mines (Wyndham *et al* 1970) The increase in max $\dot{V}O_2$ was linearly related with increase in barometric pressure between 630—865 mm Hg, which further supports the hypothesis of the circulatory system as the main limiting factor for the oxygen transport to the working muscles.

The occurrence of hypoxia in resting muscle as well has been demonstrated by Bolme and Novotny (1969) with canine gastrocnemius-plantaris preparation. By stimulating the sympathetic cholinergic vasodilator nerve they observed increased blood flow as well as increased O_2 uptake. Similar findings were obtained by Whalen and Nair (1969) who using an O electrode in resting feline skeletal muscle, reported an average PO of 6.6 (range 0—45) mm Hg 56 of 371 of their measurements on 17 animals did not significantly differ from zero. These findings should provide a satisfactory explanation of the lactate formation and release in resting canine gracilis muscle specimens

observed by Costin *et al.* (1971) and Karlsson, Rosell and Saltin (1971) as being due to partial hypoxia in resting muscle tissue. However the final explanation why there is a certain lactate concentration in the resting muscle and release to extracellular compartments cannot be provided at present. It might be that lactic acid is a significant constituent of the metabolic vasodilator mechanism suggested by Renkin and Rosell (1962)

Relation between muscle lactate concentration and the perception of exhaustion

Exhaustion during brief maximal exercise has been thought to be caused by high muscle lactate concentrations (Asmussen, Döbeln and Nielsen, 1948) and also from considerations of the results of animal experiments (Fletcher and Hopkins 1907 Meyerhof 1925) From the present studies, it can be concluded that the highest muscle lactate concentrations are found after exhaustive exercise of 1—7 min duration and that repeated maximal work periods do not increase muscle lactate concentration further In two subjects participating in three of the present studies, each consisting of maximal work, muscle lactate concentration reached 20.2—22 0 mmoles × kg⁻ and 21 1—24 8 mmoles × kg^{-1} wet muscle respectively

Similar high values have been reported after brief maximal isometric contractions, which brought the subject to exhaustion within 5 min (Bergström *et al* 1971) and after exhaustive leg extensions with heavy weights (Ollander personal communication) In contrast to this, muscle lactate concentrations greater than 7 mmoles × kg wet muscle have not been observed in exhaustion after eccentric work (sometimes referred to as negative work) (Bonde Petersen, personal communication) It seems reasonable to conclude, therefore that a close correlation exists between maximal muscle lactate concentration and perception of exhaustion during brief maximal concentric exercise, indicating that muscle lactate concentration or secondary changes are direttly or indirectly responsible for muscular fatigue.

Since proportionately lower muscle and blood lactate concentrations are found when the time to exhaustion is approximately 16 min, any explanation which demands a causal relationship between muscle lactate concentration and exhaustion is difficult to accept

A plausible explanation to exhaustion after 15—20 min exercise might be that maximal muscle temperature here in contrast to brief maximal exercise is reached (Saltin, Gagge and Bergh 1971) Similar high values (40—41 C) have been observed after brief maximal eccentric exercise, which then would

lactate concentrations obtained after brief maximal running in seven subjects are presented. Muscle lactate concentration averaged 15 6 mmoles × kg^{-1} wet muscle and blood lactate 9.3 mmoles × l indicating the same relationship as illustrated in Fig 22

Chapter II presented results concerning lactate concentration gradients from the muscle fiber structures to extra fiber structures surrounding the fiber bundles, indicating some impediment to the immediate translocation of the lactic acid molecule from the muscle cell to the interstitial space. In experiments on dog heart and skeletal muscle respectively Kübler *et al* (1966) and Hirsche Langohr and Wacker (1970) showed that the lactic acid translocation process was evidently not simple diffusion, and Kübler *et al.* postulated that an active transport mechanism might be involved. Similar experimental results have been found (Karlsson Rosell and Saltin 1971) when studying muscle, arterial and venous lactate concentrations in canine gracilis muscle specimens at rest and during electrically stimulated contractions.

The findings in intermittant exercise with supramaximal bursts of activity may be explained by the circumstance that muscle lactate concentration reached the highest value sustainable with each exercise period. Blood lactate concentration, on the other hand, increased during the interval periods until it reached equality with or a value close to the plateau muscle lactate concentration. Thus, a higher blood lactate concentration would be expected to occur with repeated maximal or near maximal, brief work loads than with a single load.

The present results make it possible to conclude that the highest blood lactate concentration reflects muscle lactate concentration fairly well after brief single exhaustive dynamic work and is, therefore, useful as an indicator of maximal exercise in similar experimental situations.

After extremely brief exercise of 10—20 sec duration at supramaximal work intensities, high muscle lactate concentrations are not indicated by high blood lactate concentrations (Saltin and Essén 1971) This has also been found to be true after exhaustive isometric exercise for 1—4 min (Ollander personal communication)

Elevated blood lactate concentrations have previously been used as an indicator of anaerobic metabolism in working muscle Even though this has been questioned (Huckabee 1958) blood lactate concentration has been of practical value in evaluations of whether or not oxygen transport has been stressed to a maximum (Astrand 1952) In the present material an almost linear relationship was found between the highest blood lactate concentration and the muscle lactate concentration obtained immediately after cessation of work (Fig 23) However there was a difference on submaximal work loads

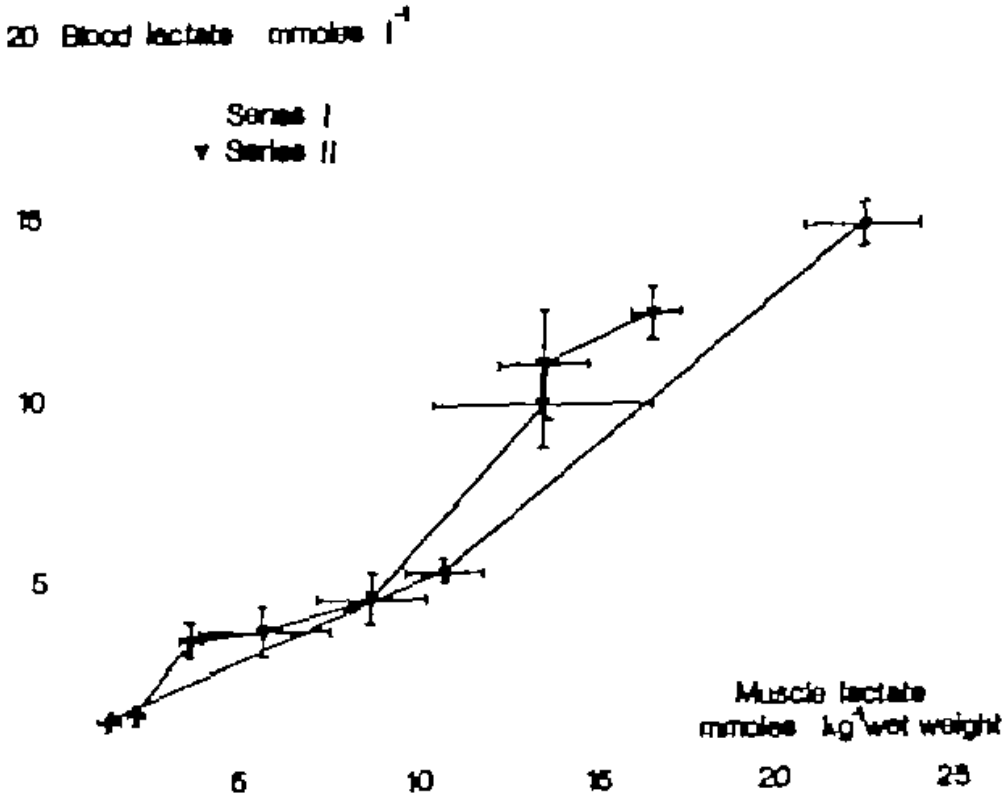


Fig. 23 The highest observed blood lactate concentration after work related to the muscle lactate concentration in Series I (trained subjects) and Series II (untrained subjects) respectively The mean value ± SE is presented.

between the two groups of subjects studied which indicated a comparatively lower blood lactate concentration in trained subjects versus the muscle lactate concentration. This occurred despite longer work durations at submaximal loads by untrained subjects (10 min compared to 6 min) The difference in training condition, in terms of a higher max $\dot{V}O_2$, might explain the difference between the two subject groups. At the maximal work load blood lactate concentration was higher ($p < 0.05$) in the trained subjects (14 6 mmoles × l⁻ compared with 12.3 mmoles × l⁻ in the trained subjects) but the differen ce was less than might be expected from the muscle lactate concentration (22.7 and 16.9 mmoles × kg⁻ wet muscle in the trained compared to the untrained subjects respectively)

Glycogen depletion and anaerobic glycolysis

Glucose G-6-P and lactate are the only glycolytic intermediary metabolites found to accumulate in the muscle in significant amounts (Paper II) But none of these except lactate is found in concentrations greater than approx

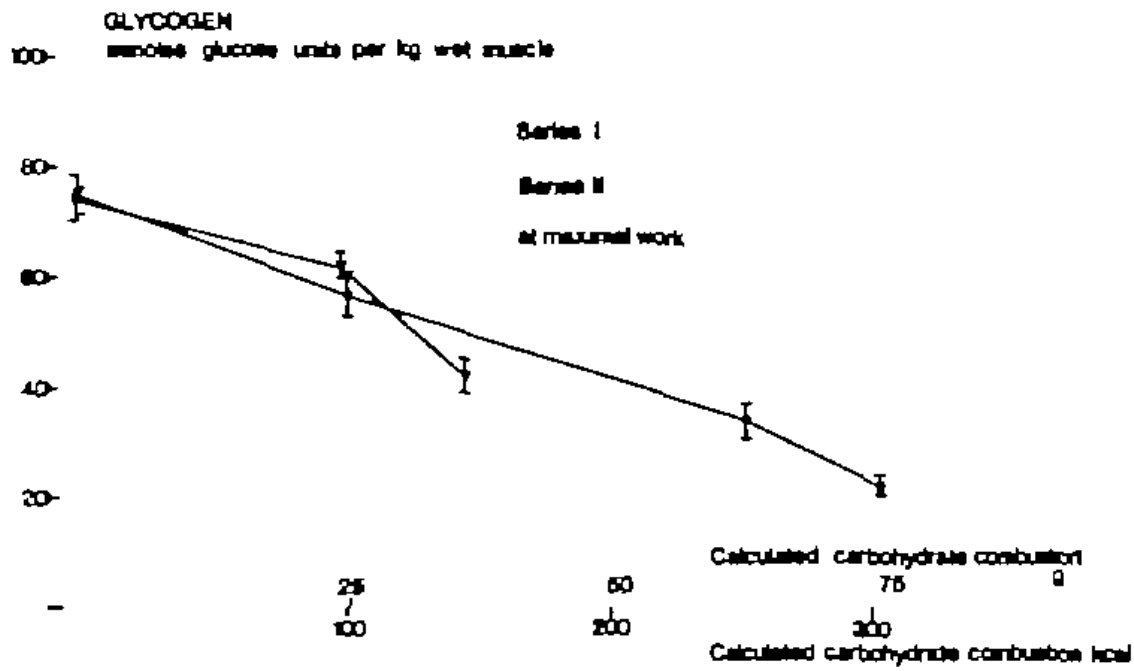


Fig. 24 Glycogen depletion (mean value ± SE) in the working muscle in relation to the carbohydrate utilization expressed in kcal or g of glycogen. Series I (trained subjects) and Series II (untrained subjects) respectively. These latter values are calculated from the oxygen uptake and RQ values, assuming a caloric output of 4.1 kcal × g glycogen

imately 4 mmoles × kg wet muscle while the lactate concentration in similar *situations ranges between 15—30 mmoles × kg wet muscle.*

After brief maximal exercise glucose concentration amounts to approximately 2 mmoles × kg wet muscle and G-6-P concentration to approximately 4 mmoles × kg. As indicated by the demonstrated release of glucose (Wahren 1970) glucose concentration might reach values corresponding to or somewhat greater than that in the blood.

Glycogen content at rest and after the separate work loads is presented in Table V for Series I and Series II and shows a significant depletion with each load. The depletion is further presented in Fig. 24 in relation to the calculated amount of carbohydrate oxidized. The calculations are based on $\dot{V}O_2$ and ventilatory exchange ratio (RQ) data. Similar curves were obtained for the two groups of subjects, illustrating a tendency for enhanced glycogen depletion in relation to the amount oxidized in brief maximal exercise and indicating relatively increased anaerobic glycolysis.

No quantitative relationship between glycogen depletion and the accumulation of different glycolytic intermediates could be established since the magnitude of glucose release and accumulation of F-6-P were not determined in

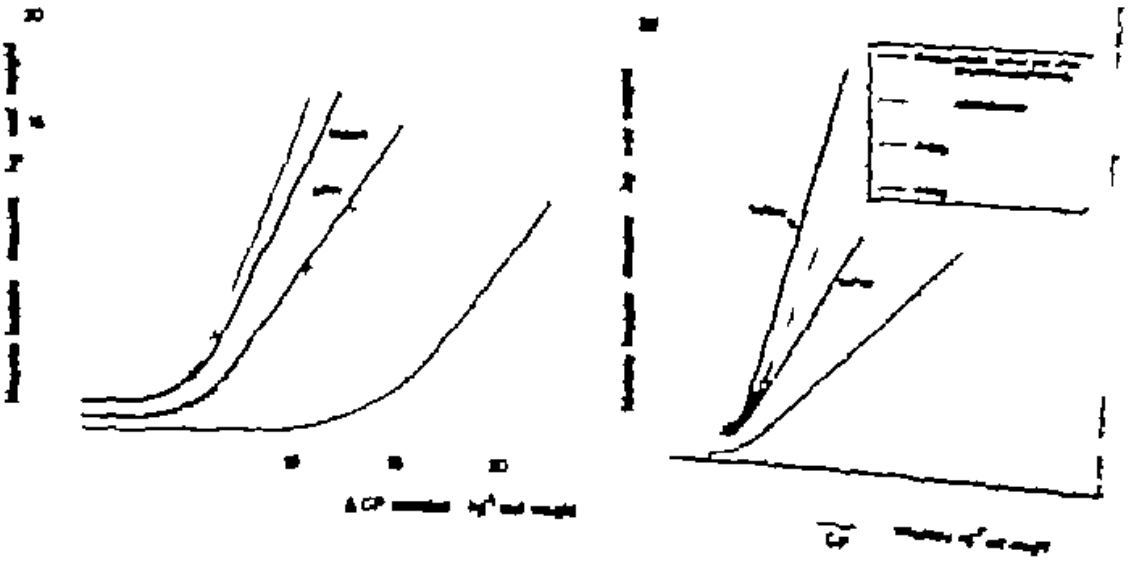


Fig. 25. Left graph: Muscle lactate concentration in relation to CP [illegible] during exercise or stimulations to contractions in man (Saltin and Karlson [illegible]) in boys (Eriksson, Karlsson and Saltin 1971) in dog (Karlsson, Rowell and Sa[illegible]) and in frog (Danforth 1965). Right graph. Muscle lactate concentration in [illegible] the corresponding reciprocal CP concentration.

the present studies. In isolated canine gracilis muscle, on the other hand, good agreement has been found between glycogen breakdown and the contribution, accumulation, uptake and release of different glycolytic intermediary metabolites (Karlsson, Rosell and Saltin 1971).

Possible factors regulating glycolytic activity

The present results indicate that the increase in lactate concentration starts after the initial decrease in phosphagen stores. Whether there is a causal relationship between these two processes, possibly triggered by the production of metabolites e.g. P is questionable.

A similar relationship has been described for muscle lactate and CP in frog sartorius muscles stimulated to contractions and frozen at different intervals after the onset of work (Danforth 1965). This is illustrated in Fig. 25 a (concentration values recalculated in relation to wet weight). Similar data obtained in the dog, adolescent boys and adult men before and after physical conditioning are included in the same figure. These data were obtained in various experiments with different work intensities as contrast to the experiments with frog sartori in which the same standardized procedure was used throughout. The same tendency was apparent in all these different muscles: a given CP depletion precedes an increase in lactate concentration

Further depletion caused different muscle lactate concentrations, suggesting that different kinetics were involved in the activation of glycolysis. The presence of different rates in the separate muscles is further emphasized by plotting muscle lactate concentration versus reciprocal CP concentration (Fig. 25 b)

Two separate glycolytic enzymes have been postulated to regulate the activity of the breakdown phosphorylase and phosphofructokinase (for ref see Danforth 1965) Phosphorylase b is transformed into its a form by means of a phosphorylation catalysed by the enzyme phosphorylase kinase, (Helmreich and Cori 1965) In 1956 Cori suggested that this reaction might be facilitated by the increase in cellular pH caused by the initial CP depletion. This increase in pH is then changed into a decrease as soon as lactic acid starts to accumulate in the cell (Karpatkin, Helmreich and Cori 1965) The importance of tissue pH as a regulatory factor has been further documented by Danforth (1965)

Rulon, Schottelius and Schottelius (1961) demonstrated that phosphorylase is activated at a sufficient rate to mobilize substrate to meet the increased energy demand. This was confirmed by Danforth, Helmreich and Cori (1962) who showed a 100 % activation of the enzyme in frog sartorius muscle 3 sec *after the onset of stimulation. Danforth and his associates suggested further* on the basis of their experiments, that two mechanisms were involved in activating the enzyme muscle contractions per se and adrenalin. Drummond Harwood and Powell (1969) showed that the mechanism of phosphorylase activation by muscle contractions is basically different from that involving adrenergic amines which would function via activation of an enzyme phosphorylase kinase. Thus, during exercise in man there seems to be two different mechanisms operating to activate phosphorylase muscle contractions and increased sympathetic tone (von Euler and Hellner 1952, Häggendahl, Hartley and Saltin 1970) presumably at a rate which is sufficient to furnish the cell with glucose residues. It seems reasonable to assume, therefore that the enzyme phosphorylase is not rate-governing during exercise but of significance in the initiation of glycolysis at start of contractions.

The other enzyme phosphofructokinase was suggested by Cori in 194 as being the most important regulatory factor in glycolysis. Passonneau and Lowry (1962) presented studies which showed how conditions similar to those present in the anaerobic cell activated the enzyme. It has also been shown that enzyme activity is inhibited by ATP in high concentrations (Passonneau and Lowry 1962) citrate in high concentrations (Randle Denton and England 1967) or by decreased pH (Danforth 1965) Thus, the enzyme seems to possess some of the characteristics necessary to the main regulatory system of glycolysis.

In the present studies (Paper II and IV) findings have been made which indicate that a regulatory function similar to that described above also holds true for human skeletal muscle, as an accumulation of G-6-P could be demonstrated during brief exhaustive exercise. Similar findings have been reported in heavily stimulated frog sartorius muscle (Karpatkin, Helmreich and Cori 1964) and, recently in man (Bergström *et al.* 1971). The latter authors also found an increase in F-6-P but not in F 1 6-P which is in agreement with our own findings (Karlsson, unpublished results) and further supports the hypothesis that phosphofructokinase is the most important regulatory mechanism during exercise. The present studies were not directly aimed at establishing whether or not true equilibrium states, as defined by Guldberg Waage's law or flows" of substrates through different metabolic pathway determine concentrations of metabolites in the cell. Some of the findings, however such as the G-6-P accumulation with brief maximal exercise and the concentration gradient for lactate between muscle and blood during exercise support the view that flows" rather than equilibria determine concentrations of muscle metabolites, as suggested by Olson (1963) and Lowry and Passonneau (1964).

SUMMARY

Methods have been adapted to determine muscle metabolites such as e.g. ATP CP glycogen and lactate in small muscle specimens (10—15 mg wet weight) obtained in the lateral portions of quadriceps femoris muscle of man.

Methodological studies in respect to the chemical analysis and the extent to which the sampling technique was representative of the muscle investigated have shown that the methods applied are adequate.

Thus, it has been possible to study the effect of exercise on these metabolites in the working muscle and to evaluate in greater detail the relative importance of the alactacid and lactacid portions of anaerobic energy yield with submaximal and brief, maximal work loads. These studies have been applied to trained as well as to untrained subjects thus covering a considerable range of individual maximal oxygen uptakes (41—72 ml × kg⁻ × min).

It was demonstrated that phosphagen concentration and the accumulation

of lactate were the same at identical relative, submaximal work loads even if great differences in absolute load existed.

With test loads greater than 90—100 % of the individual maximal oxygen uptake phosphagen stores seemed to be maximally reduced as early as 2—3 min after the start of exercise there being no further decrease over the next 10—15 min.

The highest muscle lactate concentrations were observed at exhaustion. On a per l of water basis they were approximately twice as great as the blood lactate concentrations determined simultaneously indicating that the translocation process for lactate does not immediately equilibrate the lactate concentration of the muscle cell with the lactate concentration of the extra-cellular space.

It was demonstrated that the observed increase in blood pyruvate concentration with exercise did not reflect a corresponding increase in muscle pyruvate concentration. On the contrary at rest as well as after brief maximal exercise, muscle pyruvate concentrations were obtained which were lower than blood concentrations.

Phosphagen depletion as well as muscle lactate concentration were closely related to the calculated O_2 deficit. An estimate of the total breakdown of ATP and CP and the amount of ATP resynthesis by the glycolysis corresponding to the lactate content in muscle and blood agrees in magnitude with the O deficit expressed in ATP equivalents.

Since venous oxygen tension during exercise may reach a critically low level it can be concluded that O deficit at the start of work reflects a true anaerobic state. This deficit is not due to anaerobic energy output resulting from an overflow of glycolysis with concomitant pyruvate and lactate formation. Thus, lactate formation during exercise is caused by the necessity to reoxidize extramitochondrially formed NADH in order to avoid a complete arrest of glycolysis.

With standardized work loads performed after a prolonged heavy exercise period, blood lactate concentrations were lower than in a control study after prolonged rest. On the other hand muscle lactate concentrations were unchanged. The most plausible explanation for these findings is that the preceding prolonged heavy exercise induced an increased utilization of blood-borne lactate. The lowered blood lactate concentrations might enhance lactate flux from the working muscle to the blood.

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EXTRACELLULAR OSMOLALITY AND VASCULAR SMOOTH MUSCLE ACTIVITY

BY
OLOF JONSSON

GÖTEBORG 1970

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GÖTEBORG 1970

ORSTADIUS BOKTRYCKERI AKTIEBOLAG
GÖTEBORG 1973

This summary is based on studies reported in the following papers

I. Cell volume as a factor influencing electrical and mechanical activity of vascular smooth muscle. Johansson, B and O Jonsson, *Acta physiol. scand* 1968. *72* 456—468

II *Effects of hyperosmolarity on the volume of vascular smooth muscle* cells and the relation between cell volume and muscle activity Arvill, A., B Johansson and O Jonsson, *Acta physiol scand* 1969 *75* 484—495

III Changes in the activity of isolated vascular smooth muscle in response to reduced osmolarity Jonsson, O., *Acta physiol. scand* 1969 *77* 191—200

IV Changes in the cell volume of isolated vascular smooth muscle in response to reduced osmolarity Jonsson, O., *Acta physiol scand* 1969 *77* 201—211

V The distribution of sodium potassium and chloride in the smooth muscle of the rat portal vein. *Haljamäe*, H., B Johansson, O Jonsson and H Röckert, *Acta physiol scand.* 1970 *78* 255—268

VI. Effects of variations in the extracellular osmolality on the permeability to non-electrolytes of vascular smooth muscle. Jonsson, O., *Acta physiol. scand* In press.

VII Effects of variations in the extracellular osmolality on the ionic permeability of vascular smooth muscle. Jonsson, O., *Acta physiol. scand.* In press.

The papers are referred to by their Roman numerals in text.

Introduction

Alterations in osmolality of extracellular fluid occur under physiological conditions. For instance, enhanced metabolism in different tissues causes an increased release of osmotically active substances which, in turn, results in local hypertonicity (Barcroft and Kato 1915 Hill and Kupalov 1930) Only recently however have such increases in tissue osmolality been suggested to be involved in the metabolic control of blood flow In a symposium on skeletal muscle circulation in 1966 Mellander proposed, for the first time, a possible role of hypertonicity in exercise hyperemia. (see Mellander 1968) Staunby and Fregly (1968) also discussed the importance of osmolality in blood flow autoregulation in skeletal muscle at that time.

The involvement of hyperosmolality in exercise hyperemia has later been investigated in a series of studies by Mellander and collaborators (Mellander *et al.* 1967 Gray Lundvall and Mellander 1968 Lundvall 1969 Lundvall Mellander and White 1969 Mellander and Lundvall 1970) In cat skeletal muscle, exercise was found to be associated with an increase in the osmolality of the venous effluent by up to 40 mOsm/kg Also in man clearcut increases in osmolality were found in cubital venous blood during forearm exercise. Hyperosmolality of similar degrees, produced by intra arterial infusions of inert hypertonic solutions, elicited marked dilatation of the resistance vessels and the pattern of vascular response resembled also in other respects that produced by exercise. Earlier studies (*e g* Marshall and Shepherd 1959 Overbeck, Molnar and Haddy 1961) had demonstrated blood flow increases in response to arterial infusions of hyperosmotic solutions but had not clarified whether this was due to passive dehydration of the vascular wall or to inhibition of smooth muscle tone. The observation by Mellander *et al.* (1967) that hyperosmolality failed to increase blood flow if superimposed on a supramaximal dose of papaverine indicated, however that passive dehydration is not the essential mode of action

The mechanisms by which increased osmolality interferes with vascular tone remained to be clarified. From studies of other types of muscle it was known that hyperosmolality could dissociate electrical and mechanical activity (*e g* Hodgkin and Horowicz 1957 Tomita 1966 a) and interfere

with myogenic intercellular propagation (*e g* Barr Dewey and Berger 1965 Johansson and Ljung 1967) but very high tonicities had then been used. It was therefore important to analyse the actions of more moderate osmolality variations on isolated vascular smooth muscle, if possible taken from resistance vessels. These, however are not easily prepared for direct studies. It was for this reason necessary to use as an experimental model a more conveniently isolated vessel which exhibited functional similarities to the resistance vessels The rat portal vein, with its spontaneous myogenic activity (Funaki and Bohr 1964) seemed suitable from these points of view

In a first series of experiments on the portal vein (Mellander *et al* 1967), we observed that hyperosmolality markedly inhibited the spontaneous electrical and mechanical activity evidently as a result of membrane hyperpolarization. A more complete analysis indicated the existence of an intimate relationship between the volume of the smooth muscle cells and their level of activity (I) This and the subsequent series of studies (II—VII), summarized in the present survey are concerned with the nature and background of this relation between cell volume and cell activity

Aim of the present studies

The first step in the present series of studies was to investigate in more detail the effects of alterations in extracellular osmolality on the electrical and mechanical activity of the rat portal vein. Indirect evidence was thereby obtained for a close relationship between cell volume and degree of spontaneous activity of the smooth muscle osmotic procedures which could be expected to cause cell shrinkage led to inhibition, and *vice versa* (I) Further penetration of this problem required however quantitative information concerning the influence of altered tonicity on muscle cell volume. Studies on other types of smooth muscle have revealed that the cells adapt their volume almost like perfect osmometers on exposure to moderately hyperosmotic solutions, while the changes caused by lowered tonicity seem to be more complex (Bozler 1965 Brading and Setekleiv 1968)

A possible explanation for the effects of altered osmolality on portal vein activity might be secondary changes in the membrane potential as a result of an ionic redistribution across the cell membranes. This hypothesis could be tested in experiments where both the osmolality and the ionic composition of the extracellular medium were varied. An alternative or perhaps, additional explanation would be a change in membrane permeability as a result of osmotically induced cell shrinkage or swelling. For example, if hypo-osmolality led to a more pronounced increase in the permeability constant for sodium (P_{Na}) as compared with that for potassium (P_K), this would contribute to the depolarization observed in hypo-osmotic solutions. However before such a hypothesis could be tested experimentally information on the ionic distribution in the vascular smooth muscle was required.

The purpose of the present studies was thus to find answers to the following questions:

1) How is the spontaneous electrical and mechanical activity of the isolated rat portal vein influenced by alterations in extracellular osmolality?

2) Do the smooth muscle cells beha e like perfect osmometers on tonicity changes?

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1) How is the spontaneous electrical and mechanical activity of the isolated rat portal vein influenced by alterations in extracellular osmolality?

2) Do the smooth muscle cells behave like perfect osmometers on tonicity changes?

3) How are sodium, potassium and chloride ions distributed in this vascular smooth muscle?

4) Can the osmolality effects on the spontaneous muscle activity be explained solely on the basis of altered ionic concentration gradients across the cell membranes?

5) Do alterations in P_{Ir} and P_K contribute to the changes in spontaneous activity that are elicited by altered osmolality?

Methodological considerations

Preparations The experiments carried out in the present series of studies were all performed *in vitro* which has the following advantages as compared to an *in vivo* situation 1) Stable environmental conditions can be maintained. 2) *Single* factors in the surrounding environment can be changed. 3) Repeated determinations of wet weight can be made on one and the same preparation. 4) Both electrical and mechanical activity can easily be recorded simultaneously 5) Tracer techniques can conveniently be applied to problems of ionic concentrations and membrane permeability

Even though the *in vitro* situation is basically "unphysiological" a portal vein preparation will "survive" and exhibit normal activity for at least 10 hours in normal Krebs solution at 37°C.

The rat portal vein was chosen as a test preparation for these studies since its smooth muscle exhibits characteristics similar to those of the functionally important precapillary resistance vessels with regard to myogenic activity spread of excitation, *etc* Thus, observations on this "single unit" preparation might be relevant also for the vessels that are mainly responsible for the important control of flow resistance and flow distribution. It should, however be stressed that from the pharmacological point of view for instance with regard to the responses to acetylcholine and histamine, the isolated portal vein is not a representative model of resistance vessels. A spontaneously active portal vein is found in many vertebrates, *e g* rat (Funaki and Bohr 1964), rabbit (Holman, Kasby and Suthers 1967) guinea pig (Nakajima and Horn 1967) baboon (Cuthbert 1966) It is interesting to note that in the primitive myxine there exists a true portal heart (Fänge, Bloom and Östlund 1963)

The rat was chosen for the present series of investigations since it is easily available, relatively cheap and supplies a portal vein preparation of suitable size.

As the methods used in each study are described in detail in the corresponding Methods sections, only some points of general interest will be discussed in this connection.

Solutions. A modified Krebs solution was used as standard incubation medium. Osmolality was increased by adding sucrose or other nonelectrolytes and decreased by reducing the NaCl concentration. In I and II ap-

proximate changes in osmolality were deduced on the basis of changes in concentrations. In IV a manual device for determination of the freezing point was available which allowed direct measurements of osmolality. In VI and VII it was replaced by an automatically recording osmometer which gave highly reproducible results.

Mechanical activity The mechanical activity of the preparations was recorded isometrically in the longitudinal direction of the veins with a passive tension of approximately 400 dynes. Histological examination has revealed that the muscle tissue in the part of the vein used for the present studies is mainly longitudinally oriented (Johansson *et al* 1970).

Electrical activity The membrane events were recorded extracellularly with the sucrose gap technique. This metod was originally described by Stampfli (1954) for recording on nerves and was later modified for smooth muscle by Burnstock and Straub (1958). It has certain advantages in comparison to the microelectrode technique in that it is more convenient to handle and allows a more undisturbed recording of the mechanical events. It does not, however permit exact measurements of the membrane potential since the degree of short-circuiting between the recording electrodes in the extracellular space is not known. Action potentials and *relative* changes of the membrane potential can, however readily be recorded. The consistent correlation in time between electrical and mechanical events (I, III) suggests that the phasic mechanical activity is normally triggered by the bursts of electrical activity. It also implies that a simple mechanical recording can be used as an indicator for changes in the electrical events.

Extracellular space In general, two different procedures have been used to determine the extracellular space, 1) estimations from electron microscopic sections and 2) measurements in uptake experiments with extracellular markers. The former method tends to yield lower values than the latter one (for ref. see Goodford 1968) which is more often used. The value for the extracellular space, obtained in tracer uptake studies, varies with the size of the tracer molecule — large molecules giving smaller spaces (Barr and Malvin 1965). For instance, in circular intestinal muscle of the dog the sucrose space exceeds the inulin space by 4 ml/100 g wet weight (Barr and Malvin 1965) and in the guinea pig taenia coli the sorbitol space exceeds that of inulin by 6 ml/100 g wet weight (Brading and Jones 1969). This apparent inhomogeneity of the interstitial space might be due to an inability of larger tracer molecules to fully penetrate the extracellular mucopolysaccharide lattice (Ogston 1958, Ogston and Phelps 1960). Furthermore, the size of the preparation is of importance for the measured

space, as the influence of the superficial film of solute becomes increasingly important in smaller preparations due to their larger surface to volume ratio Also the relative proportion of damaged cells probably increases in small preparations, leading to an overestimation of the extracellular space For example, Goodford (1968) found that the sorbitol space decreased by 0.7 ml/100 g wet weight per mg increase in weight of the taenia coli preparation

In the present experiments, sucrose (mol. wt. 342) was chosen as the extracellular marker As distinguished from inulin, another commonly used tracer substance, sucrose is a single molecular species. Uptake experiments show that ^{14}C sucrose is almost completely equilibrated in the portal vein within 15 min (II) Therefore, the "15 min sucrose space" has been taken to represent the extracellular volume, expressed in ml/100 g wet weight and calculated in the following way

$$\frac{\text{cpm/mg tissue} \times 100\,000}{\text{cpm/ml incubation medium}}$$

With respect to a recently introduced tracer for extracellular space, Co EDTA, a tendency for adsorption has been noted, manifesting itself as a decrease in space with increasing tracer concentrations (Brading and Jones 1969) Preliminary experiments indicated a similar tendency concerning the ^{14}C sucrose space of the rat portal vein, but only when the tracer concentration was below 0.2 mmoles/l. No such correlation was found in the range of concentrations applied in the present studies (0.2—0.6 *mmoles/l*)

Rinsing and drying In II and IV the veins were rapidly rinsed in inactive solution after exposure to radioactive media. The purpose of the procedure was to remove the surface film of active solution and to immediately interrupt the exposure which is of importance when shorter periods of incubation are used. In later studies (V VI and VII) this rinsing procedure was omitted, mainly to allow a comparison between the results obtained in ^{24}Na and ^{42}K uptake experiments and the results of Na and K determinations with flame photometry Thus figures for the ^{14}C sucrose space in V VI and VII *are somewhat higher than those in II and IV* However the calculations of cell water content in different environmental situations, presented in II and IV would not be significantly altered if the sucrose space had been determined in unrinsed veins.

Before weighing, all preparations used in tracer exchange experiments were gently blotted between two pieces of filter paper Such a "drying" procedure was necessary due to the greatly varying amounts of adhering

proximate changes in osmolality were deduced on the basis of changes in concentrations. In IV a manual device for determination of the freezing point was available which allowed direct measurements of osmolality In VI and VII it was replaced by an automatically recording osmometer which gave highly reproducible results.

Mechanical activity The mechanical activity of the preparations was recorded isometrically in the longitudinal direction of the veins with a passive tension of approximately 400 dynes. Histological examination has revealed that the muscle tissue in the part of the vein used for the present studies is mainly longitudinally oriented (Johansson *et al* 1970)

Electrical activity The membrane events were recorded extracellularly with the sucrose gap technique. This method was originally described by Stampfli (1954) for recording on nerves and was later modified for smooth muscle by Burnstock and Straub (1958). It has certain advantages in comparison to the microelectrode technique in that it is more convenient to handle and allows a more undisturbed recording of the mechanical events. It does not, however permit exact measurements of the membrane potential since the degree of short-circuiting between the recording electrodes in the extracellular space is not known. Action potentials and *relative* changes of the membrane potential can, however readily be recorded. The consistent correlation in time between electrical and mechanical events (I III) suggests that the phasic mechanical activity is normally triggered by the bursts of electrical activity It also implies that a simple mechanical recording can be used as an indicator for changes in the electrical events.

Extracellular space In general, two different procedures have been used to determine the extracellular space, 1) estimations from electron microscopic sections and 2) measurements in uptake experiments with extracellular markers. The former method tends to yield lower values than the latter one (for ref see Goodford 1968) which is more often used. The value for the extracellular space, obtained in tracer uptake studies, varies with the size of the tracer molecule — large molecules giving smaller spaces (Barr and Malvin 1965) For instance, in circular intestinal muscle of the dog the sucrose space exceeds the inulin space by 4 ml/100 g wet weight (Barr and Malvin 1965) and in the guinea pig taenia coli the sorbitol space exceeds that of inulin by 6 ml/100 g wet weight (Brading and Jones 1969) This apparent inhomogeneity of the interstitial space might be due to an inability of larger tracer molecules to fully penetrate the extracellular mucopolysaccharide lattice (Ogston 1958 Ogston and Phelps 1960) Furthermore, the size of the preparation is of importance for the measured

space, as the influence of the superficial film of solute becomes increasingly important in smaller preparations due to their larger surface to volume ratio. Also the relative proportion of damaged cells probably increases in small preparations, leading to an overestimation of the extracellular space. For example, Goodford (1968) found that the sorbitol space decreased by 0.7 ml/100 g wet weight per mg increase in weight of the taenia coli preparation.

In the present experiments, sucrose (mol. wt. 342) was chosen as the extracellular marker. As distinguished from inulin, another commonly used tracer substance, sucrose is a single molecular species. Uptake experiments show that ^{14}C sucrose is almost completely equilibrated in the portal vein within 15 min (II). Therefore, the "15 min sucrose space" has been taken to represent the extracellular volume, expressed in ml/100 g wet weight and calculated in the following way

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Rinsing and drying. In II and IV the veins were rapidly rinsed in inactive solution after exposure to radioactive media. The purpose of the procedure was to remove the surface film of active solution and to immediately interrupt the exposure which is of importance when shorter periods of incubation are used. In later studies (V, VI and VII) this rinsing procedure was omitted, mainly to allow a comparison between the results obtained in ^{24}Na and ^{42}K uptake experiments and the results of Na and K determinations with flame photometry. Thus figures for the ^{14}C sucrose space in V, VI and VII are somewhat higher than those in II and IV. However, the calculations of cell water content in different environmental situations, presented in II and IV, would not be significantly altered if the sucrose space had been determined in unrinsed veins.

Before weighing, all preparations used in tracer exchange experiments were gently blotted between two pieces of filter paper. Such a "drying" procedure was necessary due to the greatly varying amounts of adhering

fluid. Another commonly used drying technique is to drag the tissue across a plexiglass surface until no trace of moisture is seen A comparison between these procedures reveals that tissues blotted with the filter paper method are "drier" (Hagemeijer Ronve and Schoffeniels 1965 a, Brading and Setekleiv 1968) This method appears to be more appropriate for a flat preparation like the slit portal vein since it would probably coil during the dragging procedure. Accordingly the latter procedure gives highly unreliable results for strips of rat aorta but not when cylindrical" preparations, like the taenia coli, are used (Hagemeijer Ronve and Schoffeniels 1965 a, Brading and Setekleiv 1968)

Cell water Changes in cell water were calculated from direct measurements of wet weight, dry weight and ^{14}C sucrose space in II In IV and VI changes in the wet weight were indirectly estimated from alterations in dry weight and extracellular space on the assumption that the weight of intracellular solids and solutes remains essentially constant during exposure to the different test solutions. Comparisons of the directly and indirectly estimated values revealed good agreement between the two methods. By combining the different steps of calculations into a single equation, the standard deviation and significance could be estimated for the variations in cell water as a result of changes in tonicity

Ionic contents The ionic contents were determined by flame photometry (Na, K) (V) and by studying the uptake of ^{24}Na, ^{42}K and ^{36}Cl (V VII) Both for sodium and potassium the radioactive uptake technique gave somewhat lower values than the flame photometry determinations (see further below)

Wash-out experiments The efflux of the tracer substances was determined in VI and VII by transferring the "loaded" preparations through a succession of flasks containing inactive solution The different components of the curve representing the decrease in tissue activity were analysed mathematically with a calculator (Hewlett Packard 9100 A) Permeability coefficients in cm/sec for the nonelectrolytes were estimated by Fick's law of diffusion applying the rate constants for the transmembrane effluxes and the ratio between cell volume and cell surface. When the permeability coefficients for ions were calculated, a correction had to be included in the equation for the influence of the membrane potential Since the membrane potential was not measured directly the correction had to be approximate

Damaged preparations. Some of the preparations in V and VII were frozen and thawed or subjected to poisoning by cyanide and iodoacetate in order to destroy the barrier function of the cell membranes. The purpose

of these procedures was to provide a homogenous fluid phase throughout the preparation in equilibrium with the external medium. The use of such damaged preparations should facilitate the study of the "bound" ionic fractions as to their magnitude and exchange kinetics. The observation that sucrose as well as ^{24}Na and ^{42}K were equilibrated with the *total* water content of these preparations strongly indicates that their normal membrane function had really been lost. The finding that the size of the bound Na and K fractions were similar in the two types of damaged preparations despite the fact that the procedures applied to impair the cell membrane function were quite different, might indicate that the characteristics of the binding sites were not affected by these manipulations. If so, results obtained with these frozen and thawed or poisoned veins should be relevant even for normal tissues. In these damaged preparations, all binding sites should be equilibrated with the ionic composition of the incubation medium, that is, in normal Krebs, the sequestration of Na should be favoured in comparison to that of K. A multiplication of *i. e* the Na concentration in the Krebs solution with that part of the distribution space of ^{24}Na that exceeds the volume of total water should thus yield the bound sodium component in meq/unit wet weight in this kind of preparation. However evidence was obtained that in normal tissues a fraction of the binding sites is equilibrated with the intracellular fluid which thus should favour the sequestration of potassium. Since the ratio between the sites in contact with the intra and extracellular fluid, respectively is not known, the estimation of the bound amounts of ions in normal portal veins becomes quite complex. These problems will be further discussed below

Results and Comments

The effects of altered extracellular osmolality on the spontaneous activity of vascular smooth muscle.

The normal, spontaneous electrical activity of the portal vein consists of bursts of spike potentials, lasting 5 to 10 sec and appearing at a rate of 2—3 per min. Strictly correlated to these electrical events, phasic mechanical contractions of the longitudinal muscles occur with peak tensions of 400—800 dyn (I and III) Increasing the extracellular osmolality by adding sucrose to normal Krebs solution reduced the contractile activity with regard to both amplitude and frequency (I) Although most pronounced during the first min, this effect was sustained until osmolality was normalized again When on the other hand, extracellular osmolality was instead increased by urea, the inhibition lasted only 2—3 min after which normal activity was regained in spite of the continuous presence of urea. Thus sucrose led to a sustained inhibition whereas urea did not. Another clearcut difference in the muscle responses to hyperosmotic urea and sucrose media became apparent on return to normal osmolality After exposure to urea, an intense shortlasting increase of electrical and mechanical activity was seen, whereas only a moderate sluggish excitation was obtained after exposure to sucrose (I and II).

The interpretation was that shrinkage of the smooth muscle cells inhibits their spontaneous activity while they are stimulated by swelling. The difference between the responses to the hyperosmotic urea and sucrose media would then be due to a more rapid penetration of the smaller urea molecule through the cell membranes, which thus makes it unable to exert a maintained osmotic influence on the muscle cells. Sucrose, on the other hand, was considered to be effectively excluded from the intracellular domain. The excitation obtained when extracellular osmolality was normalized after urea exposure would then be due to a shortlasting cell swelling until urea had again left the cells by diffusion. This interpretation was supported by the observation that the excitatory phase was abolished by replacing extracellular urea with equiosmolal amounts of sucrose instead of returning to normal Krebs (I)

Furthermore, when extracellular osmolality was reduced by decreasing

the NaCl concentration of the Krebs solution a marked and sustained excitation was obtained (I and III) If the subtracted amounts of NaCl were replaced by an equiosmolal quantity of sucrose, no excitation was obtained indicating that it was due to the low osmolality and not to the reduced ionic concentration. If however NaCl was replaced by equiosmolal urea, an excitation followed as during hypo-osmolality again suggesting that urea penetrates the cell membranes.

These suggestions concerning the distribution of urea and sucrose in the portal vein were confirmed in II by uptake studies of ^{14}C urea and ^{14}C sucrose. Urea quickly equilibrated in a space corresponding to the volume of the total tissue water i. e about 79 ml/100 g wet weight whereas the sucrose space was less than 50 ml/100 g wet weight (see further below)

The duration of exposure to hyperosmotic urea and sucrose media was also varied to explore its effect on the excitation that followed upon normalization of the osmolality (II) For urea, this excitation increased with the preceding period of exposure, but only as long as it was shorter than the equilibration time for ^{14}C urea in the total water The excitation obtained after incubation in hyperosmotic urea therefore seems to depend on the shortlasting cell swelling bound to occur upon normalization of the osmolality In the case of sucrose, on the other hand the excitation increased with the duration of the preceding hypertonicity exposure without any correlation to the equilibration time for ^{14}C sucrose uptake. Therefore, another type of mechanism seemed to be involved in this case. In V it was found that the muscle cells lose potassium on longer exposures (60 min) to hypertonic sucrose. Normalization of the cell volume upon return to isotonicity could then lead to a subnormal ratio of $[K^+]_i$ to $[K^+]$ which, in turn, would cause depolarization and increased activity

Further evidence for a correlation between smooth muscle cell volume and activity was obtained in II and IV where it was demonstrated that the cells actually shrink in a hyperosmotic sucrose solution and swell in hypo-osmotic media. These volume alterations will be considered from a quantitative point of view in the next section.

Osmotic reduction of cell volume is thus associated with hyperpolarization and decreased frequency and duration of the spike bursts. Conversely osmotic swelling leads to depolarization and increased spike activity It is conceivable that these reactions to alterations in cell volume represent manifestations of a general mechano-electrical coupling. This response to osmotic deformation might thus be related to that induced by passive stretch, seen in many smooth muscles of the "single unit" type, e g ureter (Bozler 1947) taenia coli (Bülbring 1953) portal mesenteric

veins (Sparks 1964 Axelsson, Johansson and Jonsson 1966 Holman *et al.* 1968) small arteries (Johansson and Bohr 1966) and cerebral and umbilical arteries (Sparks 1964) Reactions to passive stretch and to osmotic deformation have also been discussed for stretch receptors as manifestations of a unitary mechanism of mechano-electrical coupling (Ottosson 1965) As to the details of the cellular events behind the mechano-electrical transduction, some interesting theories have been proposed (see *e g* Teorell 1966) but much remains to be elucidated in this respect.

Beside the influences of hyperosmolality on membrane potential and spike activity the portal vein also shows an impaired intercellular propagation, referred to above (Johansson and Ljung 1967) and a negative inotropic response (Mellander *et al* 1967) when osmolality is doubled or more. As to this inotropic effect of altered tonicity many studies have been carried out on skeletal and cardiac muscle preparations. Hodgkin and Horowicz (1957) reported that single action potentials failed to elicit a mechanical twitch in the frog semitendinosus muscle when extracellular tonicity was 2.5 times normal while trains of action potentials could still initiate weak contractions. Later studies indicate that this impaired contractility of the striated muscle in strongly hypertonic media is, in part, due to impaired electro-mechanical coupling This is suggested for instance by the finding that caffein, assumed to act directly on intracellular Ca^{++} stores, is able to induce a contraction even in a hyperosmotic medium while an increment in $[K^+]_o$, acting *via* the membrane potential, fails to do so (Caputo 1966)

It has been shown, however that increased osmolality beside this uncoupling effect exerts a direct influence on the contractile machinery This is indicated by the observation made by Howarth (1958) and Podolsky and Sugi (1967) that the shortening of striated muscle fibres in a hypertonic environment is extremely slow compared to control. The authors ascribed their findings to, alternatively an increased intracellular viscosity or to an inhibition of the myofibrillar ATP ase activity as a result of raised intracellular ionic strength

The inotropic effects of hyperosmolality on cardiac muscle are somewhat more complex as indicated by the fact that a pronounced raise in tonicity elicits quite the opposite type of response as that induced by a more moderate degree of hyperosmolality For instance Koch-Weser (1963) and Wildenthal Skelton and Coleman (1969) reported a depressed contractile strength in isolated cat papillary muscles exposed to two times the normal tonicity and more powerful contractions than in an isotonic medium when the same tissues were incubated at osmolalities up to 100—

150 mOsm/kg above normal. Moreover the latter authors found that the rate of shortening was changed in a similar manner *i e* a faster velocity of shortening as compared to control at the moderate degree of hyper osmolality and *vice versa* at a tonicity of two times the normal or more. By a thorough analysis of the mechanisms involved Wildenthal, Skelton and Coleman (1969) could demonstrate that these effects of hyperosmolality were exerted through influences on both the contractile and the series elastic elements of the myocardial fibres. It has been suggested that the increase in contractile force and velocity of shortening observed at lower degrees of hypertonicity is the result of an increase in the Ca^{++} concentration near the muscle membrane. The opposite effects observed at higher osmolalities would then be due to a dominating role of increased intracellular viscosity and raised interfilamentary friction (Koch-Weser 1963 Wildenthal Skelton and Coleman 1969)

In summary then, the results referred to in this section point to a correlation between the volume of the vascular smooth muscle cells and their degree of activity Shrinkage is associated with inhibition and swelling with excitation. Possible explanations for the mechanisms involved will be discussed in the following sections but the changes in the volume of the smooth muscle cells caused by alterations in tonicity will first be considered from a quantitative point of view

The osmotic behaviour of vascular smooth muscle cells.

The effects of changes in extracellular osmolality on the intracellular water content were analysed under different experimental conditions in II IV and VI to elucidate whether the muscle cells behave as osmometers. If so their volume would adapt to alterations in the osmolality according to the equation given by Ruck6 and McCutcheon (1932)

$$P\,(V-b) = P_o(V-b) = \text{constant} \qquad (1)$$

P denotes the original external osmotic pressure, V the original cell volume while P and V denote the same parameters in the new environment. The non solvent volume of the cell is represented by b which is assumed to be constant throughout. Thus, for a perfect osmometer V plotted against $\frac{1}{P}$ should yield a straight line with an intercept at b when $\frac{1}{P}$ approaches 0

The concept of the muscle cell as an osmometer assumes that it is surrounded by a semipermeable membrane, which separates an intracellular isosmotic solution of colloids and crystalloids from the extracellular medium. This membrane theory" is, however not generally accepted (see further below)

The volume of cell water (w) at different tonicities was analysed in the present series of experiments. If muscle cells adapt themselves to an altered osmotic environment like perfect osmometers and their entire water content participates as a solvent (no "bound" water) the experimentally determined w values would equal V — b in the equation of Lucké and McCutcheon In II it was demonstrated that w decreased to 66 per cent of control on exposure to Krebs plus 150 mmoles sucrose/l, in good agreement with the value of 64 per cent calculated from equation (1) It may be concluded, therefore, that the vascular smooth muscle cells behave as almost perfect osmometers on exposure to hyperosmolality of this degree. A similar osmotic behaviour has been reported for intestinal smooth muscle (Brading and Setekleiv 1968) as well as for amphibian skeletal muscle (Boyle and Conway 1941 Dydynska and Wilkie 1963 Blinks 1965 Bozler 1965) These uniform results in different preparations thus seem to support the concept of the cell as a semipermeable bag" filled with a fixed amount of relatively impermeant solutes and solids. Furthermore, they allow calculations of the concomitant alterations in transmembrane ionic concentration gradients that are bound to occur on shrinkage of the muscle cells. Such data will be used below when the mechanism responsible for the correlation between cell volume and degree of muscle activity is discussed

The fact that the cells behave as if surrounded by semipermeable membranes on increases of osmolality does not mean that the membranes are permeable to water alone Such an osmotic behaviour should be expected also if the cell membranes were permeable to ions as long as their inward and outward fluxes were equal, thus keeping the internal solute amount constant.

The alterations in the cell water of the vascular smooth muscle induced by reductions in the extracellular tonicity are more complex, as demonstrated in IV and VI At 37°C the cell water increased by 14 per cent in muscles stretched to their *in situ* length but by 35 per cent in unstretched preparations on exposure to Krebs minus 60 mmoles NaCl/l. When the same experiment was performed at such a low temperature (1°C) that muscle activity was virtually abolished the cell water increased by approximately the same value (35—40 per cent) in both stretched and

unstretched veins. These results indicate that the increased active tension induced by hypo-osmolality at 37°C could oppose cell swelling in the stretched veins where the contractile response should be more pronounced than in unstretched preparations, according to the length-tension relationship (Speden 1960 Sparks and Bohr 1962) However this factor cannot alone explain the restricted swelling since the cell water did not increase to the extent expected for a perfect osmometer on exposure to Krebs minus 60 mmoles NaCl/l even at 1 C (40 compared to 58 per cent) This might indicate that tension develops in the cell membranes so that a hydrostatic pressure difference becomes established at more pronounced swellings of the cells. However since muscle cells exposed to this drastically hypotonic medium were found to lose ions, they appear to obtain transmembrane osmotic equilibrium despite a fairly restricted swelling

On the other hand, on exposure to more moderately hypo-osmotic environments (Krebs minus 30 mmoles NaCl/l) at 1°C, the cell water increased to an extent expected for a perfect osmometer and, moreover remained essentially constant during prolonged exposure. Thus, when the restricting influence of the contractile response on the swelling of the cells is eliminated, the cells exhibit an almost perfect osmotic behaviour on reductions in osmolality as long as these are not too drastic.

In summary the results presented in II IV and VI concerning the adaption of the portal vein cell volume to alterations in the extracellular tonicity demonstrate that the cells shrink in hyperosmotic media and swell in hypo-osmotic ones which thus supports the conclusions drawn in I II and III. The extent of volume change is in accordance with what could be expected for perfect osmometers on increases in osmolality and also on moderate decreases in osmolality provided the contractile response is hampered. Even on drastic reductions in tonicity the results do not contradict the concept of muscle cells as semipermeable bags filled with an aqueous solution of crystalloids and colloids, if it is assumed that they then adapt to hypo-osmolality by losing ions when they cannot swell any more.

Concerning other types of muscle cells exposed to hypo-osmolality Blinks (1965) reported a perfect osmotic behaviour for amphibian *skeletal muscle* while Bozler (1965) stated that they swell less than expected. Bozler explained this as due to a loss of ions and to the presence of bound, osmotically inactive water Most authors seem to agree that amphibian and mammalian *smooth muscle* do not swell to an extent predicted from the osmotic laws, at least not when the tonicity reductions are pronounced (e g Bozler 1965 Brading and Setekleiv 1968). In both these studies a

certain loss of cellular ions was observed which is in agreement with the present findings on the portal vein Bozler proposed that the restriction in cell swelling was due to the development of strong cross-linked gels in the cytoplasm on exposure to hypotonicity while Brading and Setekleiv suggested that it depended on limited distensibility of the cell membranes.

As mentioned earlier the membrane model for the cell is not generally accepted. As an alternative, the "sorptional theory" has been advocated particularly by a group of Russian authors (for review see Troshin 1966) According to this view cell permeability is not a function of the membrane but of the sorptional activity of the protoplasm This concept includes the solubility of substances in the protoplasm as well as their adsorptional and chemical binding by cellular colloids. The sorptional activity of the protoplasm is considered to be regulated by the cell metabolism A substance penetrating the cell would then be either in a dissolved or in a bound state depending on the actual sorptional ability of the protoplasm Normally the greater part of cellular potassium, magnesium and calcium would be bound and therefore incapable of free diffusion while the binding and, consequently the cellular content of sodium would be small. Upon excitation or damage of the cells, a shift in the sorptional activity would occur leading to a decrease in the binding of some substances like potassium to the cell colloids, while that of others, like sodium, would be increased Consequently the permeability to sodium would then be increased and that to potassium decreased.

Further according to Troshin s concept most of the cell water would prevail in an organized "bound" form implying that its properties would be different from those it has in a normal aqueous solution. The alterations in cell water content, induced by exposure to hyperosmotic media, are ascribed to a decrease in the hydration of the cellular colloids as a result of solute penetration into the cells. Thus, according to this sorptional theory cell shrinkage upon exposure to hypertonicity is ascribed to the penetration of the extra" solute into the cells while, according to the membrane theory it is explained by the inability of the solute to penetrate into the cells.

Troshin enumerates several investigations performed on different kinds of tissues where results have been obtained that support the sorptional theory For instance, Nasonov and Aizenberg (1937) found that the ability of a Ringer solution, made hypertonic by addition of non-electrolytes to withdraw water from the frog leg muscle, increased with the molecular weight of the added solute. Even urea and glycerol, known to penetrate muscle cells easily (Overton 1902) could make them shrink

permanently However when isovolumetric (*i. e* a constant ratio between volume of solutes and volume of solvent) instead of isosmotic concentrations were applied, equal reductions in muscular volume were caused irrespective of the mol. wt. Similar results were obtained on egg cells by Aizenberg (1939) In accordance with these results, Troshin (1953) found that the water content of erythrocytes, suspended in hypertonic galactos-Ringer solutions, decreased simultaneously with and in proportion to the entrance of the solute Troshin concluded that the erythrocytes lost water not because the sugar did not penetrate them, but because it did.

These results, which no doubt are difficult to explain on the basis of the membrane model alone, do not seem to be relevant for the portal vein For instance, in II it was demonstrated that a hyperosmotic urea Krebs medium is unable to permanently withdraw water from the smooth muscle cells, as no change in the wet weight of the preparation could be measured. In general, the sorptional theory has been challenged by several authors on the basis of considerable experimental evidence incompatible with it. For instance, Hodgkin and Keynes (1953) demonstrated that the self diffusion coefficient and ionic mobility of potassium ions inside a cuttlefish axon is only a few per cent lower than the corresponding values in an aqueous solution. These findings contradict the hypothesis that the greater part of the cellular K prevails in a bound form. Furthermore, electrical measurements of the cable constants of nerve and striated muscle fibres have demonstrated that the cytoplasm behaves as a good electrolytic conductor surrounded by an insulating membrane (Hodgkin and Rushton 1946, Katz 1948) Similar electrical properties have been revealed for bundles of smooth muscle fibres (Tomita 1966 b, 1967 Abe and Tomita 1968).

It thus seems justified to discuss the present results as to the effects of altered osmolality on the vascular smooth muscle cells on the basis of the membrane model The present results thus indicate a perfect osmotic behaviour on increases of tonicity while the swelling as a result of hypotonicity was less than expected. This could, at least partly be ascribed to a restricting influence of the contractile response When prevented from adapting themselves to hypo-osmolality by swelling, the cells seem to be able to do so by adjusting their intracellular ionic amounts.

The distribution of ions in the vascular smooth muscle

The total amounts of sodium and potassium in the isolated portal vein were determined in V by flame photometry The tissue contents of these

elements remained essentially constant for considerable periods (270 min) of incubation in normal Krebs solution at 37°C. A comparison between values obtained with flame photometry and the distribution volume of ^{24}Na after different periods of exposure to the radioactive medium showed that ^{24}Na after only a few min equilibrated in a space corresponding to about 90 per cent of the total Na content. No significant increase in the distribution volume of ^{24}Na was obtained with longer periods of exposure. A similar comparison for K showed that the ^{42}K uptake was much slower and after six hours of exposure to radioactivity the ^{42}K distribution space was still significantly lower than expected from the total K content suggesting the existence of a slowly exchanging potassium fraction. The total content of chloride was not determined in the present series of studies. The plateau value of the ^{36}Cl uptake curve which was reached within 15 min, was therefore used for calculations on chloride distribution.

If these values for *total* Na, K and Cl amounts and the values for tissue water and extracellular space were used to calculate the intracellular ionic concentrations on the basis of a two compartment tissue model the following values, in meq/l cell water were obtained $[Na^+]_i$ 90 $[K^+]_i$ 163 and $[Cl^-]_i$ 114. These concentrations are, however too high to allow an osmotic equilibrium across the cell membranes, which led to the assumption that some fraction of the total ionic contents prevails in a bound osmotically inactive form.

This possibility was investigated in V by two different approaches. In the first series of experiments the absolute amounts of Na and K in the portal vein were determined by flame photometry after 5, 30 and 60 min of incubation in an isotonic sucrose solution containing 2.5 mmoles $CaCl_2$/l as its only ionic constituent. The tissue content of Na decreased rapidly in this medium and after one hour it was below the measurable level. With the method used, this implies a Na content below 2 meq/kg wet weight. The decrease in the potassium content was less pronounced, and even after 60 min approximately 40 per cent of the original K content remained in the tissue. These results speak against the existence of any *sizeable* component of *firmly* bound Na. The more gradual decline in the potassium content was probably due to the fact that this ion is mainly located intracellularly and thus has to pass the cell membranes. No definite conclusions concerning bound fractions of K can therefore be drawn from these results.

The second series of experiments in V intended to give information on bound ions, involved studies of the uptake of ^{24}Na, ^{42}K and ^{36}Cl in portal

veins after interference with the cell membrane function by freezing and thawing or by poisoning. In such preparations the entire fluid phase of the tissue appeared to be homogenous and equilibrated with the external medium. *The ability of the tissue to bind ions would then be revealed by* a distribution volume of the tracer substances greater than the volume of total water The effectiveness of these procedures to bring about the intended effect on the cell membranes was indicated by the findings in the damaged veins that sucrose could equilibrate in the entire fluid phase and that the distribution volumes of ^{24}Na and ^{42}K were approximately equal. The existence of bound fractions of sodium, potassium and chloride were indicated by the observation that the spaces of ^{24}Na and ^{42}K and that of ^{36}Cl in these damaged preparations exceeded total water by approximately 13 13 and 7 ml/100 g wet weight, respectively The binding sites *did not seem to discriminate between the two cations since the* ^{42}K *space* was essentially unaltered when the uptake experiments were performed in a medium where NaCl had been replaced by KCl.

If the characteristics of the binding sites in normal veins were identical to those of the damaged preparations and their localization in the tissue were known, it would be possible to calculate the amounts of bound ions in the former kind of tissue by multiplying the "excess" electrolyte space with the ionic concentration in that fluid compartment with which the binding sites are considered to be equilibrated. However since no information was available concerning the localization of the binding sites it *was simply assumed that the ratios of free* Na^+ K^+ *and* Cl^- *to the total* amounts of the respective ions in normal veins would be reflected in the damaged preparations by the ratio of tissue water to total space of tracer ion. The intracellular ion concentrations obtained by such correction for bound fractions were for $[Na^+]$ 44.8 meq/l, $[K^+]_i$ 138 meq/l and $[Cl^-]_i$ 86.5 meq/l. Similar calculations based on an assumption that all binding sites are equilibrated with the extracellular solution gave the following intra *cellular concentrations in meq/l cell water* $[Na^+]_i$ *13.7* $[K^+]_i$ *163 and* $[Cl^-]_i$ 83

In VII the efflux of ^{24}Na and ^{42}K was studied under different experimental conditions. By extrapolating to zero time that part of the washout curve thought to represent the transmembrane efflux, an estimate of the intracellular concentration of the actual ion could be made. The value for $[K^+]_i$ calculated in this way agreed satisfactorily with those found in V *However for* $[Na^+]_i$ *a value of 13 meq/l* was obtained in the washout experiments which is considerably lower than the figures reported in V as referred to above. Furthermore, this low estimate of $[Na^+]_i$

was obtained at 15°C and since a gain of sodium and loss of potassium on cooling have been reported for other types of smooth muscle (e g Freeman Narrod and Goodford 1962, Friedman and Friedman 1967) it is possible that the corresponding value at 37°C would be somewhat lower still. However since the spontaneous activity of the portal vein was affected to a surprisingly small extent by lowering the temperature to 15°C it seemed unlikely that more drastic alterations in the transmembrane ionic concentration gradients had occurred and 13 meq/l might therefore be an approximate estimate of $[Na^+]_i$ even at 37°C.

The reason for this discrepancy between the values for the intracellular sodium concentration obtained in V and VII is probably that, in the former study $[Na^+]_i$ was calculated by subtracting the extracellular and bound amounts of Na determined in ^{24}Na uptake experiments from the total sodium content determined by flame photometry. If there is a second sequestered sodium fraction for which the exchange with ^{24}Na is very incomplete over the actual exposure periods it would, with this mode of calculation, become included in the value for the free intracellular sodium ions. In fact, the observation that the ^{24}Na uptake curve did not quite reach the plateau predicted from flame photometry does suggest the occurrence of a small slowly exchanging fraction.

For this reason it might be more appropriate to use the total *exchangeable* Na fraction instead of the values from flame photometry when $[Na^+]_i$ values are estimated by subtracting extracellular and "bound" Na from the total amounts. "Bound" Na in this context would not correspond to the sequestered, "non-exchangeable" pool but would represent a more loosely adsorbed fraction exchanging freely with Na^+ in the fluid phases. Such calculations have recently been performed by Bäth, Johansson and Jonsson (1971) in a study where the sodium distribution in the portal vein was more closely examined. The distribution volumes of ^{24}Na and the tissue water content as well as the ^{14}C sucrose spaces were investigated in normal and in frozen-thawed preparations of portal vein at different concentrations of sodium in the incubation medium. It was demonstrated that the maximal capacity of the damaged preparations to bind sodium was approximately 22 meq/kg wet weight, a value reached at an $[Na^+]$ of 212 meq/l. The corresponding figure obtained when the binding sites were equilibrated with the $[Na^+]$ of the normal Krebs solution was 18 meq/kg wet weight. The total amount of exchangeable bound Na in intact preparations will evidently depend on the relative distribution of the binding sites between extracellular structures exposed to the Na rich medium, and intracellular structures being in contact with

Na low fluid. These results indicate that all Na binding sites are not equilibrated with [Na^+] since the amounts of bound and extracellular Na would then exceed the total exchangeable Na content. Therefore it seems probable that Na binding occurs both intra and extracellularly This conclusion is in good agreement with the results in VII which indicate the presence of a small (0.3 meq/kg wet weight) slowly exchanging, sequestered Na fraction located inside the cells, and a larger one which was equilibrated with [Na^+] and washed out together with the extra cellular solutes.

An integrated interpretation of the results reported in V VII and in the study by Bäth, Johansson and Jonsson (1971) suggests the following pattern of Na distribution in the portal vein

1) Extracellular ionized Na 68 meq/kg wet weight.
2) Intracellular ionized Na: 3—4 meq/kg wet weight.
3) Intracellularly bound, slowly exchanging Na 0.3 meq/ kg wet weight.
4) Extracellularly bound, rapidly exchanging Na: 8—9 meq/kg wet weight.
5) Possibly a non-exchangeable Na fraction not exceeding 10 meq/kg wet weight. This fraction, whose localization is unknown, might, after all *be of the same nature as 3*

The calculated values for the potassium chloride and sodium equilibrium potentials are —84 —12 and +62 mV respectively when the intra cellular concentrations given in V are used for the former two ions and 13 1 meq/l cell water for [Na^+] Since the membrane potential in the portal vein is approximately —50 mV (Nakajima and Horn 1967) these estimates indicate that it is mainly but not solely determined by the distribution of the potassium ion across the cell membrane. Furthermore, the chloride ion may not be distributed according to a passive Donnan equilibrium, but the presence of spontaneous activity makes this question difficult to decide.

This picture of the ionic composition of the portal vein will now be considered in the light of similar studies on other types of smooth muscle.

Concerning arterial smooth muscle, several authors have reported the occurrence of sequestered fractions of ions (Headings, Rondell and Bohr 1960, Friedman and Sréter 1963 Garrahan Villamil and Zadunaisky 1965 Friedman and Friedman 1967 Friedman *et al* 1968 Friedman Gustafson and Friedman 1968 Keannge 1968 Villamil *et al* 1968 Schoffeniels 1969 Ronive 1969) Headings, Rondell and Bohr (1960) found that the dog carotid artery had a considerable capacity to bind cations, Na^+ K^+ and H^+ appearing to compete for the anionic binding

sites. They concluded that these sites were equilibrated with the extracellular sodium-rich medium and that therefore, in the normal state, only Na binding occurred. It was proposed that this binding occurred in the mucopolysaccharides of the ground substance.

The assumption that the sequestered Na was located extracellularly was supported by the results of Garrahan, Villamil and Zadunaisky (1965) who studied washout of ^{22}Na from dog carotid artery They found that the first, rapid component in the curve representing the decline in remaining activity contained considerably more Na than could be extracellularly dissolved and concluded that the excess represented bound Na. Analyses performed later have indicated the occurrence of four or five different Na compartments in arterial wall (Friedman and Friedman 1967 Friedman *et al.* 1968 Friedman, Gustafson and Friedman 1968 Rorive 1969) of which two to three represent sequestered Na. The opinions concerning their magnitude and characteristics do however vary considerably as reflected in the scatter of the values for $[Na^+]_i$ reported by different authors 7 mmoles/l (Keatinge 1968) 20—30 mmoles/l (calculated from Friedman *et al* 1968), 44 mmoles/l (Schoffeniels 1969) 50 mmoles/l (Garrahan, Villamil and Zadunaisky 1965), 84 mmoles/l (Hagemeijer Rorive and Schoffeniels 1965 a) Binding of Cl and of smaller K components have also been reported (Friedman and Friedman 1967 Villamil *et al* 1968 Surprisingly high $[Cl^-]_i$ values have been given suggesting again that this ion is not passively distributed according to a Donnan equilibrium in vascular smooth muscle (Villamil *et al* 1968) The $[K^+]_i$ estimates are somewhat more uniform than in the case of sodium: 125 meq/l (Headings, Rondell and Bohr 1960), 114 meq/l (Hagemeijer Rorive and Schoffeniels 1965 a) 151 meq/l (Villamil *et al* 1968) 99 meq/l (Schoffeniels 1969)

Also for intestinal smooth muscle the ionic content does not seem to be distributed according to a simple two compartment model (Durbin and Jenkinson 1961 Durbin and Monson 1961 Goodford and Hermansen 1961 Freeman Narrod and Goodford 1962, Goodford 1962, 1964 1965 1966 1970 Buck and Goodford 1966, Brading, Bülbring and Tomita 1969 Brading and Jones 1969) The occurrence of bound fractions of both cations and anions have been established. For instance Goodford has demonstrated a competition between Na^+ K^+ Mg^{++} and Ca^{++} for superficial anionic charges, probably located at the cell membranes (Goodford 1965 1966, 1970) Besides these rapidly exchanging fractions of "counter cations" the occurrence of more firmly bound ions in the intestinal smooth muscle has been established (*e g* Goodford and

Hermansen 1961 Buck and Goodford 1966) As for vascular smooth muscle, the values reported by different authors for $[Na^+]_i$ and $[K^+]_i$ vary due to divergent interpretations of the magnitude of the sequestered fractions and of the amounts of the cellular fluid (Goodford and Hermansen 1961 Casteels 1969) Casteels and Kuriyama (1966) reported rather high values for $[Cl^-]_i$ in the taenia coli and concluded that Cl^- is not passively distributed according to a Donnan equilibrium in the smooth muscle. However in taenia coli preparations made quiescent by cooling, the distribution of chloride may be represented by such a passive equilibrium as pointed out by Buck and Goodford (1966) These observations indicate that the equilibrium potential for Cl^- ought to be compared to a mean potential during a period of spontaneous activity rather than to the highest polarization observed. Although such an analysis no doubt would cause the Cl^- distribution in smooth muscle to deviate less from a Donnan equilibrium, the findings made by Casteels and Kuriyama (1966) that $[Cl^-]_i$ is not increased on exposure of the tissue to high potassium concentrations until E_{Cl^-} is less positive than the membrane potential, suggest a more complex mechanism For instance, an active inward movement of Cl^- was proposed by Casteels and Kuriyama (1966) to explain the high values of $[Cl^-]_i$ in the normal state. The question whether Cl^- is passively distributed in smooth muscle is intimately connected to the estimate of $[Cl^-]_i$ which in turn is dependent on the evaluation of the magnitude of the bound chloride fraction Since the latter has not been as extensively studied as is the case for the cations, more information is required before it can be definitely decided whether a Donnan equilibrium prevails for Cl^-

It may thus be concluded that the ionic distribution in vascular as well as intestinal smooth muscle is best represented by a multicompartment model. When due attention is paid to the sequestered amounts of electrolytes the calculated values for the intracellular ionic concentrations seem to conform to the concept of an osmotic equilibrium across the cell membranes.

The importance of altered ionic concentration gradients for the muscle responses induced by osmolality variations.

Evidence has been presented above for a correlation between cell volume and degree of activity in vascular smooth muscle. The alterations of spontaneous contractile activity caused by variations in extracellular tonicity could be ascribed essentially to changes in the pattern of electrical

activity (I and III) Therefore, in looking for the underlying mechanisms behind these effects, attention should be paid to factors which regulate the electrical behaviour of the cell membranes.

As pointed out above the membrane potential of the portal vein is, in regard to ionic distribution, principally influenced by the equilibrium potential for potassium. However due to the relatively high permeability of smooth muscle to sodium (Kuriyama 1963) this ion will also influence the membrane potential. Furthermore, since the chloride distribution may not be passive in smooth muscle (Casteels 1965 1969 Casteels and Kuriyama 1966, V) this ion must be taken into account as well.

In a system where ions move across a membrane only under the influence of potential and concentration gradients, and where the voltage gradient through the membrane is constant, the "net" potential (E) caused by the combined influence of K^+ Na^+ and Cl^- is given by the "constant field equation" (Goldman 1943 Hodgkin and Katz 1949)

$$E = \frac{RT}{F} \ln \frac{[K^+]_i + \alpha[Na^+]_i + \beta[Cl^-]}{[K^+] + \alpha[Na^+] + \beta[Cl^-]_i}$$

α and β denote the relative permeabilities for Na^+ and Cl^- compared to that for K^+ taken as unity Thus, if the sodium pump of smooth muscle cells was electrically neutral, *i. e.* if it transferred Na^+ outwards and K^+ inwards in a one to one fashion, the membrane potential of the portal vein would be given by this formula. However evidence has been presented for an electrogenic function of the Na pump in smooth muscle, at least under some circumstances, implying that it extrudes a net amount of positive charges and thus contributes to the membrane potential directly (Casteels, Hendrickx and Droogmans 1969 Taylor Paton and Daniel 1969) Furthermore, increased activity of such an electrogenic pump has been proposed to explain the hyperpolarization of intestinal smooth muscle induced by adrenaline (Burnstock 1958) If so the actual membrane potential would be determined by some combination of "passive" diffusion potential and the rate of "active net transfer of positive charges out from the cells.

Casteels (1969) obtained a value for the membrane potential of —37 mV in the taenia coli by inserting into the constant field equation the ionic concentrations and the permeability constants for the ions deduced from estimated fluxes of ^{42}K, ^{24}Na and ^{36}Cl. This value is approximately 20 mV lower than that measured by the microelectrode technique. The difference was ascribed either to the presence of a partly electrogenic sodium pump or to "single file diffusion" of K^+ as was suggested to occur in giant axons from *Sepia officinalis* by Hodgkin and Keynes (1955)

Such single file diffusion of K^+ would lead to an underestimation of the permeability constant for this ion.

In spite of the possibility of an electrogenic Na pump and of the lack of information concerning the voltage profile in the cell membranes of smooth muscle, the "constant field" equation might still be used as a starting point for the discussion of osmolality-induced effects on the electrical activity

A shrinkage of the muscle cells caused by increased extracellular tonicity should result in raised intracellular ionic concentrations. The question arises whether the changed transmembrane ionic gradients are the cause of the inhibited spontaneous activity. According to the constant field equation, an increase of $[K^+]_i$ and $[Na^+]_i$ should lead to hyperpolarization, and an increase of $[Cl^-]_i$ to depolarization. Since the electrical activity of the portal vein is relatively slightly influenced by changes in the $[K^+]_i$ to $[Na^+]_i$ ratio when induced by changes in $[K^+]_i$ (Axelsson *et al* 1967 I and III) and since a raised $[Cl^-]_i$ would tend to cause excitation, it is probably sufficient to consider the potassium ion alone in this particular context. The question can then be simplified as to whether the increased $[K^+]_i$ to $[K^+]_o$ ratio can entirely explain the suppressed spontaneous activity in hypertonic media. Brading and Sneddon (1968) measured the changes in the ionic content of taenia coli on alterations in osmolality and concluded that the hyperpolarization of 10—15 mV recorded by Tomita (1966) on exposure to two times the normal tonicity could be fully explained by the concomitant increase in $[K^+]_o$. Furthermore, for striated muscle, Adrian (1956) reported that the membrane potential was altered to an extent that could be expected for a potassium electrode when the extracellular tonicity was raised. Direct measurements of the resting membrane potential have not been carried out in the present series of studies and the following discussion of the importance of the potassium gradient is therefore based on observations concerning the pattern of phasic electrical activity. The discussion thus rests on the assumption that the relationship between the level of the membrane potential and the spontaneous burst activity is not materially affected by osmolality.

In I, III and in a preceding article (Mellander *et al* 1967) the role played by an altered $[K^+]_i$ to $[K^+]_o$ ratio in the activity responses of portal veins as a result of variations in osmolality was elucidated by making combined changes in $[K^+]_o$ and in tonicity. The purpose of this procedure was to investigate whether a normalization of the $[K^+]_i$ to $[K^+]_o$ ratio could outbalance the effects of the anisosmotic media on the spontaneous activity. If so, the K^+ concentration gradient

adequately and completely account for the relationship between cell volume and degree of activity It could be clearly demonstrated, however that such a simple relationship does not prevail, at least not at the normal $[K^+]$ level. For instance, $[K^+]$ had to be raised from the normal value 5 9 meq/l by two to three times to compensate for the inhibitory effect exerted by a 30 per cent tonicity increase (I) In this case a $[K^+]$ rise to about 8 meq/l would have been sufficient to normalize the transmembrane concentration gradient. Since a far more pronounced increment was required to restore normal activity this strongly suggests the involvement of additional mechanisms. A similar conclusion was drawn in III concerning the excitation induced by hypo-osmolality at the normal range of potassium concentrations. In fact, here it was even more obvious that the excitation could not be solely ascribed to an altered $[K^+]_i$ to $[K^+]$ ratio. For instance, the response to a tonicity reduction of 55 mOsm/kg was stronger than that caused by a fourfold $[K^+]$ increment. As judged by the changed amount of cell water obtained on exposure to such a lowered tonicity the $[K^+]_i$ to $[K^+]$ ratio would decrease only some 10—20 per cent (IV VI)

However at higher levels of extracellular potassium ion concentrations, the effects of anisosmotic media are cancelled by roughly proportional alterations in $[K^+]$ (I III) The inhibitory effect induced by the addition of 100 mmoles sucrose/l to a medium containing 24 meq K^+/l could then be completely eliminated by an increment of the latter constituent to 30—36 meq/l. Thus, in this case, a tonicity rise of 30 per cent could be completely compensated by a corresponding increase of $[K^+]_o$, just as would be expected for a potassium electrode exhibiting a perfect osmotic behaviour

A similar increase in the importance of the potassium concentration gradient for the responses to lowered osmolality was demonstrated in III The reason for this shift in the relation between the effects of altered $[K^+]$ and changed osmolality when the potassium concentration in the control situations is high, may be the fact that the membrane potential of smooth muscle cells is more influenced by the $[K^+]_i$ to $[K^+]$ ratio at higher levels of $[K^+]$ (Holman 1958 Marshall 1962, Axelsson *et al.* 1967) It has been proposed that this "potassium electrode — like behaviour" of smooth muscle in K rich media is due to a relatively higher membrane permeability for K under these circumstances.

It can thus be concluded that the concomitant changes in the ratio of $[K^+]_i$ to $[K^+]$ on alterations in cell volume, can fully explain the activity responses when $[K^+]$ is initially supranormal, but that some

additional mechanism must contribute to the events at the normal range of $[K^+]_o$ which, of course, is most relevant for the *in vivo* situation. According to the constant field equation, not only changes in the ionic concentration gradients but also alterations in ionic membrane permeability can influence the membrane potential The question now arises as to whether such alterations occur upon osmotically induced shrinkage or swelling of the smooth muscle cells. These problems were investigated in VI and VII and are discussed below

Permeability properties of smooth muscle cell membranes at different osmolalities

As discussed above the changes in portal vein activity produced by alterations in osmolality could not be solely ascribed to the concomitant variations in transmembrane ionic concentration gradients. An additional factor might be variations in the passive permeability properties of the cell membranes associated with alterations in the cell volume. For instance, it seems possible that stretching of the cell membranes in connection with osmotic swelling, might lead to a thinning of the lipoid membrane structures, or to widening of the aqueous pathways ("the pores") Furthermore, it is conceivable that such modifications in the membrane structures might facilitate the passage of a larger penetrating ion (*e g* the hydrated Na^+) relatively more than that of a smaller one (*e g* K^+) If so, the ratio P_{Na}/P_K would increase during osmotically induced swelling of the muscle cells, an adjustment which would lead to depolarization according to the "constant field equation" The hyperpolarization caused by raised tonicity may also be explained along these lines.

The possibility of such alterations in permeability upon swelling and shrinkage of the smooth muscle cells was examined in VI and VII. In VI some general aspects of membrane permeability were considered by investigating the effects of tonicity on the exchange kinetics of two non-electrolytes, urea and erythritol. In VII the specific permeabilities for Na^+ and K^+ under different environmental conditions were examined. The methods used involved "loading" of the portal veins in radioactive medium until the actual tracer substance had equilibrated in the tissue, followed by examination of the decay in tissue radioactivity during exposure to a non-active medium. The washout curves were analysed with the purpose of identifying a monoexponential term that could be considered to represent the transmembrane exchange. It was then assumed that the cell membranes exert a rate-limiting barrier function which

predominantly determines the permeability for the different penetrating substances. This view is the most widely accepted one although some authors consider the permeability characteristics of a cell as a property of the cytoplasm rather than of the membranes. Such approaches are represented by "the sorptional theory of cell permeability" (Troshin 1966), referred to above and Ling's "association-induction hypothesis" discussed for smooth muscle by Jones (1970). According to the latter approach, cellular ions are either associated with polarized groups of the proteins (fixed charges, mainly negative) or dissolved in the *interstitial* water. The association of ions to the fixed charges is selective and metabolically dependent. The exchange kinetics of the dissolved ions are those of bulk diffusion, exhibiting little temperature dependence in contrast to the "adsorption-desorption" mechanism governing the exchange of the "associated" ions. The efflux of Na from the cells is considered to be governed mainly by bulk diffusion processes and that of K by the adsorption-desorption kinetics. In accordance with this hypothesis, Brading and Jones (1969) reported that 98—99 per cent of the ^{24}Na efflux from taenia coli can be described by bulk diffusion, thus indicating that the cell membranes do not exert a barrier function with respect to this ion.

However other authors have found that the component in the ^{24}Na washout curve considered to represent transmembrane efflux in taenia coli (Buck and Goodford 1966) and in arterial smooth muscle (Garrahan, Villamil and Zadunaisky 1965, Hagemeijer, Ronive and Schoffeniels 1965 b) is, in fact, quite sensitive to alterations in temperature and to ouabain. With regard to the washout curves of ^{14}C urea and ^{14}C erythritol from the portal vein (VI) the data could after some min be fitted by simple exponentials whose intercepts with the ordinate corresponded to spaces which agreed fairly well with the volume of intracellular water as calculated from determinations of total water and extracellular (^{14}C sucrose) space. These different results, obtained with various kinds of smooth muscle seem to support the concept of cell membranes as rate-limiting barriers and to justify the application of the membrane model in the interpretation of the results in VI and VII.

The results obtained in the experiments where the efflux of ^{14}C urea and ^{14}C erythritol were studied did not indicate any change in the permeability constants for these non-electrolytes in connection with increases or moderate decreases in extracellular tonicity. However at more pronounced reductions (—109 mOsm/kg) in osmolality a clear-cut increase in the permeability for both substances was observed. The fact that the P value for the smallest and most lipid soluble molecule of the two, i.e.

urea, increased more than that for erythritol, suggests that the reduction in the barrier function of the cell membranes was not due to widening of water filled pores since such an alteration would probably favour the larger and more water-soluble molecule. Some other mechanism, for instance thinning of the lipoid membrane, may therefore be considered.

The question as to whether the permeability coefficients for sodium and potassium (P_{Na} and P_K, respectively) are differently influenced by anisosmotic media, was elucidated by studying the fluxes of ^{24}Na and ^{42}K (VII) The washout of ^{42}K in normal Krebs solution at 37°C could, after the first few min, be described by a simple exponential, the ordinate intercept of which corresponded to a value for $[K^+]_i$ that agreed satisfactorily with that calculated by the "analytical" method in V Since the exchange of ^{24}Na at 37°C was too rapid to permit detailed compartmental analysis, the comparative study concerning tonicity effects on P_K and P_{Na} was performed at 15 C. At this low temperature the decline in tissue ^{42}K could not be strictly fitted by a single exponential term until 40—60 min had elapsed, probably due to a reduction in P_K at prolonged exposure to 15 C. However the earlier part of the washout curve must also to a great extent represent efflux of cellular potassium Further the decline in ^{42}K activity over the 7—30 min washout interval was essentially monoexponential and since this particular time interval represented transmembrane efflux of ^{24}Na in the sodium experiments, it was considered most appropriate to use this period for calculating cellular efflux of potassium as well With this approach the P_K and P_{Na} values would be estimated under quite comparable conditions as regards time of exposure to the anisosmotic media and to 15°C. With the assumption that the membrane potential under these circumstances averages 40 mV in normal Krebs solution, the calculated value for P_K amounts to 5.3 10^{-8} cm/sec, which is in fairly good agreement with that reported by Casteels (1969) for the taenia coli.

Since a considerable fraction of the Na content in the portal vein prevails in a bound, osmotically inactive form (V), ^{24}Na washout experiments were not only performed with intact portal veins but also with the damaged "membrane-deprived" tissues, described above. This approach made it possible to distinguish that component of the ^{24}Na washout curve of normal tissues which represented exchange of $[Na^+]_i$ from that representing washout of sequestered Na. This method gave a value for the transmembrane Na efflux at 15 C of 1.2 10^{-11} M/cm² sec. However due to the action of the postulated Na K pump the passive permeability for sodium should be investigated by studying the kinetics of the

uptake which is considered to be a solely passive process. Yet, since the total exchangeable Na amount remained essentially constant during the washout period, influx and efflux of Na^+ should equal each other in the experimental situation. The value given above for the transmembrane sodium efflux may therefore be used to estimate P_x and a figure of 4.3 10^{-8} cm/sec is then obtained. A P_{x_a}/P_k ratio of 0.081 would then prevail in portal vein, assuming a membrane potential of 40 mV at 15°C.

Similar determinations were made in hyper- and hypo-osmotic media, but since the change in membrane potential induced by these alterations in the environment was not known, no definite values for the actual permeability coefficients could be calculated. Instead, a set of P_K and P_x values were deduced for different possible levels of membrane potential. On the basis of these data it was concluded that the ratio P_{x_a}/P_K increases in connection with osmotically induced swelling of the smooth muscle cells. This effect can, according to the discussion above, contribute to the depolarization caused by hypo-osmolality and thus help to explain the strong excitation seen under such conditions. Concerning the effects of raised osmolality no evidence for changes in either P_K or P_x was obtained. It should, however, in this context be stressed that determination of permeability constants in smooth muscles in general is technically exceedingly difficult for several reasons, implying that given values must always be judged with caution.

The hypothesis that an increase in the P_{x_a}/P_K ratio contributes to the excitation induced by exposure to hypotonicity is supported by some observations made in III. The marked increase in activity evoked by a reduction in osmolality by approximately 60 mOsm/kg was almost completely abolished when the NaCl concentration of the hypo-osmotic solution was reduced from 92 to 32 mmoles/l. This inability of hypotonicity to cause excitation in NaCl-deficient medium was shown to be due to the low $[Na^+]$ and not to the reduced $[Cl^-]_o$. Moreover, no similar "anti-hypo-osmolality" effect of reduced $[K^+]$ could be demonstrated. These results suggest that the sodium ion is of particular importance for the response elicited by reduced tonicity, just as would be expected on the basis of the above hypothesis for its underlying mechanism.

Since no convincing evidence could be obtained for any alterations in the P_x to P_k ratio upon cell shrinkage and since the subsequent changes in the $[K^+]_i$ to $[K^+]_o$ ratio apparently cannot alone explain the inhibition of activity, some additional mechanism must be sought. In this context the observation made by Sperelakis and Schneider (1968) that the Cl^- conductance/K conductance ratio of the frog sartorius muscle

decreases on exposure to hyperosmotic media is of interest. If this also *holds true for the portal vein it offers a possible explanation for the* hyperpolarization caused by raised tonicity since the equilibrium potential for chloride seems to be much more positive than the membrane potential (V) Another possibility might be tonicity effects on an electrogenic sodium pump.

General Discussion

Since vascular smooth muscle presents marked individualities with regard to its physiological characteristics depending on its localization in the vascular tree (*e g* Bohr 1965) it is important to consider to what extent the present results with the portal vein are relevant for other types of vascular smooth muscle. Unfortunately there seems to be very little information concerning the effects of osmolality on preparations from other vessels. However in a study on strips of canine aorta, Yamabayashi and Hamilton (1959) reported the occurrence of phasic spontaneous contractions in this normally quiescent preparation when the tonicity of the incubation medium was increased by adding NaCl or sucrose. Furthermore, the amplitude of these contractions rose with the degree of hypertonicity In accordance with these observations, Somlyo and Somlyo (1970) found that rabbit and canine pulmonary arteries are contracted by hyperosmolality They conclude that these findings on different types of "Windkessel" vessels, where the smooth muscle exhibits essentially "multi-unit" behaviour indicate that the portal vein responses to tonicity variations may not be representative for the osmometric properties of *all* types of vascular *smooth muscle*

However there is much experimental evidence indicating that the functionally more important resistance vessels in many systemic circuits behave like the portal vein with respect to artificially induced osmolality variations. For instance, close intra arterial injections or infusions of hypertonic solutions of NaCl or of different nonelectrolytes, cause increased regional blood flow in dog limb (Marshall and Shepherd 1959 Read *et al* 1960 Overbeck, Molnar and Haddy 1961 Haddy and Scott 1965) cat skeletal muscle (Mellander *et al* 1967) dog skeletal muscle (Stainsby and Fregly 1968) dog myocardium (Read *et al* 1960) dog brain (Buyniski and Rapela 1968) dog kidney (Lucas and Read 1966, Gazitua *et al* 1969) and human forearm (Lundvall, Mellander and White 1969) Analysis of the underlying mechanisms indicate that the hyperemia caused by hyperosmolality is neither due to any passive widening of the vessels as a result of dehydration of the vascular walls, nor due to an altered blood viscosity but is caused by a locally elicited inhibition of vascular smooth muscle activity (Mellander *et al.* 1967)

Furthermore, arterial infusions of hypotonic solutions lead to *increases* of flow resistance in the dog forelimb (Overbeck, Molnar and Haddy 1961), dog kidney (Gazitua *et al.* 1969), dog skeletal muscle (Stainsby and Fregly 1968) as well as the human forearm (Overbeck, Daugherty and Haddy 1966) The mechanism behind these latter effects has not been studied as extensively as in the case of hyperosmolality

The present *in vitro* results concerning osmotic effects on myogenic activity in single unit vascular smooth muscle have quite recently been supplemented by an interesting *in vivo* study concerning the effects of close arterial infusions of hypo- iso- and hyperosmotic solutions of NaCl, urea or sucrose on the vascular resistance of the dog kidney (Gazitua *et al* 1969) These investigators found that hypertonic sucrose solutions elicited sustained vasodilatation whereas urea and NaCl only brought about a transient blood flow increase. When the infusion of urea and NaCl was stopped, a shortlasting pronounced rise in vascular resistance was obtained Similar characteristics in the vascular responses to hyperosmotic urea were found also in the dog forelimb. Furthermore, infusions of hypo-osmotic NaCl, sucrose and urea or of iso-osmotic urea caused an increase in renal vascular resistance while no such reaction was induced by iso-osmotic sucrose solutions. These results obtained with the blood perfused dog kidney can be fully explained, as pointed out by the authors, if it is assumed that the vascular smooth muscle of the renal resistance vessels exhibit osmometric properties similar to those shown by the isolated rat portal vein.

However this pattern of response to osmolality variations is apparently not typical for *all* kinds of vascular smooth muscles or vascular regions as pointed out earlier in this chapter For example, in the pulmonary circuit, blood flow *decreases* as a response to strongly hypertonic solutions (Binet and Burstein 1951 Eliakim, Rosenberg and Braun 1958 Semler Shepherd and Swan 1959 Read *et al* 1960 Eliakim, Stern and Nathan 1961 Braun 1966) by many of the investigators proposed to be due to an osmotically induced *vasoconstriction*. Semler Shepherd and Swan (1959) and Read *et al* (1960) on the other hand, suggested that it is caused by a transient red cell agglutination, because hypertonic injections did not increase pulmonary flow resistance when the isolated dog lung was perfused with dextran instead of blood. Further evidence for the occurrence of erythrocyte agglutination as a result of hyperosmolality was obtained in studies using vital microscopy (Read *et al* 1960). Different opinions prevail concerning the responses to hypertonicity of the pulmonary vascular bed when the role played by sludging of the blood is

eliminated. According to some early reports (Semler Shepherd and Swan 1959 Read *et al.* 1960 Haddy and Scott 1965) no vasodilatation ensues while later studies have indicated a pattern of response that closely resembles that exhibited by the systemic circuits (Bo and Hauge 1969) It should in this context be stressed that the pulmonary precapillary vessels react in the *opposite* direction to the systemic ones to a whole series of vasoactive agents (*e g* Hauge, Lunde and Waaler 1966)

The importance of osmolality variations as a factor in the physiological regulation of vascular tone in skeletal muscle was suggested by Mellander in 1966 (see Mellander 1968) and has been extensively studied by his group (Mellander *et al.* 1967 Gray Lundvall and Mellander 1968 Lundvall 1969 Lundvall Mellander and White 1969 Mellander and Lundvall 1970) They have demonstrated that the osmolality of the regional venous blood increases in direct proportion to the intensity of the work performed and can reach a value of 40 mOsm/kg above control during heavy exercise. When comparable degrees of hyperosmolality are induced by intra arterial infusions of concentrated solutions, a pattern of response within the various series-coupled sections of the vascular bed is elicited which in all respects, resembles that observed during muscle exercise. Furthermore, interstitial deposits of ^{133}Xe in resting muscle are cleared at faster rates when the tracer is dissolved in a hypertonic instead of an iso-tonic medium These results indicate that experimental hyperosmolality which primarily involves the extravascular space is also able to elicit vasodilatation It appears clear from these studies by Mellander *et al* that the exercise hyperemia in skeletal muscle in cat as well as in man is, at least partly caused by an increased release of osmotically active particles. It is, of course, possible that a similar blood flow regulating mechanism exists also in other metabolically active tissues.

It is conceivable that increased tissue osmolality and hence local vasodilatation, can result also from other processes than release of metabolites. For instance, events like resorption and secretion in an organ may obviously be accompanied by alterations in the regional osmolality Such a mechanism is indeed suggested by the results of van Heerden Wagner and Kaihara (1968) upon studying the effects on jejunal blood flow of intraintestinal administrations of concentrated glucose solutions. They demonstrated that perfusion of one part of the jejunum with a hypertonic solution caused vasodilatation in that particular intestinal section while blood flow in adjacent unperfused segments was unaffected. Furthermore, it has been suggested that the systemic vasodilatation seen in the dumping syndrome of patients after gastric resection is due to a raised osmotic

pressure of the blood following absorption of materials that are more concentrated than when a normal pyloric function is present (Lucas and Read 1966) These authors provoked a typical dumping syndrome in dog and man by intraduodenal administration of 50 per cent glucose as previously described by Machella (1949) The time course of the general vasodilatation elicited in this way turned out to be strictly correlated to the concomitant increase in blood osmolality Moreover intravenous administration of hypertonic glucose elicited a similar type of vascular response, indicating that the vasodilatory stimulus is not of intestinal origin but the result of blood hypertonicity It is further interesting to consider what happen to the intestinal circulation on drinking large amounts of water To the knowledge of the author this interesting topic has not yet been subjected to experimental study

Angiography in clinical practice often implies infusion of strongly hypertonic solutions. Giammona, Lurie and Segar (1963) reported that plasma osmolality could rise as much as 10 per cent in children with a variety of cardiac abnormalities undergoing selective angiocardiography The sensation of warmth that patients often feel at angiographic examination has been ascribed to a peripheral vasodilatation (Deterling 1952) which apparently might be due to the subsequent raise in blood tonicity In this context it may be of interest to note that repeated intravenous injections of hypertonic solutions have been reported to give successful results in the treatment of *e g* thromboangiitis obliterans (Demel and Sgalitzer 1933 Silbert 1935)

Another aspect of the present results that might have clinical connection concerns the effects of lowered osmolality on the portal vein. It has been claimed that, in hypertensive disease, the arterial walls contain more water than under normal conditions. These observations were originally made by Tobian and Binion (1952) on renal arteries from patients with essential hypertension Such waterlogging of the arterial wall would, as these authors pointed out, lead to a passive increase in the peripheral resistance. Furthermore, if the extra water was located intracellularly and thus the vascular smooth muscle cells were swelled, the active tone might also be increased according to the results presented in I and III Since these original findings of Tobian and Binion were published, several studies have been performed on water content and ionic distribution in arteries from animals with experimental hypertension (for ref see Schoffenels 1969) Most authors seem to agree that hypertension is accompanied by a rise in the sodium content of the arterial wall However a great discrepancy prevails in the opinions concerning the exact localization of

these extra amounts of sodium in the tissue. Redleaf and Tobian (1958) concluded that $[Na^+]_i$ is increased in the hypertensive state. They based this conclusion on the fact that the ratio of Na to Cl was above normal and on the assumption that Cl^- is mainly located extracellularly. Such a shift in the Na distribution might lead to accumulation of water inside the cell which, in turn, should stimulate the spontaneous activity as outlined above. In contrast to this, some authors claim that the excess sodium found in arteries from hypertensive animals is bound extracellularly and then the muscle cells would not swell (Friedman and Friedman 1967, Schoffeniels 1969). This hypothesis is supported by the observation made by the former authors that even in the absence of functional cellular membranes, the contents of cations is higher in aortae isolated from hypertensive animals than in normal aortae. Thus, the question concerning the relations that may exist between the excess of vascular sodium and the hypertensive condition are, at present, far from settled.

In summary, the activity responses of the portal vein on variations in the osmolality studied in I—III are similar to those of the resistance vessels in many different vascular regions. It therefore seems reasonable that the results of the examinations concerning the underlying mechanisms, summarized in the preceding sections, are relevant also for the latter kinds of vessels. Since alterations in the regional tonicity, as discussed above, occur under physiological conditions it is conceivable that the demonstrated relation between volume and degree of activity in "single unit" vascular smooth muscle cells constitutes an important local blood flow regulating mechanism, also in other situations than muscle exercise.

Summary

A study has been performed concerning the effects of altered extracellular osmolality on the spontaneously active, isolated rat portal vein. This vascular smooth muscle preparation has functional characteristics that, in many aspects, resemble those of the systemic resistance vessels. The results and conclusions can be summarized as follows

1 Evidence has been obtained for a correlation between muscle cell volume and degree of activity swelling being associated with muscle excitation and shrinkage with inhibition of the spontaneous electrical and mechanical activity Thus, incubation of the portal vein in hypo-osmotic media greatly enhances the spontaneous activity In contrast, exposure to a hyperosmotic environment, induced by supplementing the normal Krebs solution with sucrose, which does not penetrate the cell membranes, leads to a *sustained* inhibition of the spontaneous activity When, on the other hand, hyperosmolality is created by addition of urea, which penetrates the cell membranes at a moderate rate and hence equilibrates in the total tissue water only a *transient* inhibition is obtained with rapid return to control activity despite the continued presence of urea. Normalization of the osmolality after urea exposure is associated with a transient excitation whose intensity is closely related to the duration of the previous period of urea hyperosmolality up to the point when ^{14}C urea reaches its plateau value in the uptake curve.

Determinations of the cell water content at different tonicities indicate that the muscle cells are, in fact, continuously shrunken in hyperosmotic sucrose solutions and swelled in hypo-osmotic media. In contrast, exposure to a membrane penetrating substance like urea, apparently leads to only a transient shrinkage and, on return to the control solution to a shortlasting swelling

An analysis of the mechanisms responsible for this correlation between cell olume and degree of cell activity requires quantitative information concerning the osmometric behaviour of the cells and the ionic distribution in the portal vein.

2. When the osmolality is raised by addition of sucrose to the incubation medium, the muscle cells shrink to an extent expected for perfect osmo-

meters. The alterations in cell water content upon exposure to hypo-osmolality are, however more complex since the cell swelling is to some extent hampered by the concomitantly enhanced active tension development of the muscle cells. If this activity is suppressed by cooling the cells behave like perfect osmometers up to a 20 per cent reduction in osmolality but not at still more drastic levels of hypo-osmolality It is, however even in this latter situation possible that the muscle cells approach an osmotic equilibrium with the medium, since a loss of intracellular ions occurs under these circumstances.

3 Concerning the ionic distribution in the portal vein, a simple two compartment model cannot be applied for either sodium, potassium or chloride, since there exists bound osmotically inactive fractions of the electrolytes. As in other types of excitable tissues, $[K^+]_i$ is higher than $[Na^+]_i$ The membrane potential is predominantly determined by E_{K^+} but also influenced to an appreciable extent by E_{X^+} The distribution of Cl^- does not seem to conform to a passive Donnan equilibrium.

4 The possibility that the relation between muscle cell volume and degree of activity is mediated by variations in the transmembrane ionic concentration gradients has been elucidated by making simultaneous alterations in tonicity and in the extracellular electrolyte concentrations. The results indicate that, in the *normal* range of $[K^+]_o$, a restoration of the original ionic concentration gradients is not alone sufficient to cancel the osmolality effects on the spontaneous muscle activity However at supranormal levels of $[K^+]_o$, alterations in tonicity and thus in the intracellular electrolyte concentrations, seem to be quantitatively more comparable to changes in the extracellular ionic composition.

5 Studies of the exchange kinetics of ^{22}Na and ^{42}K across the cell membranes at different osmolalities suggest that the permeability constant for sodium increases more than that for potassium during hypo-osmolality Such an effect of swelling on the membrane permeability may help to explain the subsequent depolarization and increase of phasic activity During cell shrinkage, on the other hand, there was no evidence of any change in the ratio P_X to P_K and alternative mechanisms must therefore be considered to explain the inhibitory responses.

6. The application of the present *in vitro* results, concerning osmotic effects on the active tension development of isolated vascular smooth muscle of the "single unit" type to the *in vivo* situation is discussed from physiological and pathophysiological points of view

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uptake which is considered to be a solely passive process. Yet, since the total exchangeable Na amount remained essentially constant during the washout period, influx and efflux of Na^+ should equal each other in the experimental situation. The value given above for the transmembrane sodium efflux may therefore be used to estimate P_X and a figure of 4.3 10^{-9} cm/sec is then obtained. A P_{X_a}/P_K ratio of 0.081 would then prevail in portal vein, assuming a membrane potential of 40 mV at 15°C.

Similar determinations were made in hyper- and hypo-osmotic media, but since the change in membrane potential induced by these alterations in the environment was not known, no definite values for the actual permeability coefficients could be calculated. Instead, a set of P_K and P_X values were deduced for different possible levels of membrane potential. On the basis of these data it was concluded that the ratio P_{X_a}/P_K increases in connection with osmotically induced swelling of the smooth muscle cells. This effect can according to the discussion above, contribute to the depolarization caused by hypo-osmolality and thus help to explain the strong excitation seen under such conditions. Concerning the effects of raised osmolality no evidence for changes in either P_K or P_X was obtained. It should however in this context be stressed that determination of permeability constants in smooth muscles in general is technically exceedingly difficult for several reasons, implying that given values must always be judged with caution.

The hypothesis that an increase in the P_{X_a}/P_K ratio contributes to the excitation induced by exposure to hypotonicity is supported by some observations made in III. The marked increase in activity evoked by a reduction in osmolality by approximately 60 mOsm/kg was almost completely abolished when the NaCl concentration of the hypo-osmotic solution was reduced from 92 to 32 mmoles/l. This inability of hypotonicity to cause excitation in NaCl-deficient medium was shown to be due to the low $[Na^+]$ and not to the reduced $[Cl^-]_o$. Moreover no similar anti-hypo-osmolality" effect of reduced $[K^+]$ could be demonstrated. These results suggest that the sodium ion is of particular importance for the response elicited by reduced tonicity just as would be expected on the basis of the above hypothesis for its underlying mechanism.

Since no convincing evidence could be obtained for any alterations in the P_{X_a} to P_K ratio upon cell shrinkage and since the subsequent changes in the $[K^+]_i$ to $[K^+]_o$ ratio apparently cannot alone explain the inhibition of activity some additional mechanism must be sought. In this context the observation made by Sperelakis and Schneider (1968) that the Cl^- conductance/K^+ conductance ratio of the frog sartorius muscle

decreases on exposure to hyperosmotic media is of interest. If this also holds true for the portal vein, it offers a possible explanation for the hyperpolarization caused by raised tonicity since the equilibrium potential for chloride seems to be much more positive than the membrane potential (V). Another possibility might be tonicity effects on an electrogenic sodium pump.

sites. They concluded that these sites were equilibrated with the extracellular sodium-rich medium and that therefore, in the normal state, only Na binding occurred. It was proposed that this binding occurred in the mucopolysaccharides of the ground substance.

The assumption that the sequestered Na was located extracellularly was supported by the results of Garrahan Villamil and Zadunaisky (1965) who studied washout of ^{22}Na from dog carotid artery They found that the first, rapid component in the curve representing the decline in remaining activity contained considerably more Na than could be extracellularly dissolved and concluded that the excess represented bound Na. Analyses performed later have indicated the occurrence of four or five different Na compartments in arterial wall (Friedman and Friedman 1967 Friedman *et al* 1968 Friedman, Gustafson and Friedman 1968 Rorive 1969) of which two to three represent sequestered Na. The opinions concerning their magnitude and characteristics do however vary considerably as reflected in the scatter of the values for $[Na^+]_i$ reported by different authors 7 mmoles/l (Keatinge 1968) 20—30 mmoles/l (calculated from Friedman *et al* 1968) 44 mmoles/l (Schoffeniels 1969) 50 mmoles/l (Garrahan, Villamil and Zadunaisky 1965) 84 mmoles/l (Hagemeijer Rorive and Schoffeniels 1965 a) Binding of Cl and of smaller K components have also been reported (Friedman and Friedman 1967 Villamil *et al* 1968 Surprisingly high $[Cl^-]_i$ values have been given suggesting again that this ion is not passively distributed according to a Donnan equilibrium in vascular smooth muscle (Villamil *et al.* 1968) The $[K^+]_i$ estimates are somewhat more uniform than in the case of sodium 125 meq/l (Headings, Rondell and Bohr 1960) 114 meq/l (Hagemeijer Rorive and Schoffeniels 1965 a) 151 meq/l (Villamil *et al.* 1968) 99 meq/l (Schoffeniels 1969)

Also for intestinal smooth muscle the ionic content does not seem to be distributed according to a simple two compartment model (Durbin and Jenkinson 1961 Durbin and Monson 1961 Goodford and Hermansen 1961 Freeman Narrod and Goodford 1962, Goodford 1962, 1964 1965 1966 1970 Buck and Goodford 1966 Brading, Bülbring and Tomita 1969 Brading and Jones 1969) The occurrence of bound fractions of both cations and anions have been established For instance, Goodford has demonstrated a competition between Na^+ K^+ Mg^{++} and Ca for superficial anionic charges, probably located at the cell membranes (Goodford 1965 1966 1970) Besides these rapidly exchanging fractions of "counter cations" the occurrence of more firmly bound ions in the intestinal smooth muscle has been established (*e g* Goodford and

Hermansen 1961 Buck and Goodford 1966) As for vascular smooth muscle, the values reported by different authors for $[Na^+]_i$ and $[K^+]_i$ vary due to divergent interpretations of the magnitude of the sequestered fractions and of the amounts of the cellular fluid (Goodford and Hermansen 1961 Casteels 1969) Casteels and Kuriyama (1966) reported rather high values for $[Cl^-]_i$ in the taenia coli and concluded that Cl^- is not passively distributed according to a Donnan equilibrium in the smooth muscle. However in taenia coli preparations made quiescent by cooling, the distribution of chloride may be represented by such a passive equilibrium as pointed out by Buck and Goodford (1966) These observations indicate that the equilibrium potential for Cl^- ought to be compared to a mean potential during a period of spontaneous activity rather than to the highest polarization observed. Although such an analysis no doubt would cause the Cl^- distribution in smooth muscle to deviate less from a Donnan equilibrium, the findings made by Casteels and Kuriyama (1966) that $[Cl^-]_i$ is not increased on exposure of the tissue to high potassium concentrations until E_{Cl^-} is less positive than the membrane potential suggest a more complex mechanism For instance, an active inward movement of Cl^- was proposed by Casteels and Kuriyama (1966) to explain the high values of $[Cl^-]_i$ in the normal state. The question whether Cl^- is passively distributed in smooth muscle is intimately connected to the estimate of $[Cl^-]_i$ which in turn is dependent on the evaluation of the magnitude of the bound chloride fraction. Since the latter has not been as extensively studied as is the case for the cations, more information is required before it can be definitely decided whether a Donnan equilibrium prevails for Cl^-

It may thus be concluded that the ionic distribution in vascular as well as intestinal smooth muscle is best represented by a multicompartment model When due attention is paid to the sequestered amounts of electrolytes the calculated values for the intracellular ionic concentrations seem to conform to the concept of an osmotic equilibrium across the cell membranes.

The importance of altered ionic concentration gradients for the muscle responses induced by osmolality variations.

Evidence has been presented above for a correlation between cell volume and degree of activity in vascular smooth muscle. The alterations of spontaneous contractile activity caused by variations in extracellular tonicity could be ascribed essentially to changes in the pattern of electrical

activity (I and III) Therefore, in looking for the underlying mechanisms behind these effects, attention should be paid to factors which regulate the electrical behaviour of the cell membranes.

As pointed out above the membrane potential of the portal vein is, in regard to ionic distribution principally influenced by the equilibrium potential for potassium. However due to the relatively high permeability of smooth muscle to sodium, (Kuriyama 1963) this ion will also influence the membrane potential Furthermore, since the chloride distribution may not be passive in smooth muscle (Casteels 1965 1969 Casteels and Kuriyama 1966 V) this ion must be taken into account as well.

In a system where ions move across a membrane only under the influence of potential and concentration gradients, and where the voltage gradient through the membrane is constant, the "net" potential (E), caused by the combined influence of K^+ Na^+ and Cl^- is given by the "constant field equation" (Goldman 1943 Hodgkin and Katz 1949)

$$E = \frac{RT}{F} \ln \frac{[K^+]_i + \alpha[Na^+]_i + \beta[Cl^-]}{[K^+] + \alpha[Na^+] + \beta[Cl^-]_i}$$

α and β denote the relative permeabilities for Na^+ and Cl^- compared to that for K taken as unity Thus, if the sodium pump of smooth muscle cells was electrically neutral, *i e* if it transferred Na^+ outwards and K inwards in a one to one fashion, the membrane potential of the portal vein would be given by this formula. However evidence has been presented for an electrogenic function of the Na pump in smooth muscle at least under some circumstances, implying that it extrudes a net amount of positive charges and thus contributes to the membrane potential directly (Casteels, Hendrickx and Droogmans 1969 Taylor Paton and Daniel 1969) Furthermore increased activity of such an electrogenic pump has been proposed to explain the hyperpolarization of intestinal smooth muscle induced by adrenaline (Burnstock 1958) If so the actual membrane potential would be determined by some combination of "passive" diffusion potential and the rate of "active net transfer of positive charges out from the cells.

Casteels (1969) obtained a value for the membrane potential of —37 mV in the taenia coli by inserting into the constant field equation the ionic concentrations and the permeability constants for the ions deduced from estimated fluxes of ^{42}K, ^{24}Na and ^{36}Cl This value is approximately 20 mV lower than that measured by the microelectrode technique. The difference was ascribed either to the presence of a partly electrogenic sodium pump or to "single file diffusion" of K^+ as was suggested to occur in giant axons from *Sepia officinalis* by Hodgkin and Keynes (1955)

Such single file diffusion of K^+ would lead to an underestimation of the permeability constant for this ion.

In spite of the possibility of an electrogenic Na pump and of the lack of information concerning the voltage profile in the cell membranes of smooth muscle, the "constant field" equation might still be useful as a starting point for the discussion of osmolality induced effects on the electrical activity

A shrinkage of the muscle cells caused by increased extracellular tonicity should result in raised intracellular ionic concentrations. The question arises whether the changed transmembrane ionic gradients are the cause of the inhibited spontaneous activity According to the constant field equation, an increase of $[K^+]_i$ and $[Na^+]_i$ should lead to hyperpolarization, and an increase of $[Cl^-]_i$ to depolarization. Since the electrical activity of the portal vein is relatively slightly influenced by changes in the $[Na^+]_i$ to $[Na^+]$ ratio when induced by changes in $[Na^+]$ (Axelsson *et al.* 1967 I and III) and since a raised $[Cl^-]_i$ would tend to cause excitation, it is probably sufficient to consider the potassium ion alone in this particular context. The question can then be simplified as to whether the increased $[K^+]_i$ to $[K^+]$ ratio can entirely explain the suppressed spontaneous activity in hypertonic media. Brading and Setekleiv (1968) measured the changes in the ionic content of taenia coli on alterations in osmolality and concluded that the hyperpolarization of 10—15 mV recorded by Tomita (1966) on exposure to two times the normal tonicity could be fully explained by the concomitant increase in $[K^+]_i$. Furthermore, for striated muscle, Adrian (1956) reported that the membrane potential was altered to an extent that could be expected for a potassium electrode when the extracellular tonicity was raised Direct measurements of the resting membrane potential have not been carried out in the present series of studies and the following discussion of the importance of the potassium gradient is therefore based on observations concerning the pattern of phasic electrical activity The discussion thus rests on the assumption that the relationship between the level of the membrane potential and the spontaneous burst activity is not materially affected by osmolality

In I III and in a preceding article (Mellander *et al.* 1967) the role played by an altered $[K^+]_i$ to $[K^+]$ ratio in the activity responses of portal veins as a result of variations in osmolality was elucidated by making combined changes in $[K^+]_o$ and in tonicity The purpose of this procedure was to investigate whether a normalization of the $[K^+]_i$ to $[K^+]_o$ ratio could outbalance the effects of the anisosmotic media on the spontaneous activity If so, the K^+ concentration gradient would

adequately and completely account for the relationship between cell volume and degree of activity It could be clearly demonstrated, however that such a simple relationship does not prevail at least not at the normal $[K^+]$ level For instance, $[K^+]$ had to be raised from the normal value 5 9 meq/l by two to three times to compensate for the inhibitory effect exerted by a 30 per cent tonicity increase (I) In this case a $[K^+]$ rise to about 8 meq/l would have been sufficient to normalize the transmembrane concentration gradient. Since a far more pronounced increment was required to restore normal activity this strongly suggests the involvement of additional mechanisms. A similar conclusion was drawn in III concerning the excitation induced by hypo-osmolality at the normal range of potassium concentrations. In fact, here it was even more obvious that the excitation could not be solely ascribed to an altered $[K^+]_i$ to $[K^+]$ ratio For instance, the response to a tonicity reduction of 55 mOsm/kg was stronger than that caused by a fourfold $[K^+]$ increment. *As judged by the changed amount of cell water* obtained on exposure to such a lowered tonicity the $[K^+]_i$ to $[K^+]$ ratio would decrease only some 10—20 per cent (IV VI)

However at higher levels of extracellular potassium ion concentrations, the effects of anisosmotic media are cancelled by roughly proportional alterations in $[K^+]$ (I III) The inhibitory effect induced by the addition of 100 mmoles sucrose/l to a medium containing 24 meq K^+/l could then be completely eliminated by an increment of the latter constituent to 30—36 meq/l Thus, in this case, a tonicity rise of 30 per cent could be completely compensated by a corresponding increase of $[K^+]_e$, just as would be expected for a potassium electrode exhibiting a perfect osmotic behaviour

A similar increase in the importance of the potassium concentration gradient for the responses to lowered osmolality was demonstrated in III The reason for this shift in the relation between the effects of altered $[K^+]$ and changed osmolality when the potassium concentration in the control situations is high, may be the fact that the membrane potential of smooth muscle cells is more influenced by the $[K^+]_i$ to $[K^+]$ ratio at higher levels of $[K^+]$ (Holman 1958 Marshall 1962, Axelsson *et al.* 1967) It has been proposed that this potassium electrode — like behaviour of smooth muscle in K rich media is due to a relatively higher membrane permeability for K under these circumstances.

It can thus be concluded that the concomitant changes in the ratio of $[K^+]_i$ to $[K^+]$ on alterations in cell volume, can fully explain the activity responses when $[K^+]$ is initially supranormal but that some

additional mechanism must contribute to the events at the normal range of $[K^+]$ which, of course, is most relevant for the *in vivo* situation. According to the constant field equation, not only changes in the ionic concentration gradients but also alterations in ionic membrane permeability can influence the membrane potential. The question now arises as to whether such alterations occur upon osmotically induced shrinkage or swelling of the smooth muscle cells. These problems were investigated in VI and VII and are discussed below.

Permeability properties of smooth muscle cell membranes at different osmolalities

As discussed above the changes in portal vein activity produced by alterations in osmolality could not be solely ascribed to the concomitant variations in transmembrane ionic concentration gradients. An additional factor might be variations in the passive permeability properties of the cell membranes associated with alterations in the cell volume. For instance, it seems possible that stretching of the cell membranes in connection with osmotic swelling, might lead to a thinning of the lipoid membrane structures, or to widening of the aqueous pathways ("the pores"). Furthermore, it is conceivable that such modifications in the membrane structures might facilitate the passage of a larger penetrating ion (*e g* the hydrated Na^+) relatively more than that of a smaller one (*e g* K^+). If so the ratio P_{Na}/P_K would increase during osmotically induced swelling of the muscle cells, an adjustment which would lead to depolarization according to the "constant field equation". The hyperpolarization caused by raised tonicity may also be explained along these lines.

The possibility of such alterations in permeability upon swelling and shrinkage of the smooth muscle cells was examined in VI and VII. In VI some general aspects of membrane permeability were considered by investigating the effects of tonicity on the exchange kinetics of two non electrolytes, urea and erythritol. In VII the specific permeabilities for Na^+ and K^+ under different environmental conditions were examined. The methods used involved "loading" of the portal veins in radioactive medium until the actual tracer substance had equilibrated in the tissue, followed by examination of the decay in tissue radioactivity during exposure to a non-active medium. The washout curves were analysed with the purpose of identifying a monoexponential term that could be considered to represent the transmembrane exchange. It was then assumed that the cell membranes exert a rate-limiting barrier function which

predominantly determines the permeability for the different penetrating substances. This view is the most widely accepted one although some authors consider the permeability characteristics of a cell as a property of the cytoplasm rather than of the membranes. Such approaches are represented by the sorptional theory of cell permeability (Troshin 1966) referred to above and Ling's "association induction hypothesis" discussed for smooth muscle by Jones (1970). According to the latter approach, cellular ions are either associated with polarized groups of the proteins (fixed charges, mainly negative) or dissolved in the *interstitial* water. The association of ions to the fixed charges is selective and metabolically dependent. The exchange kinetics of the dissolved ions are those of bulk diffusion, exhibiting little temperature dependence in contrast to the "adsorption-desorption" mechanism governing the exchange of the "associated" ions. The efflux of Na from the cells is considered to be governed mainly by bulk diffusion processes and that of K by the adsorption desorption kinetics. In accordance with this hypothesis, Brading and Jones (1969) reported that 98—99 per cent of the ^{24}Na efflux from taenia coli can be described by bulk diffusion, thus indicating that the cell membranes do not exert a barrier function with respect to this ion.

However other authors have found that the component in the ^{24}Na washout curve considered to represent transmembrane efflux in taenia coli (Buck and Goodford 1966) and in arterial smooth muscle (Garrahan, Villamil and Zadunaisky 1965 Hagemeijer Ronve and Schoffeniels 1965 b) is, in fact, quite sensitive to alterations in temperature and to ouabain. With regard to the washout curves of ^{14}C urea and ^{14}C erythritol from the portal vein (VI) the data could after some min be fitted by simple exponentials whose intercepts with the ordinate corresponded to spaces which agreed fairly well with the volume of intracellular water as calculated from determinations of total water and extracellular (^{14}C sucrose) space. These different results, obtained with various kinds of smooth muscle seem to support the concept of cell membranes as rate-limiting barriers and to justify the application of the membrane model in the interpretation of the results in VI and VII.

The results obtained in the experiments where the efflux of ^{14}C urea and ^{14}C erythritol were studied did not indicate any change in the permeability constants for these non-electrolytes in connection with increases or moderate decreases in extracellular tonicity. However at more pronounced reductions (—109 mOsm/kg) in osmolality a clear-cut increase in the permeability for both substances was observed. The fact that the P value for the smallest and most lipid soluble molecule of the two i.e.

urea, increased more than that for erythritol suggests that the reduction in the barrier function of the cell membranes was not due to widening of water filled pores since such an alteration would probably favour the larger and more water-soluble molecule. Some other mechanism, for instance thinning of the lipoid membrane, may therefore be considered.

The question as to whether the permeability coefficients for sodium and potassium (P_{Na} and P_K, respectively) are differently influenced by anisosmotic media, was elucidated by studying the fluxes of ^{24}Na and ^{42}K (VII) The washout of ^{42}K in normal Krebs solution at 37°C could, after the first few min, be described by a simple exponential, the ordinate intercept of which corresponded to a value for $[K^+]_i$ that agreed satisfactorily with that calculated by the "analytical" method in V Since the exchange of ^{24}Na at 37°C was too rapid to permit detailed compartmental analysis, the comparative study concerning tonicity effects on P_K and P_{Na} was performed at 15 C. At this low temperature the decline in tissue ^{42}K could not be strictly fitted by a single exponential term until 40—60 min had elapsed, probably due to a reduction in P_K at prolonged exposure to 15°C. However the "earlier" part of the washout *curve must also to a great extent represent efflux of cellular potassium* Further the decline in ^{42}K activity over the 7—30 min washout interval was essentially monoexponential and since this particular time interval represented transmembrane efflux of ^{24}Na in the sodium experiments, it was considered most appropriate to use this period for calculating cellular efflux of potassium as well. With this approach the P_K and P_{Na} values would be estimated under quite comparable conditions as regards time of exposure to the anisosmotic media and to 15 C. With the assumption *that the membrane potential under these circumstances averages 40 mV* in normal Krebs solution, the calculated value for P_K amounts to $5.3 \cdot 10^{-8}$ cm/sec, which is in fairly good agreement with that reported by Casteels (1969) for the taenia coli.

Since a considerable fraction of the Na content in the portal vein prevails in a bound, osmotically inactive form (V) ^{24}Na washout experiments were not only performed with intact portal veins but also with the damaged "membrane-deprived" tissues, described above. This approach made it possible to distinguish that *component of the* ^{24}Na washout curve of normal tissues which represented exchange of $[Na^+]_i$ from that representing washout of sequestered Na. This method gave a value for the transmembrane Na efflux at 15 C of $1.2 \cdot 10^{-11}$ M/cm² sec. However due to the action of the postulated Na K pump the passive permeability for sodium should be investigated by studying the kinetics of the ^{24}Na

uptake which is considered to be a solely passive process. Yet, since the total exchangeable Na amount remained essentially constant during the washout period, influx and efflux of Na^+ should equal each other in the experimental situation. The value given above for the transmembrane sodium efflux may therefore be used to estimate P_{Na} and a figure of 4.3 10^{-9} cm/sec is then obtained. A P_{Na}/P_K ratio of 0.081 would then prevail in portal vein, assuming a membrane potential of 40 mV at 15°C.

Similar determinations were made in hyper and hypo-osmotic media, but since the change in membrane potential induced by these alterations in the environment was not known, no definite values for the actual permeability coefficients could be calculated. Instead, a set of P_K and P_{Na} values were deduced for different possible levels of membrane potential. On the basis of these data it was concluded that the ratio P_{Na}/P_K increases in connection with osmotically induced swelling of the smooth muscle cells. This effect can according to the discussion above, contribute to the depolarization caused by hypo-osmolality and thus help to explain the strong excitation seen under such conditions. Concerning the effects of raised osmolality no evidence for changes in either P_K or P_{Na} was obtained. It should, however in this context be stressed that determination of permeability constants in smooth muscles in general is technically exceedingly difficult for several reasons, implying that given values must always be judged with caution.

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General Discussion

Since vascular smooth muscle presents marked individualities with regard to its physiological characteristics depending on its localization in the vascular tree (*e g* Bohr 1965) it is important to consider to what extent the present results with the portal vein are relevant for other types of vascular smooth muscle. Unfortunately there seems to be very little information concerning the effects of osmolality on preparations from other vessels. However in a study on strips of canine aorta, Yamabayashi and Hamilton (1959) reported the occurrence of phasic spontaneous contractions in this normally quiescent preparation when the tonicity of the incubation medium was increased by adding NaCl or sucrose Furthermore, the amplitude of these contractions rose with the degree of hypertonicity In accordance with these observations, Somlyo and Somlyo (1970) found that rabbit and canine pulmonary arteries are contracted by hyperosmolality They conclude that these findings on different types of "Windkessel" vessels, where the smooth muscle exhibits essentially "multi-unit" behaviour indicate that the portal vein responses to tonicity variations may not be representative for the osmometric properties of *all* types of vascular smooth muscle.

However there is much experimental evidence indicating that the functionally more important resistance vessels in many systemic circuits behave like the portal vein with respect to artificially induced osmolality variations. For instance, close intra arterial injections or infusions of hypertonic solutions of NaCl or of different nonelectrolytes, cause increased regional blood flow in dog limb (Marshall and Shepherd 1959 Read *et al* 1960, Overbeck, Molnar and Haddy 1961 Haddy and Scott 1965), cat skeletal muscle (Mellander *et al* 1967) dog skeletal muscle (Staunsby and Fregly 1968) dog myocardium (Read *et al.* 1960) dog brain (Buyniski and Rapela 1968) dog kidney (Lucas and Read 1966 Gazitua *et al* 1969) and human forearm (Lundvall Mellander and White 1969) Analysis of the underlying mechanisms indicate that the hyperemia caused by hyperosmolality is neither due to any passive widening of the vessels as a result of dehydration of the vascular walls, nor due to an altered blood *viscosity but is caused by a locally elicited inhibition of* vascular smooth muscle activity (Mellander *et al* 1967)

Furthermore, arterial infusions of hypotonic solutions lead to *increases* of flow resistance in the dog forelimb (Overbeck, Molnar and Haddy 1961) dog kidney (Gazitua *et al* 1969) dog skeletal muscle (Staunby and Fregly 1968) as well as the human forearm (Overbeck, Daugherty and Haddy 1966) The mechanism behind these latter effects has not been studied as extensively as in the case of hyperosmolality

The present *in vitro* results concerning osmotic effects on myogenic activity in single unit vascular smooth muscle have quite recently been supplemented by an interesting *in vivo* study concerning the effects of close arterial infusions of hypo- iso- and hyperosmotic solutions of NaCl, urea or sucrose on the vascular resistance of the dog kidney (Gazitua *et al* 1969) These investigators found that hypertonic sucrose solutions elicited sustained vasodilatation whereas urea and NaCl only brought about a transient blood flow increase. When the infusion of urea and NaCl was stopped, a shortlasting pronounced rise in vascular resistance was obtained. Similar characteristics in the vascular responses to hyperosmotic urea were found also in the dog forelimb. Furthermore, infusions of hypo-osmotic NaCl, sucrose and urea or of iso-osmotic urea caused an increase in renal vascular resistance while no such reaction was induced by iso-osmotic sucrose solutions. These results obtained with the blood perfused dog kidney can be fully explained, as pointed out by the authors, if it is assumed that the vascular smooth muscle of the renal resistance vessels exhibit osmometric properties similar to those shown by the isolated rat portal vein.

However this pattern of response to osmolality variations is apparently not typical for *all* kinds of vascular smooth muscles or vascular regions as pointed out earlier in this chapter For example, in the pulmonary circuit, blood flow *decreases* as a response to strongly hypertonic solutions (Binet and Burstein 1951; Eliakim, Rosenberg and Braun 1958 Semler Shepherd and Swan 1959 Read *et al* 1960 Eliakim, Stern and Nathan 1961 Braun 1966), by many of the investigators proposed to be due to an osmotically induced *vasoconstriction* Semler Shepherd and Swan (1959) and Read *et al.* (1960) on the other hand, suggested that it is caused by a transient red cell agglutination, because hypertonic injections did not increase *pulmonary flow* resistance when the isolated dog lung was perfused with dextran instead of blood. Further evidence for the occurrence of erythrocyte agglutination as a result of hyperosmolality was obtained in studies using vital microscopy (Read *et al.* 1960). Different opinions prevail concerning the responses to hypertonicity of the pulmonary vascular bed when the role played by sludging of the blood is

General Discussion

Since vascular smooth muscle presents marked individualities with regard to its physiological characteristics depending on its localization in the vascular tree (*e g* Bohr 1965), it is important to consider to what extent the present results with the portal vein are relevant for other types of vascular smooth muscle. Unfortunately there seems to be very little information concerning the effects of osmolality on preparations from other vessels. However in a study on strips of canine aorta, Yamabayashi and Hamilton (1959) reported the occurrence of phasic spontaneous contractions in this normally quiescent preparation when the tonicity of the incubation medium was increased by adding NaCl or sucrose. Furthermore, the amplitude of these contractions rose with the degree of hypertonicity In accordance with these observations, Somlyo and Somlyo (1970) found that rabbit and canine pulmonary arteries are contracted by hyperosmolality They conclude that these findings on different types of "Windkessel" vessels, where the smooth muscle exhibits essentially "multi-unit" behaviour indicate that the portal vein responses to tonicity variations may not be representative for the osmometric properties of *all* types of vascular smooth muscle.

However there is much experimental evidence indicating that the functionally more important resistance vessels in many systemic circuits behave like the portal vein with respect to artificially induced osmolality variations. For instance, close intra arterial injections or infusions of hypertonic solutions of NaCl, or of different nonelectrolytes, cause increased regional blood flow in dog limb (Marshall and Shepherd 1959 Read *et al* 1960 Overbeck, Molnar and Haddy 1961 Haddy and Scott 1965) cat skeletal muscle (Mellander *et al* 1967) dog skeletal muscle (Stainsby and Fregly 1968) dog myocardium (Read *et al* 1960) dog brain (Buyniski and Rapela 1968) dog kidney (Lucas and Read 1966 Gazitua *et al* 1969) and human forearm (Lundvall Mellander and White 1969) Analysis of the underlying mechanisms indicate that the hyperemia caused by hyperosmolality is neither due to any passive widening of the vessels as a result of dehydration of the vascular walls, nor due to an altered blood viscosity but is caused by a locally elicited inhibition of vascular smooth muscle activity (Mellander *et al* 1967)

Furthermore, arterial infusions of hypotonic solutions lead to *increases* of flow resistance in the dog forelimb (Overbeck, Molnar and Haddy 1961) dog kidney (Gazitua *et al.* 1969) dog skeletal muscle (Stainsby and Fregly 1968) as well as the human forearm (Overbeck, Daugherty and Haddy 1966) The mechanism behind these latter effects has not been studied as extensively as in the case of hyperosmolality

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eliminated. According to some early reports (Semler Shepherd and Swan 1959 Read *et al.* 1960 Haddy and Scott 1965) no vasodilatation ensues while later studies have indicated a pattern of response that closely resembles that exhibited by the systemic circuits (Bo and Hauge 1969) It should in this context be stressed that the pulmonary precapillary vessels react in the *opposite* direction to the systemic ones to a whole series of vasoactive agents (*e g* Hauge, Lunde and Waaler 1966)

The importance of osmolality variations as a factor in the physiological regulation of vascular tone in skeletal muscle was suggested by Mellander in 1966 (see Mellander 1968) and has been extensively studied by his group (Mellander *et al* 1967 Gray Lundvall and Mellander 1968 Lundvall 1969 *Lundvall, Mellander and White 1969 Mellander and Lundvall* 1970) They have demonstrated that the osmolality of the regional venous blood increases in direct proportion to the intensity of the work performed and can reach a value of 40 mOsm/kg above control during heavy exercise. When comparable degrees of hyperosmolality are induced by intra-arterial infusions of concentrated solutions, a pattern of response within the various series-coupled sections of the vascular bed is elicited which, in all respects, resembles that observed during muscle exercise. Furthermore, interstitial deposits of ^{133}Xe in resting muscle are cleared at faster rates when the tracer is dissolved in a hypertonic instead of an iso-tonic medium. These results indicate that experimental hyperosmolality which primarily involves the extravascular space is also able to elicit vasodilatation. It appears clear from these studies by Mellander *et al* that the exercise hyperemia in skeletal muscle in cat as well as in man is, at least partly caused by an increased release of osmotically active particles. It is, of course, possible that a similar blood flow regulating mechanism exists also in other metabolically active tissues.

It is conceivable that increased tissue osmolality and hence local vasodilatation, can result also from other processes than release of metabolites. For instance events like resorption and secretion in an organ may obviously be accompanied by alterations in the regional osmolality Such a mechanism is indeed suggested by the results of van Heerden, Wagner and Kaihara (1968) upon studying the effects on jejunal blood flow of intraintestinal administrations of concentrated glucose solutions. They demonstrated that perfusion of one part of the jejunum with a hypertonic solution caused vasodilatation in that particular intestinal section while blood flow in adjacent unperfused segments was unaffected. Furthermore, it has been suggested that the systemic vasodilatation seen in the dumping syndrome of patients after gastric resection is due to a raised osmotic

pressure of the blood following absorption of materials that are more concentrated than when a normal pyloric function is present (Lucas and Read 1966) These authors provoked a typical dumping syndrome in dog and man by intraduodenal administration of 50 per cent glucose as previously described by Machella (1949) The time course of the general vasodilatation elicited in this way turned out to be strictly correlated to the concomitant increase in blood osmolality Moreover intravenous administration of hypertonic glucose elicited a similar type of vascular response, indicating that the vasodilatory stimulus is not of intestinal origin but the result of blood hypertonicity It is further interesting to consider what happen to the intestinal circulation on drinking large amounts of water To the knowledge of the author this interesting topic has not yet been subjected to experimental study

Angiography in clinical practice often implies infusion of strongly hypertonic solutions. Giammona, Lurie and Segar (1963) reported that plasma osmolality could rise as much as 10 per cent in children with a variety of cardiac abnormalities undergoing selective angiocardiography The sensation of warmth that patients often feel at angiographic examination has been ascribed to a peripheral vasodilatation (Deterling 1952) which apparently might be due to the subsequent raise in blood tonicity In this context it may be of interest to note that repeated intravenous injections of hypertonic solutions have been reported to give successful results in the treatment of *e g* thromboangiitis obliterans (Deibel and Sgalitzer 1933 Silbert 1935)

Another aspect of the present results that might have clinical connection concerns the effects of lowered osmolality on the portal vein. It has been claimed that, in hypertensive disease, the arterial walls contain more water than under normal conditions. These observations were originally made by Tobian and Binion (1952) on renal arteries from patients with essential hypertension. Such waterlogging of the arterial wall would, as these authors pointed out, lead to a passive increase in the peripheral resistance. Furthermore, if the extra water was located intracellularly and thus the vascular smooth muscle cells were swelled, the active tone might also be increased according to the results presented in I and III Since these original findings of Tobian and Binion were published, several studies have been performed on water content and ionic distribution in arteries from animals with experimental hypertension (for ref see Schofenels 1969) Most authors seem to agree that hypertension is accompanied by a rise in the sodium content of the arterial wall. However a great discrepancy prevails in the opinions concerning the exact localization of

these extra amounts of sodium in the tissue Redleaf and Tobian (1958) concluded that $[Na^+]_i$ is increased in the hypertensive state. They based this conclusion on the fact that the ratio of Na to Cl was above normal and on the assumption that Cl^- is mainly located extracellularly Such a shift in the Na distribution might lead to accumulation of water inside the cell which in turn, should stimulate the spontaneous activity as outlined above. In contrast to this, some authors claim that the excess sodium found in arteries from hypertensive animals is bound extracellularly and then the muscle cells would not swell (Friedman and Friedman 1967 Schoffeniels 1969) This hypothesis is supported by the observation made by the former authors that even in the absence of functional cellular membranes, the contents of cations is higher in aortae isolated from hypertensive animals than in normal aortae. Thus, the question concerning the relations that may exist between the excess of vascular sodium and the hypertensive condition are, at present, far from settled.

In summary the activity responses of the portal vein on variations in the osmolality studied in I—III are similar to those of the resistance vessels in many different vascular regions. It therefore seems reasonable that the results of the examinations concerning the underlying mechanisms, summarized in the preceding sections, are relevant also for the latter kinds of vessels. Since alterations in the regional tonicity as discussed above, occur under physiological conditions it is conceivable that the demonstrated relation between volume and degree of activity in "single unit vascular smooth muscle cells constitutes an important local blood flow regulating mechanism also in other situations than muscle exercise.

Summary

A study has been performed concerning the effects of altered extracellular osmolality on the spontaneously active, isolated rat portal vein. This vascular smooth muscle preparation has functional characteristics that, in many aspects, resemble those of the systemic resistance vessels. The results and conclusions can be summarized as follows.

1. Evidence has been obtained for a correlation between muscle cell volume and degree of activity, swelling being associated with muscle excitation and shrinkage with inhibition of the spontaneous electrical and mechanical activity. Thus, incubation of the portal vein in hypo-osmotic media greatly enhances the spontaneous activity. In contrast, exposure to a hyperosmotic environment, induced by supplementing the normal Krebs solution with sucrose, which does not penetrate the cell membranes, leads to a *sustained* inhibition of the spontaneous activity. When, on the other hand, hyperosmolality is created by addition of urea, which penetrates the cell membranes at a moderate rate and hence equilibrates in the total tissue water, only a *transient* inhibition is obtained with rapid return to control activity despite the continued presence of urea. Normalization of the osmolality after urea exposure is associated with a transient excitation whose intensity is closely related to the duration of the previous period of urea hyperosmolality, up to the point when ^{14}C urea reaches its plateau value in the uptake curve.

Determinations of the cell water content at different tonicities indicate that the muscle cells are, in fact, continuously shrunken in hyperosmotic sucrose solutions and swelled in hypo-osmotic media. In contrast, exposure to a membrane penetrating substance like urea, apparently leads to only a transient shrinkage and, on return to the control solution, to a shortlasting swelling.

An analysis of the mechanisms responsible for this correlation between cell volume and degree of cell activity requires quantitative information concerning the osmometric behaviour of the cells and the ionic distribution in the portal vein.

2. When the osmolality is raised by addition of sucrose to the incubation medium, the muscle cells shrink to an extent expected for perfect osmo-

meters. The alterations in cell water content upon exposure to hypo-osmolality are, however more complex since the cell swelling is to some extent hampered by the concomitantly enhanced active tension development of the muscle cells. If this activity is suppressed by cooling, the cells behave like perfect osmometers up to a 20 per cent reduction in osmolality but not at still more drastic levels of hypo-osmolality It is, however even in this latter situation possible that the muscle cells approach an osmotic equilibrium with the medium since a loss of intracellular ions occurs under these circumstances.

3 Concerning the ionic distribution in the portal vein, a simple two compartment model cannot be applied for either sodium, potassium or chloride, since there exists bound osmotically inactive fractions of the electrolytes. As in other types of excitable tissues, $[K^+]_i$ is higher than $[Na^+]_i$. The membrane potential is predominantly determined by E_{K^+} but also influenced to an appreciable extent by E_{X^+} The distribution of Cl^- does not seem to conform to a passive Donnan equilibrium.

4 The possibility that the relation between muscle cell volume and degree of activity is mediated by variations in the transmembrane ionic concentration gradients has been elucidated by making simultaneous alterations in tonicity and in the extracellular electrolyte concentrations. The results indicate that, in the *normal* range of $[K^+]_o$, a restoration of the original ionic concentration gradients is not alone sufficient to cancel the osmolality effects on the spontaneous muscle activity However at supranormal levels of $[K^+]_o$, alterations in tonicity and thus in the intracellular electrolyte concentrations, seem to be quantitatively more comparable to changes in the extracellular ionic composition

5 Studies of the exchange kinetics of ^{24}Na and ^{42}K across the cell membranes at different osmolalities suggest that the permeability constant for sodium increases more than that for potassium during hypo-osmolality Such an effect of swelling on the membrane permeability may help to explain the subsequent depolarization and increase of phasic activity *During cell shrinkage on the other hand, there was no evidence of any* change in the ratio P_{X_o} to P_K and alternative mechanisms must therefore be considered to explain the inhibitory responses.

6 The application of the present *in vitro* results, concerning osmotic effects on the active tension development of isolated vascular smooth muscle of the "single unit" type to the *in vivo* situation is discussed from physiological and pathophysiological points of view

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ACTA PHYSIOLOGICA SCANDINAVICA
SUPPLEMENTUM 360

Catechol- and Indolamines in some Endocrine Cell Systems

An autoradiographical, histochemical and radioimmunological study

BY

HANS TJÄLVE

STOCKHOLM 1971

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Contents

Introduction 5

Chapter 1
Uptake of DOPA and 5-HTP in the pancreatic islets of the mouse studied by autoradiography fluorescence microscopy and combined autoradiography—fluorescence microscopy 8

Chapter 2
Uptake of DOPA and 5-HTP in the parafollicular cells of the thyroid of the mouse studied by autoradiography and combined autoradiography—fluorescence microscopy 21

Chapter 3
Uptake of DOPA and 5-HTP in the mucosa of the stomach of the mouse studied by autoradiography fluorescence microscopy and combined autoradiography—fluorescence microscopy 28

Chapter 4
Electron microscope autoradiography of the mouse fundic mucosa after the injection of tritiated L-DOPA 40

Chapter 5
Distribution of DOPA 1 C (carboxyl- ^{14}C- labelled DOPA) in relation to DOPA ^{1}C in mice before and after inhibition of DOPA-decarboxylase, studied by whole-body autoradiography 52

Chapter 6
Histochemical demonstration of monoamine oxidase (MAO) and its relation to the *in vitro* uptake of serotonin- ^{14}C studied in whole-body sections of mice 69

Chapter 7
Studies on the uptake and metabolism of DOPA in the rabbit pancreas 80

Part 1 Fluorescence microscopic investigation of the *in vivo* and *in vitro* uptake of DOPA in the rabbit pancreas 80

Part 2. Chromatographic investigation of the *in vitro* metabolism of DOPA 2 ^{14}C in the rabbit pancreas 83

Chapter 8
Radioimmunological studies on the effect of catechol and indolamines and of their precursors on the insulin secretion from rabbit pancreas *in vitro* 87

Summary 108

Acknowledgements 110

References 111

Introduction

The physiological importance of the biogenic amines was early linked with neuronal transmittor functions (*cf* Guggenheim 1951) The occurrence of catecholamines in the adrenal medulla was also observed at an early stage (Meulon 1905) In recent years special interest has been focused on other extraneuronal localizations and functions of the biogenic amines. The discovery of biogenic amines as occurring in several non-neuronal tissues has been contributory to this development. This has been possible due to the introduction of sensitive histochemical and autoradiographical methods, which permit the demonstration of small amounts of biogenic amines in the tissues (see Ritzén 1967)

With the help of these methods it has been possible to demonstrate a localization of catechol- and indolamines in some endocrine cell systems such as the pancreatic islets, the parafollicular cells of the thyroid and the enterochromaffin cells of the gastrointestinal tract (*cf* Falck and Owman 1968) In species where catechol- and indolamines have not been demonstrated in the pancreatic islets and the parafollicular cells, a formation of amines has been shown to occur after the administration of the precursors in the biosynthesis of the amines dihydroxyphenylalanine (DOPA) and 5-hydroxytryptophan (5-HTP) (see Ritzén *et al.* 1965, Larsson *et al.* 1966, Cegrell 1968) In the gastrointestinal tract, where the presence of serotonin has long been known (Erspamer and Asero 195) precursor injections have been found to give an amine formation in cells, which lack endogenous storage of serotonin (Hâkanson *et al.* 1967 a)

The capability of these cell systems to store and form catechol and indolamines have led to assumptions that they are of importance for the synthesis and/or release of the polypeptide hormones, which are known to be produced by these cell systems (see Falck and Owman 1968 Pearse 1968, Ullberg and Hammarström 1969) The monoamine forming capability of the enterochromaffin cells has been taken as an indication of the possibility that they might be the producers of the gastrointestinal hormones (see Aures *et al.* 1970)

The development of radioimmunological techniques for the measurement of hormones has made it possible to show that monoamines really have an effect on the release of insulin (see Malaisse *et al* 1967 Porte 1969 Feldman and Lebovitz 1970 and Chapter 8) An effect on the release of calcitonin, which is produced by the parafollicular cells has also been shown (Bates *et al* 1969 Phillippo *et al* 1969)

Connected with the localization and function of the biogenic amines are the enzymes, which are respon ible for their formation and metabolism. The same enzyme namely L-amino acid decarboxylase is responsible for the decarboxyla tion of DOPA to dopamine and of 5-HTP to 5-hydroxytryptamine (Lovenberg *et al* 1962 a) The catabolism of catecholamines takes place through the action of the enzymes monoamine oxidase (MAO) and catechol O-methyl transferase (COMT) (see Kopin 1964) Serotonin is mainly metabolized through the action of MAO (see Erspamer 1961) While the enzymes L-amino acid decarboxylase MAO and COMT have a widespread distribution in the body both in neuronal and extraneuronal tissues, the enzymes which convert dopamine to noradrenaline (dopamine β-hydroxylase) and noradrena line to adrenaline (phenylethanolamine N methyl transferase) seem to be restricted in their localization to neuronal tissues, blood vessel walls and the adrenal medulla (see Axelrod 1966, Hartman and Udenfriend 1970)

The aim of the present investigation has been to elucidate some of the extraneuronal localizations and functions of catechol and indolamines. Special attention has been paid to the localization and function in the mentioned endocrine cell systems. Mice and rabbits ha e been used as experimental animals.

Autoradiographical and fl orescence microscopical methods ha e been used to study the localization of catechol and indolamines formed after the administration of DOPA and 5-HTP and the combination of autoradiography and fluorescence microscopy has allowed a concon itant demonstration of catechol- and indolamines in a/ the pancreatic islets, b/ the parafollicular cells of the thyroid and c/ the mucosa of the stomach in mice (Chapter 1—3) Electron microscope autoradiography has been applied to study the formation of catecholamines in the enterochromaffin cell types of the fundic part of the stomach after the administration of ^{3}H DOPA into mice (Chapter 4) Whole body autoradiography has been used to compare the distribution patterns obtained after the administration of DOPA 1 ^{14}C and DOPA 2 ^{14}C to mice (Chapter 5) The localization of MAO has been studied with a histochemical method in whole-body sections of mice (Chapter 6) The uptake and metabolism of DOPA in the rabbit pancreas has been studied by fluorescence microscopy and chromatography (Chapter 7) The effect of catechol and indol-

amines and of their precursors on the insulin secretion from rabbit pancreas *in vitro* has been studied by radioimmunological methods (Chapter 8)

Parts of Chapter 1 and Chapter 2 have been performed in co-operation with dr Premysl Slanina and Chapter 5 in co-operation with prof Sven Ullberg (Tjalve and Slanina 1971 Tjälve and Ullberg in preparation)

CHAPTER 1

Uptake of DOPA and 5-HTP in the pancreatic islets of the mouse studied by autoradiography, fluorescence microscopy and combined autoradiography — fluorescence microscopy

In the pancreatic islets of the pig, dog, cat, guinea-pig and horse an endogenous storage of dopamine and/or serotonin has been demonstrated (see Falck and Hellman 1963 Cegrell 1968) The cell types, which store the monoamines show variations between the species, as does the type of monoamine which is stored in the cells. It also seems as if dopamine and serotonin are stored together in the same islet cells in some species (see Cegrell 1968)
In other species, such as the mouse, rat, rabbit and golden hamster no endogenous occurrence of monoamines has been demonstrated in adult animals (see Cegrell 1968) Injections of DOPA or 5-HTP into mice have however been shown to result in a formation of monoamines within the islets. This has been demonstrated with fluorescence microscopy and microautoradiography (Hempel 1963 Cegrell *et al* 1964 Ritzén *et al.* 1965 Gershon and Ross 1966 a, Cegrell 1968 Ullberg and Hammarström 1969) Cegrell (1968) who studied the uptake of DOPA in the islets with fluorescence microscopy found fluorescence in all cells of the islets shortly after the injection. Later the fluorescence could be observed mainly in cells identified as α-cells.

The present investigation gives some additional data on the uptake of DOPA and 5-HTP in the pancreatic islets. The aim has been to compare the distribution patterns obtained after DOPA and 5-HTP-injections. For this purpose a combined microautoradiographic fluorescence microscopic method (Hammarström *et al.* 1966) was employed, which makes it possible to demonstrate catechol- and indolamines concomitantly in the same sections. Moreover microautoradiography was used separately to compare the distribution patterns up to 24 hours after the injections of the precursors. Some additional fluorescence microscopical data and data on the MAO-localization by means of histochemistry are also presented.

Material and methods

Animals

Albino mice of the NMRI strain of both sexes weighing about 20 g were used. The animals were kept on a complete pelleted diet (AB Ewos Sweden) at a room temperature of +23 C and were given water ad lib.

Compounds

The isotopes used were ring 2,5 6-^{3}H-labelled L-3(3 4-dihydroxyphenyl)alanine (H DOPA) specific activity 28 Ci/mM corresponding to 140 mCi/mg and generally ^{3}H-labelled 5-hydroxy DL-tryptophan (^{3}H 5-HTP) specific activity 2.56 Ci/mM corresponding to 11 6 mCi/mg. Both isotopes were obtained from The Radiochemical Centre, Amersham, England.

Non-labelled L-*β*-3 4-dihydroxyphenylalanine (L-DOPA) and 5-hydroxy-L-tryptophan (L-5-HTP) were obtained from Sigma Chemical Company USA. The monoamine oxidase inhibitor nialamide (Niamid) was obtained from Pfizer AB, Sweden. Nitro Blue tetrazolium and tryptamine hydrochloride were obtained from Sigma Chemical Company

Microautoradiography

Microautoradiography was performed essentially according to Rittén *et al.* (1965)

Three mice were injected intravenously with 0.5 mCi ^{3}H DOPA or with 0.5 mCi ^{3}H 5-HTP respectively and were killed by cervical dislocation 5 minutes, 1 hour and 24 hours after the injections (see Table 1) Specimens from the pancreas were then rapidly removed and immediately frozen in isopentane cooled with liquid nitrogen. The pieces were then freeze-dried under vacuum (10^{-4} mm Hg) at about $-40°$ C for five days in a Speedivac Pearse tissue dryer (Edwards High Vacuum Ltd., England) After the freeze drying the specimens were transferred to a glass jar containing paraformaldehyde gas at a relative humidity of about 60 % (Hamberger *et al.* 1965 a) The jar was placed in an oven at 80° C for one hour The gas fixation obtained is the same as the one used for visualization of monoamines in tissues (Falck *et al* 1962) This treatment also fixes the monoamines in the tissues and prevents their diffusion d ring the microautoradiographic and histological treatment (Rittén *et al.* 1965)

After the gas fixation the specimens were embedded in warm (60° C) paraffin (melting point 50—52° C) in vacuo and they were then subjected to conventional stripping film autoradiography (Doniach and Pelc 1950) Sections, 5 *μ* thick, were mounted on slides, deparaffinized in xylene and carried through an alcohol series to distilled water Stripping film (Kodak AR 10) was stretched on water and floated on to the sections. Exposure was carried out at $-10°$ C for 1—6 months. The films were developed for 3 minutes in Kodak D19, fixed and thereafter stained with haematoxylin and eosin and in some cases also with aldehyde-fuchsin (see Staining)

TABLE I P nureas.

Microautoradiogr phy

No. of nimals t each urvival time	Treatment (substance and dose)	Survival time
3	H DOP\, 0.5 mCi corresponding to 0.18 mg/kg	5 min, 1 h 2+ h
3	H-5-HTP 0.5 mCi corresponding to 2 16 mg/kg	5 min, 1 h, 4 h

Fluorescence nicroscopy

No. of nimals t each survival time	Treatment (substance nd dose)	Survival time
3	L-DOPA 100 mg/kg	5 min, 30 min, 1h, 2 h, 4 h
3	L-5-HTP 100 mg/kg	3 min, 30 mun, 1 h, 2 h, 4 h
3	\ialamid 500 mg/kg + L-DOP\ 100 mg, kg	2 h, 3 h, 4 h
3	Nialamide 500 mg/kg + L 5-HTP 100 mg/kg	2 h, 3 h, 4 h

Combined nacroautoradiography — fluorescence microscopy

No. of nimals t each survi al time	Treatment (substance and dose)	Survival time
2	H 5-HTP 0.5 mCi correspooding to 2 16 mg/kg + L-DOPA 100 mg/kg	1 h, 2 h
2	H DOP\, 0 5 mCi correspooding to 0 18 mg/kg + L-5-HTP 100 mg/kg	1 h, 2 h

Fluorescence nicrosc py

The fluorescence method of Falck and Hillarp (Falck *et al.* 196? Falck and Owman 1965) for demonstration of biogenic amines in tissues was used.

Mice were injected intravenously with DOPA or 5-HTP In some cases they were pre treated with the monoamine oxidase inhibitor nialamide which then was injected i p. 2 hours before the DOPA or 5-HTP injections. The

number of animals, the substances injected and the survival times are given in Table 1

The animals were killed at the stated survival times and pieces of pancreas were frozen, freeze-dried treated with paraformaldehyde gas and paraffin embedded as has been described above (see Microautoradiography) 5 μ thick sections were taken and put on warm (70 C) slides for some minutes allowing the paraffin to melt and the sections to spread. The sections were then subjected to fluorescence microscopy in a Zeiss Standard Universal microscope.

Combined microautoradiography—fluorescence microscopy

The method which has been used for the combination of microautoradiography and fluorescence microscopy is a modification of a method described by Hammarström *et al.* (1966)

A small amount of one of the precursors in labelled form was injected together with a large amount of the other precursor in non-labelled form. The specimens obtained were first treated according to the histochemical fluorescence method. Sections were then subjected to fluorescence microscopy and photographed. The same sections were then treated according to a microautoradiographic technique. A direct comparison was thus possible between the fluorescence picture, obtained through the injection of the non-labelled precursor and the autoradiogram, obtained through the injection of the labelled precursor The amount of precursor injected in labelled form was far too small to give any fluorescence of its own. This was checked by injecting the corresponding amount of precursor in non-labeled form into mice and then treat the specimens obtained for fluorescence microscopy

The isotope was injected into a tail vein with a tuberculine syringe attached to a needle with a polyethylene catheter Immediately following the isotope injection the non-labelled substance was injected in the same polyethylene catheter after replacing the first tuberculine syringe. The number of animals, the substances injected and the survival times are given in Table 1 The animals were killed at the stated survival times and pieces of pancreas were frozen, freeze-dried treated with paraformaldehyde-gas and paraffin embedded as has been described earlier (see Microautoradiography) 5 μ thick sections were then mounted on slides without cover slips and examined and photographed under a fluorescence microscope. The same sections were then passed down an alcohol series and subjected to conventional stripping film autoradiography (Doniach and Pelc 1950) as has been described earlier (see Microautoradiography) After the development comparison was possible between fluorescence pictures and autoradiograms.

Staining

A direct identification of the β-cells in the pancreatic islets on the autoradiograms with aldehyde-fuchsin staining was not possible since this staining completely removed the silver grains. This difficulty could, however be overcome if the autoradiograms were first stained with haematoxylin and eosin and passed through a series of increasing concentrations of ethanol and mounted in euparal (GBI Ltd., England) They were then examined and photographed. Thereafter the coverslips were removed and the sections were passed through absolute ethanol, 96 % ethanol and 70 % ethanol and then postfixed in 10 % formalin for 24 hours and stained with aldehydefuchsin as described by Jennings (1965) The aldehyde-fuchsin staining completely removed the haematoxylin and eosin staining as well as the silver grains.

Monoamine oxidase histochemistry

Monoamine oxidase (MAO) activity in the pancreas was determined with the method described by Glenner *et al.* (1957) 10 μ thick cryostat sections of pancreas were incubated for one hour at 37 C in a 0.1 M phosphate buffer pH 7 6 containing tryptamine hydrochloride (0 03 M) as substrate. Nitroblue tetrazolium was used as tetrazolium salt Control sections were incubated without substrate or pre incubated with nialamide (0.01 M) for 15 minutes. The technique will be described in more detail later (Chapter 6)



Fig 1 Microautoradiogram of a pancreatic islet of mouse 5 min ten after an intravenous injection of H 5-HTP A high radioactivity can be seen in all islet cells. The radioactivity in the exocrine pancreas is lower than in the islet and diffusely distributed. Hts-eosin. (×100)



Fig. 2 a. Microautoradiogram of a pancreatic islet of mouse 1 hour after an intravenous injection of H DOPA. Hx-eosin.



Fig. b. The same islet after staining with aldehyde-fuchsin. The most strongly labelled cells are a-cells (arrows) (×700)



Fig. 3 a. Microautoradiogram of pancreatic islet of mouse 1 hour after an intravenous injection of H DOPA. Hx-eosin.

It was not possible to stain sections subjected to monoamine oxidase histochemistry with aldehyde-fuchsin or silver staining techniques for identification of β- and α-cells in the pancreatic islets.

Results

Microauto adiography and fluorescence microscopy

The distribution of radioactivity in the pancreatic islets after the injection of ^{3}H DOPA was similar to the distribution seen after the injection of ^{3}H 5-HTP at the survival times studied.

Five minutes after the injection of ^{3}H DOPA or H 5-HTP there was a very high activity in all cells of the pancreatic islets (Fig 1) The activity in the endocrine pancreas exceeded somewhat that in the exocrine pancreas, where a diffuse distribution of the activity could be seen.

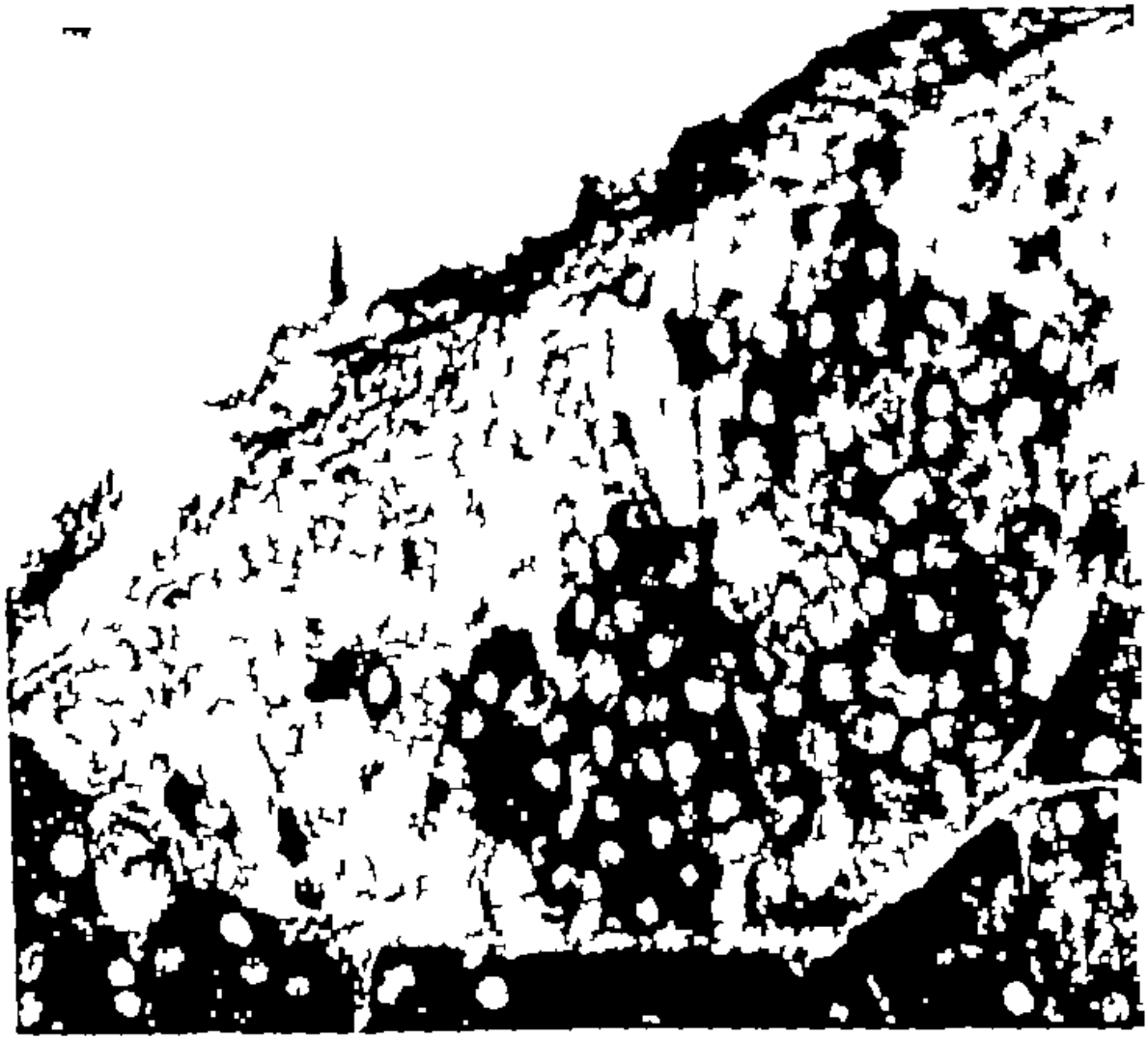


Fig. 3 b. The same islet after staining with aldehyde-fuchsin. The α-cells contain more radioactivity than the β-cells. Some α-cells are also more heavily labelled than other α-cells. (×380)

One hour after the injection of ^{3}H DOPA or ^{3}H-5-HTP some cells, mostly located at the periphery of the islets, contained a high activity (Fig. 2) The number of these cells varied between different islets. Subsequent aldehyde-fuchsin staining showed that the cells belonged to the α-cell group There were, however also α-cells with a lower amount of activity but also here the activity was considerable and seemed to exceed the activity in the β-cells to some extent (Fig. 3) In the exocrine pancreas more activity could be seen in the apical than in the basal parts of the cells. Also the excretory ducts contained a high activity

Twenty four hours after the injection of H DOPA or ^{3}H-5-HTP a high activity usually still persisted in a few scattered cells at the periphery of the islets. The number of these cells, also belonging to the α-cell group varied considerably from islet to islet and were lacking in some islets. In the exocrine

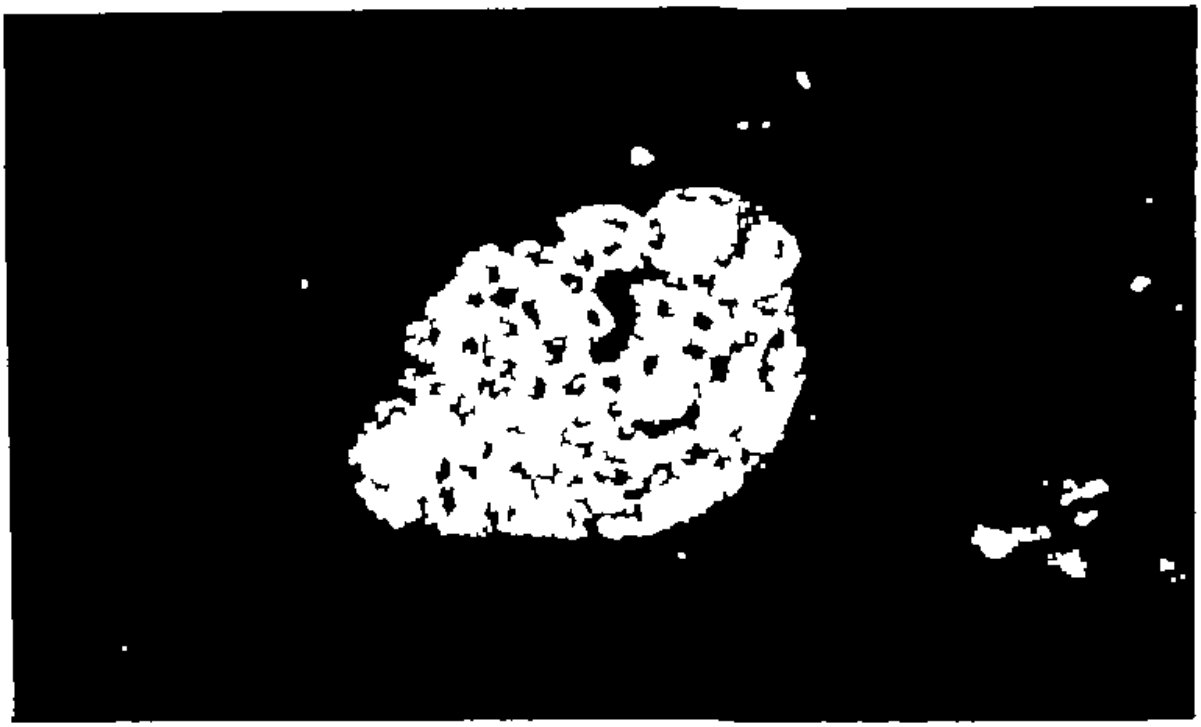


Fig. 4 Fluorescence photomicrograph of a pancreatic islet of mouse 30 minutes after an intravenous injection of DOPA. Fluorescence can be seen in all cells of the islet. Some peripherally located cells show little stronger fluorescence than the other cells. (×150)

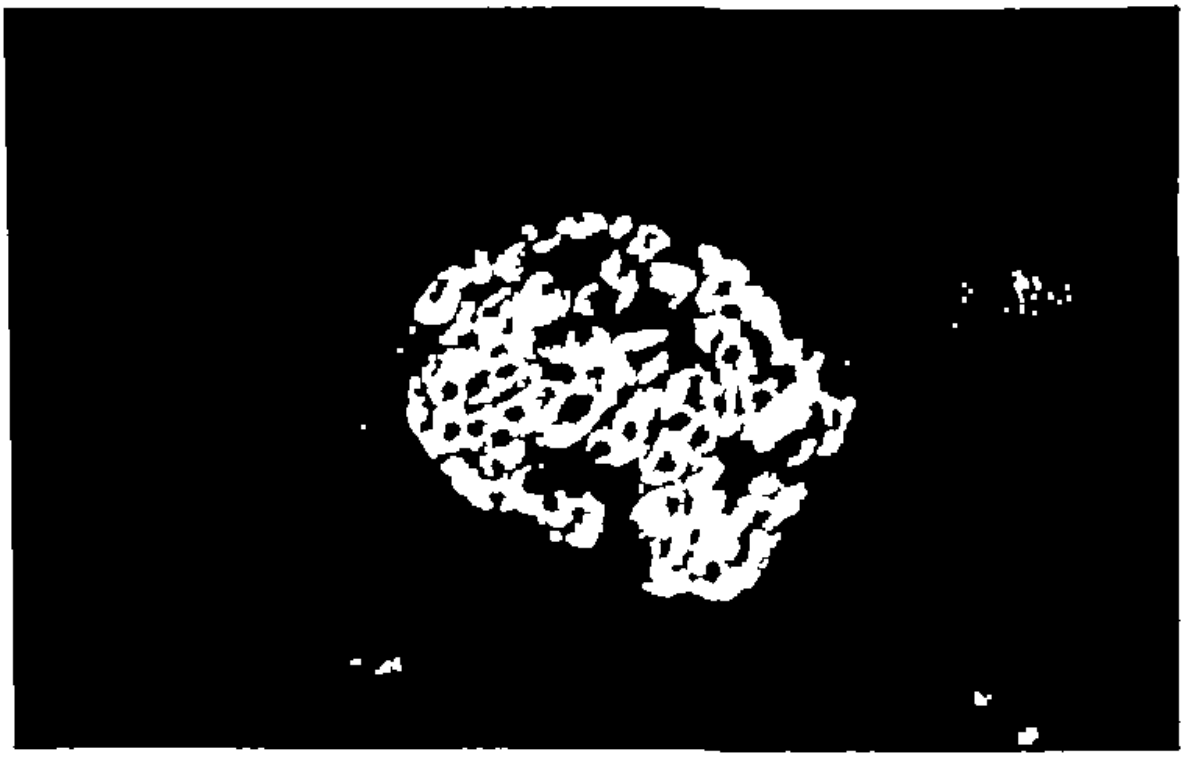


Fig. 5 Fluorescence photomicrograph of pancreatic islet of mouse 1 hou after an intravenous injection of DOPA. Some peripherally located cells show the trongest fluorescence Some cells show lower and some an even lower fluorescence. (×150)

pancreas activity could still be seen in the apical parts of acinar cells and in excretory ducts.

The fluorescence pictures obtained after injection of non-labelled DOPA and 5-HTP were also very similar to each other and showed the same general distribution patterns as seen in the autoradiograms (Fig. 4 and 5). However the lower sensitivity of the fluorescence method in comparison with the microautoradiographic method resulted in the disappearance of fluorescence 4 hours after the injection, while radioactivity could be observed in the islets 24 hours after the injection. The fluorescence pictures and the autoradiograms differed also in so far as the gradation between cells with different label was more pronounced in the fluorescent pictures, where they gave the impression of an all-or nothing effect.

If the animals were pre treated with the monoamine oxidase inhibitor nialamide there was a longer persistence of fluorescence in the islets after the injection of DOPA or 5-HTP. In addition there was no differentiation between peripherally located cells with high fluorescence and centrally located cells with lower fluorescence up to three hours after the injection (Fig. 6). Four hours after the injection the picture was similar to a picture seen after two hours without pre treatment with nialamide.

Combined microautoradiography—fluorescence microscopy

The combination of fluorescence microscopy and microautoradiography showed that the fluorescence obtained in the islets through the injection of one of the precursors (non-labelled) was in accordance with the radioactivity obtained through the injection of the other precursor (labelled) (Fig. 7). The result obtained was independent of whichever of the precursors was injected in la-

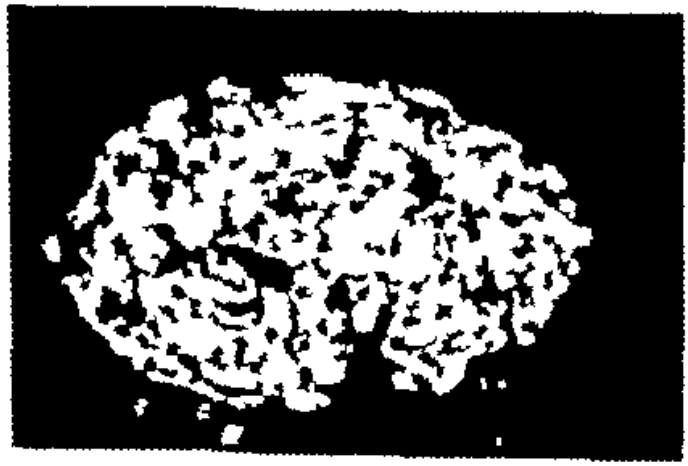


Fig. 6. Fluorescence photomicrograph of pancreatic islet 3 hours after an intravenous injection of 5-HTP into a mouse which has been pre-treated with the MAO-inhibitor nialamide. Fluorescence can be seen in all cells of the islet. ×130)

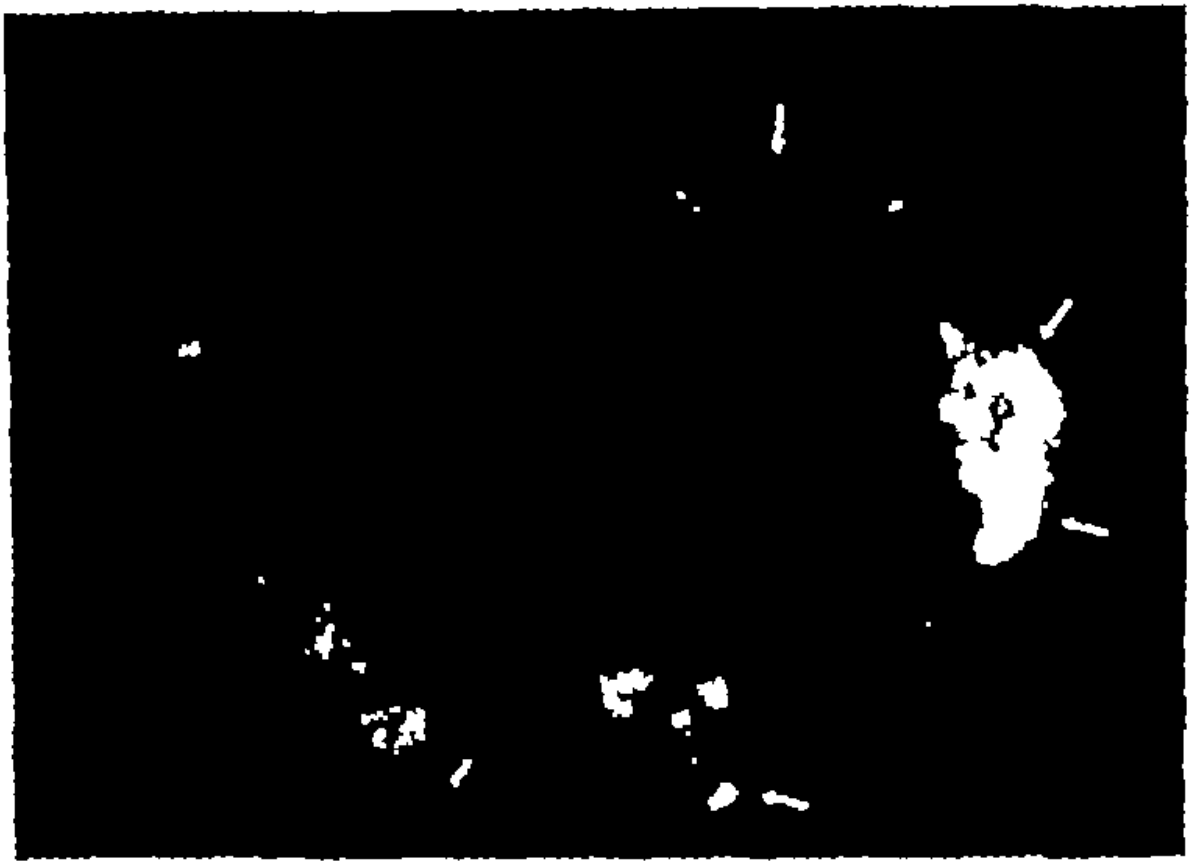


Fig. 7 a. Fluorescence photomicrograph of an islet of mouse 2 hours after an intravenous injection of non-labelled DOPA



Fig. 7 b. Microautoradiogram of the same islet obtained through the simultaneous injection of ^{3}H-5-HTP. Hæ-eosin. Cells with the strongest fluorescence have also accumulated the highest radioactivity (arrows). (×350)

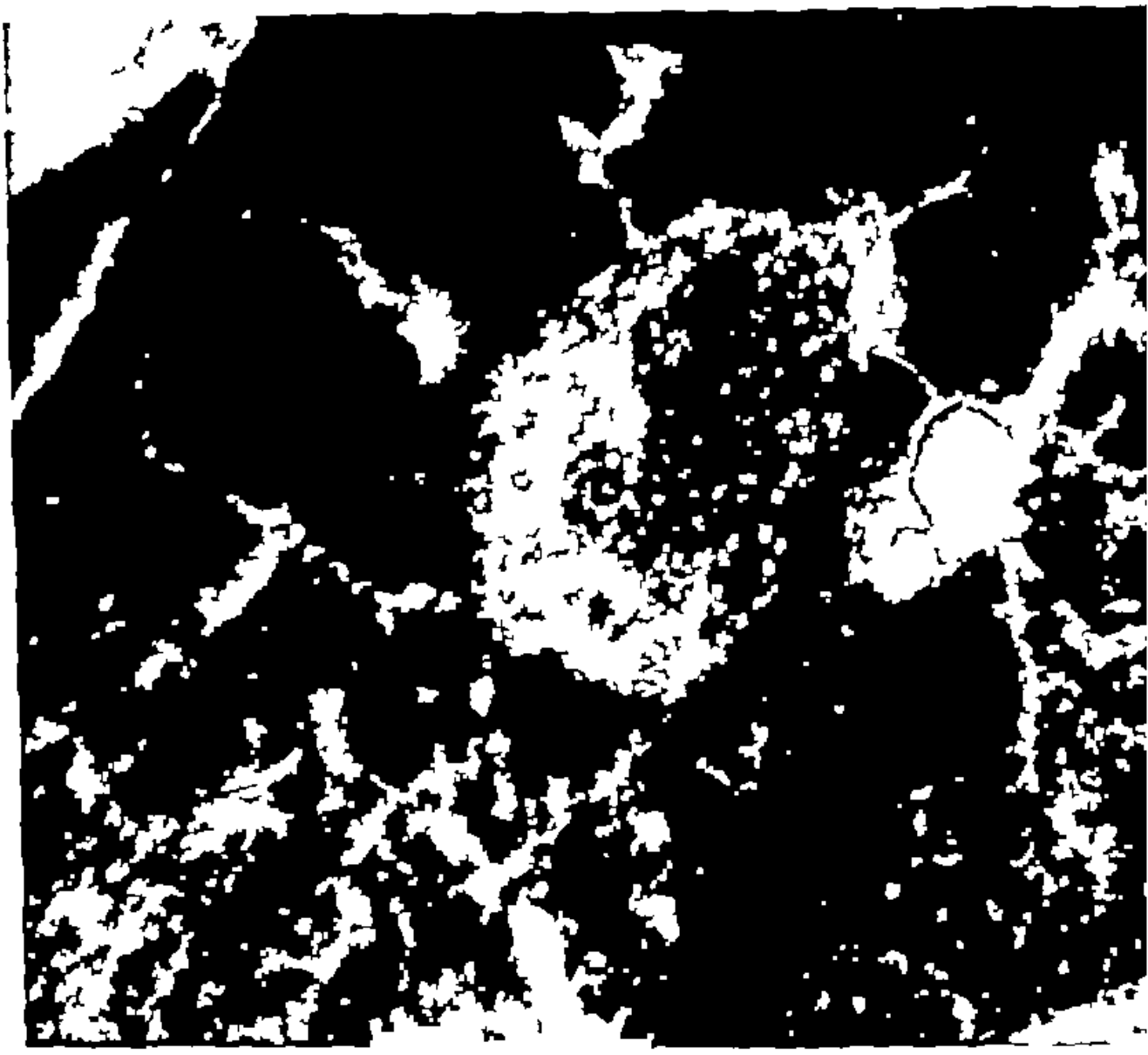


Fig. 8. MAO-activity in the pancreas of mouse. The strongest activity is seen in the exocrine pancreas. In the pancreatic islet most cells show moderate activity. Some peripherally located cells show a weak activity and some cells seem to entirely lack activity. (×140)

belled or non-labeled form. The low quantity of precursors injected the labelled form was shown to give no fluorescence of their own.

Monoamine oxidase histochemistry

The highest amount of formazan precipitate indicating the highest monoamine oxidase activity was seen in the exocrine pancreas. In the islet the majority of cells showed a moderate activity. Other cells, localized more in the periphery of the islets showed a weak activity and some cells, also peripherally localized, seemed to be entirely lacking activity (Fig. 8).

Discussion

The results obtained in the present investigation show that the localization of catechol and indolamines formed in the pancreatic islets after the injection of DOPA and 5-HTP is similar.

The accumulation of activity after the injection of the precursors is shown to appear very rapidly. This accumulation is accompanied by a decarboxylation of the precursors (Cegrell 1968). The decarboxylation is a rapid process (Udenfriend *et al.* 1957) which takes place via the action of aromatic L-amino acid decarboxylase (Lovenberg *et al.* 1962 a) an enzyme common to the two precursors. The presence of this enzyme in the pancreas has been shown (Holt *et al.* 1942, West 1958). Inhibition of the decarboxylase reduces the fluorescence induced in the islets after a DOPA injection (Cegrell 1968). This might indicate that the condition precedent for the retention of DOPA and 5-HTP in the pancreatic islets is the decarboxylation to the corresponding amine as will also be shown to be the case in the adrenal medulla (see Chapter 5). Reduced fluorescence after decarboxylase inhibition, when DOPA was injected into mice, in the parafollicular cells and in the enterochromaffin cell system has also been noted (Larsson *et al.* 1966, Hökfelt *et al.* 1967 a).

The monoamines persist in the pancreatic islets for a longer period after the inhibition of monoamine oxidase (MAO). This has been shown after the injection of DOPA (Cegrell 1968) and is now shown to be valid also for 5-HTP. The differentiation between cells with high and low monoamine content in the islets also disappears after the MAO-inhibition. As has been pointed out (Cegrell 1968) there seems to be an inverse relation between the MAO activity in and the monoamine disappearance from the islets, since the highest MAO content is present in centrally located cells, probably the β-cells, while peripherally located cells either contain a low activity or none. The low MAO activity in peripherally located cells has been observed earlier in other species (Petkov 1965). A direct identification of α- or β-cells with aldehyde fuchsin staining or silver impregnation methods on sections treated for MAO histochemistry was in the present investigation found to be impossible.

Radioactivity could in the present investigation easily be demonstrated even 24 hours after the injection. Fluorescence can be demonstrated for a much shorter time period. The inability to demonstrate an endogenous storage of monoamines in some mammals might therefore depend on the stores of monoamines being too low to be demonstrated with histochemical methods as also has been proposed (Cegrell 1968).

CHAPTER 7

Uptake of DOPA and 5-HTP in the parafollicular cells of the thyroid of the mouse studied by autoradiography and combined autoradiography fluorescence microscopy

As was the case for the pancreatic islets, the parafollicular cells of the thyroid have been shown to contain monoamines in some species, while other species lack demonstrable amounts of monoamines. Thus serotonin has been found to be endogenously occurring in the parafollicular cells of goat and sheep (Falck *et al* 1964 Falck and Owman 1968) As for the pancreatic islets, the injection of DOPA or 5-HTP leads to a formation of monoamines also in species where an endogenous monoamine storage cannot be demonstrated which has been shown with *autoradiography and fluorescence microscopy* (Falck *et al.* 1964 Rutén *et al.* 1965 Gershon and Ross 1966 a, Larsson *et al.* 1966, Pearse 1966, Owman and Sundler 1967 Ericson 1970)

In the present investigation the combined microautoradiographic—fluorescence microscopic technique has been used to investigate whether the cells which take up DOPA are identical with the cells which take up 5-HTP Some data from a separate microautoradiographic study are also presented.

Material and methods

The microautoradiographic method and the combined microautoradiographic—fluorescence microscopic method used have been described earlier (see Chapter 1) The number of animals, the substances injected and the survival times are given in Table 2.

Results

Microaut radiography

There seemed to be no difference between the microautoradiograms obtained after the ^{3}H DOPA and ^{3}H-5-HTP injections.

High activity could be seen in the parafollicular cells already five minutes after the injection (Fig 9) and was still present after 24 hours. The activity was greater than the corresponding activity in the most active cells of the pancreatic islets at the corresponding intervals.

The follicular cells contained a small amount of activity in comparison with the parafollicular cells. The activity which could be observed in them was a

TABLE II Thyroid.

Microautoradiography

No. of animals in each survival time	Treatment (substance and dose)	Survival time
5	H DOPA, 0.5 mCi corresponding to 0.18 mg/kg	5 min, 1 h, 24 h
5	H-5-HTP 0.5 mCi corresponding to 2.16 mg/kg	5 min, 1 h, 24 h

Combined microautoradiography — fluorescence microscopy

No. of animals in each survival time	Treatment (substance and dose)	Survival time
2	H 5-HTP 0.5 mCi corresponding to 2.16 mg/kg + L-DOPA 100 mg/kg	30 min, 2 h, 4 h
2	H DOPA, 0.5 mCi corresponding to 0.18 mg/kg + L 5-HTP 100 mg/kg	30 min, 2 h, 4 h

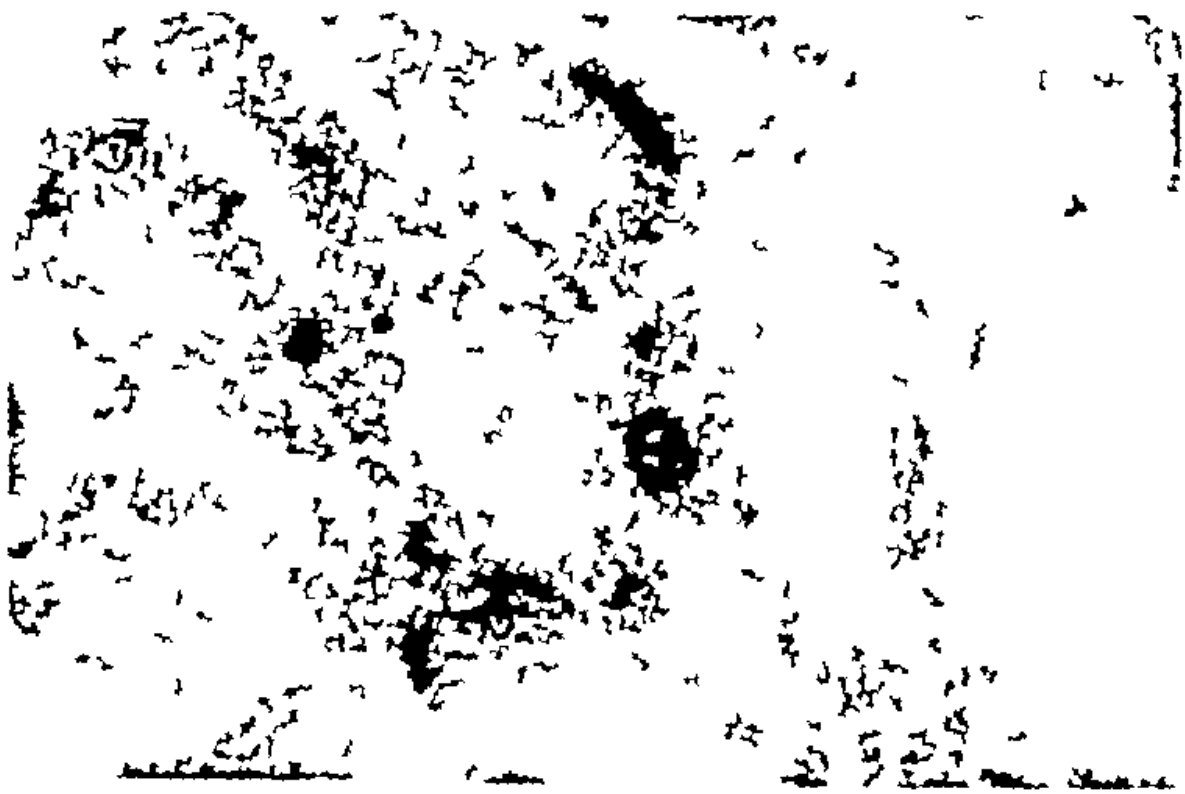


Fig. 9 Microautoradiogram of the thyroid of a mouse 5 minutes after an intravenous injection of ^{3}H 5-HTP. A strong accumulation of radioactivity can be seen in the parafollicular cells. Hæ-eosin (×360)

in the parafollicular cells restricted to the cytoplasm of the cells. The activity in the follicle cells was a little higher than the activity in the colloid.

Combined microautoradiography—fluorescence microscopy

The combination of microautoradiography and fluorescence microscopy showed that the localization of catechol- and indolamines corresponded well (Fig. 10). The strongest fluorescing cells contained the highest radioactivity and vice versa. As can be seen in Fig. 11 fluorescence is present in the parafollicular cells 4 hours after the injection, while at this time no fluorescence is present in the pancreatic islets.

Discussion

There seems to be no principal difference between the monoaminergic mechanisms in the parafollicular cells and in the pancreatic islets.

Formation of catechol- and indolamines takes place in the same parafollicular cells. The accumulation of activity occurs rapidly. Inhibition of the decarboxylase has been shown to reduce a precursor induced fluorescence in the parafollicular cells and inhibition of MAO has been shown to produce a longer persistence of the fluorescence (Larsson *et al.* 1966). These mechanisms have been discussed in Chapter 1.

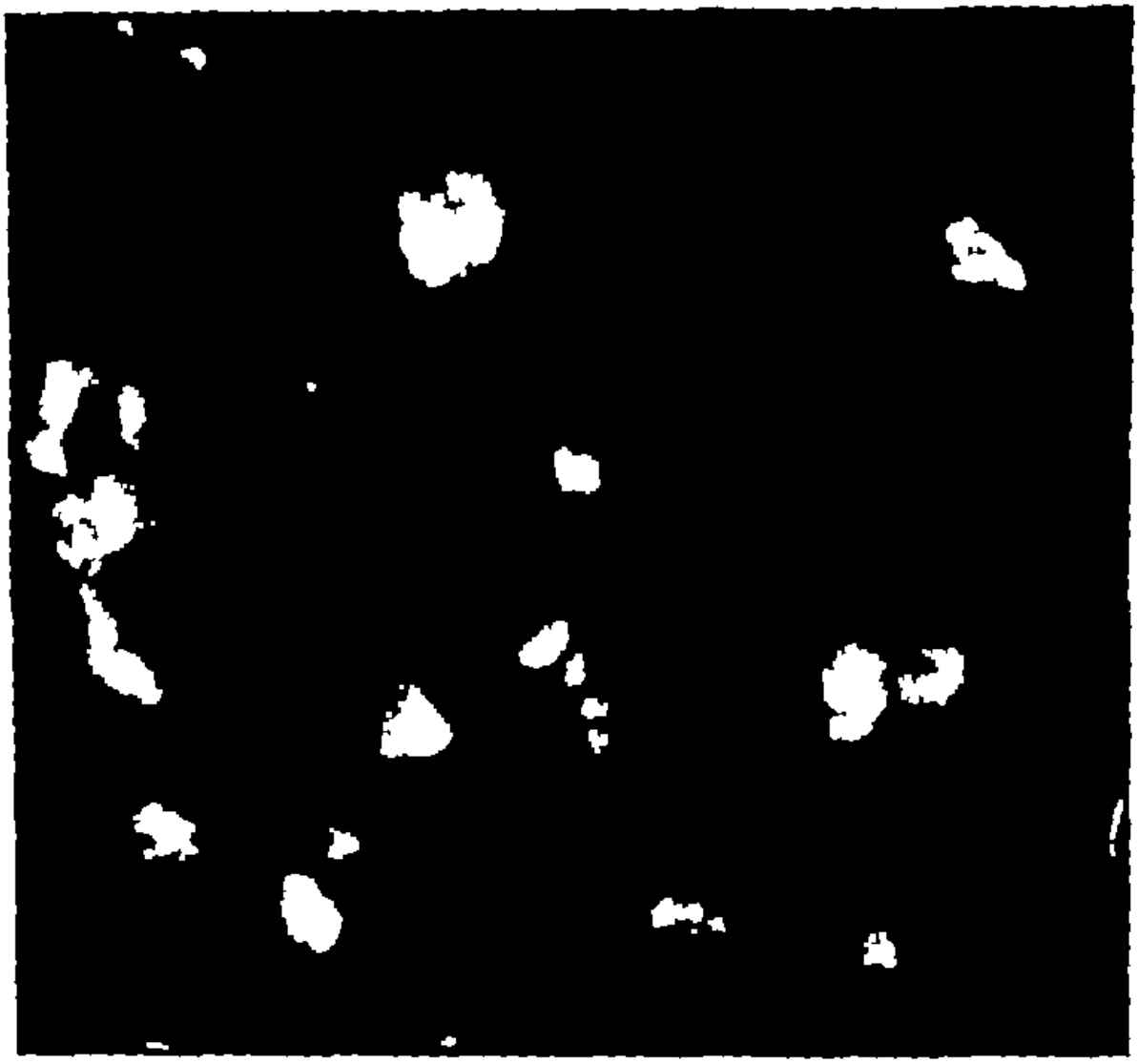


Fig. 10 a. Fluorescence photomicrograph of a section from the thyroid of mouse 30 minutes after an intr enous injection of non-labelled DOPA.

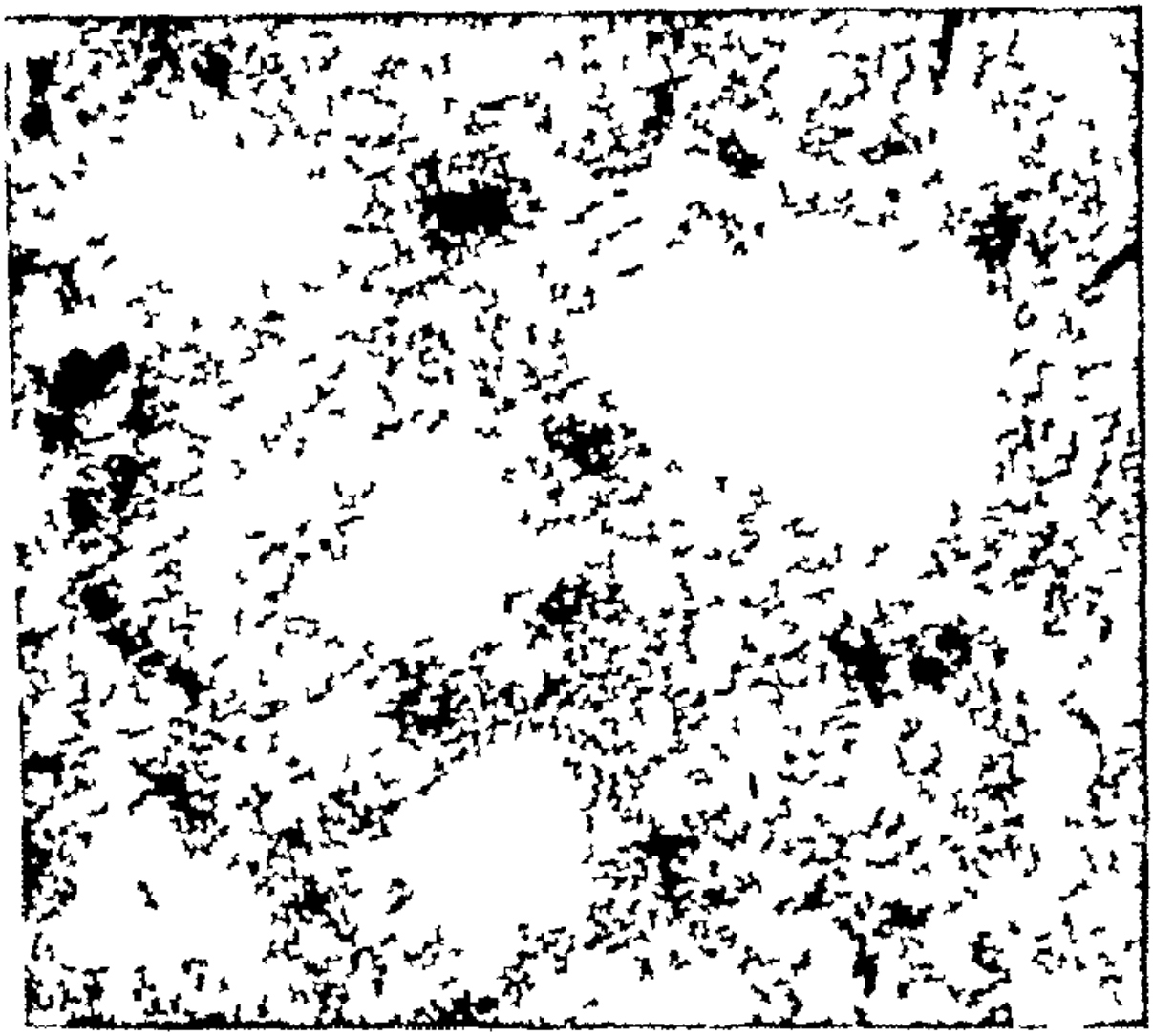


Fig. 10 b. Microautoradiogram of the same section obtained through the simultaneous injection of H 5-HTP Hex-rosin. The fluorescence in the parafollicular cells is in accordance with the accumulation of silver grains. (×410)

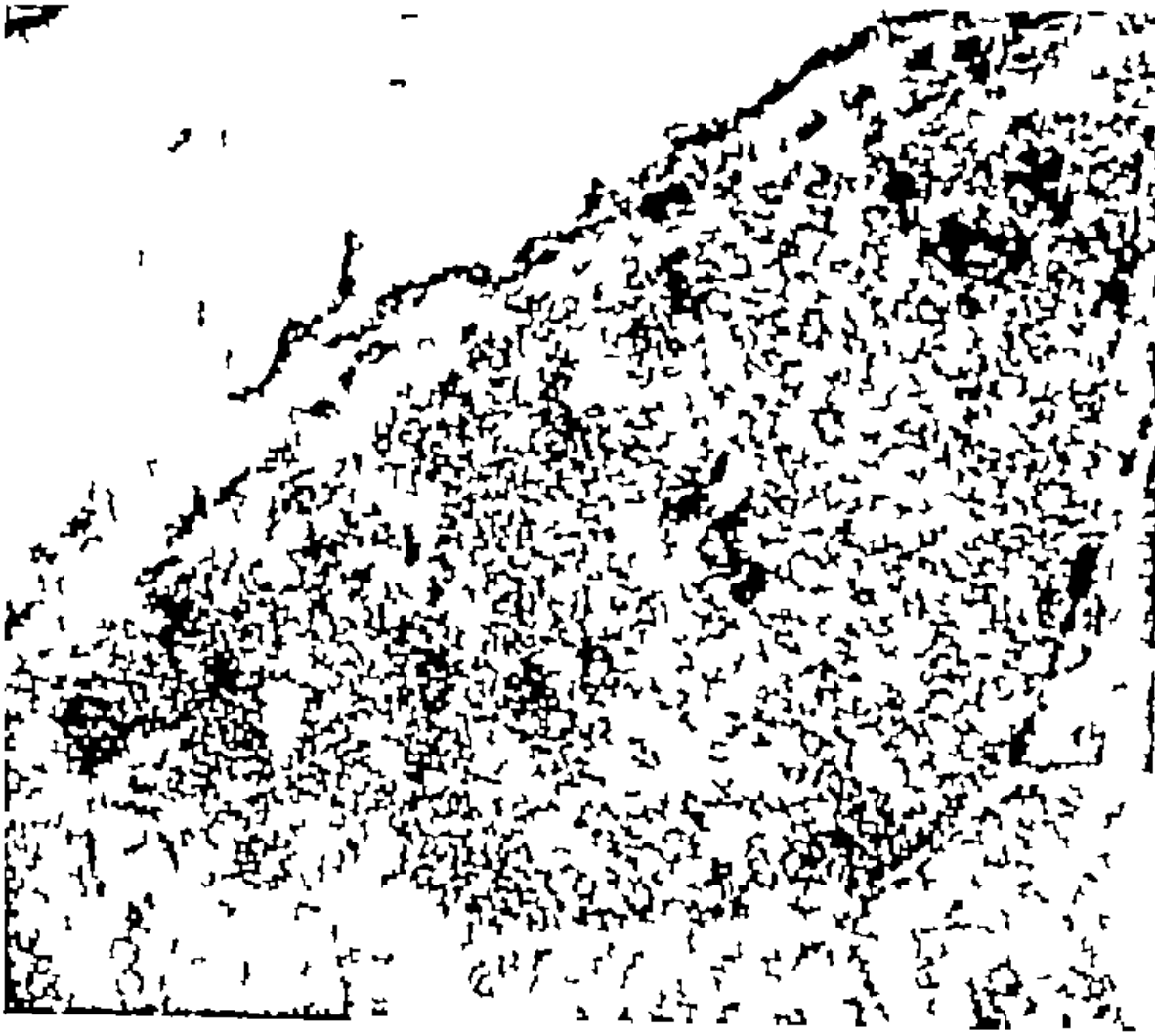


Fig. 3 a. Micro toradiogram f pancreatic islet of a mouse 1 hour after an intra enous injection of ^{3}H DOPA. Hux-eosin.

It was not possible to stain sections subjected to monoamine oxidase histochemistry with aldehyde fuchsin or silver staining techniques for identification of β- and α-cells in the pancreatic islets.

Results

Microaut adiography and fluorescence microscopy

The distribution of radioactivity in the pancreatic islets after the injection of ^{3}H DOPA was similar to the distribution seen after the injection of ^{3}H 5-HTP at the survival times studied.

Fi e minutes after the injection of ^{3}H DOPA or ^{3}H 5-HTP there was a very high activity in all cells of the pancreatic islets (Fig 1) The activity in the endocrine pancreas exceeded somewhat that in the exocrine pancreas, \ here a diffuse distribution of the activity could be seen.

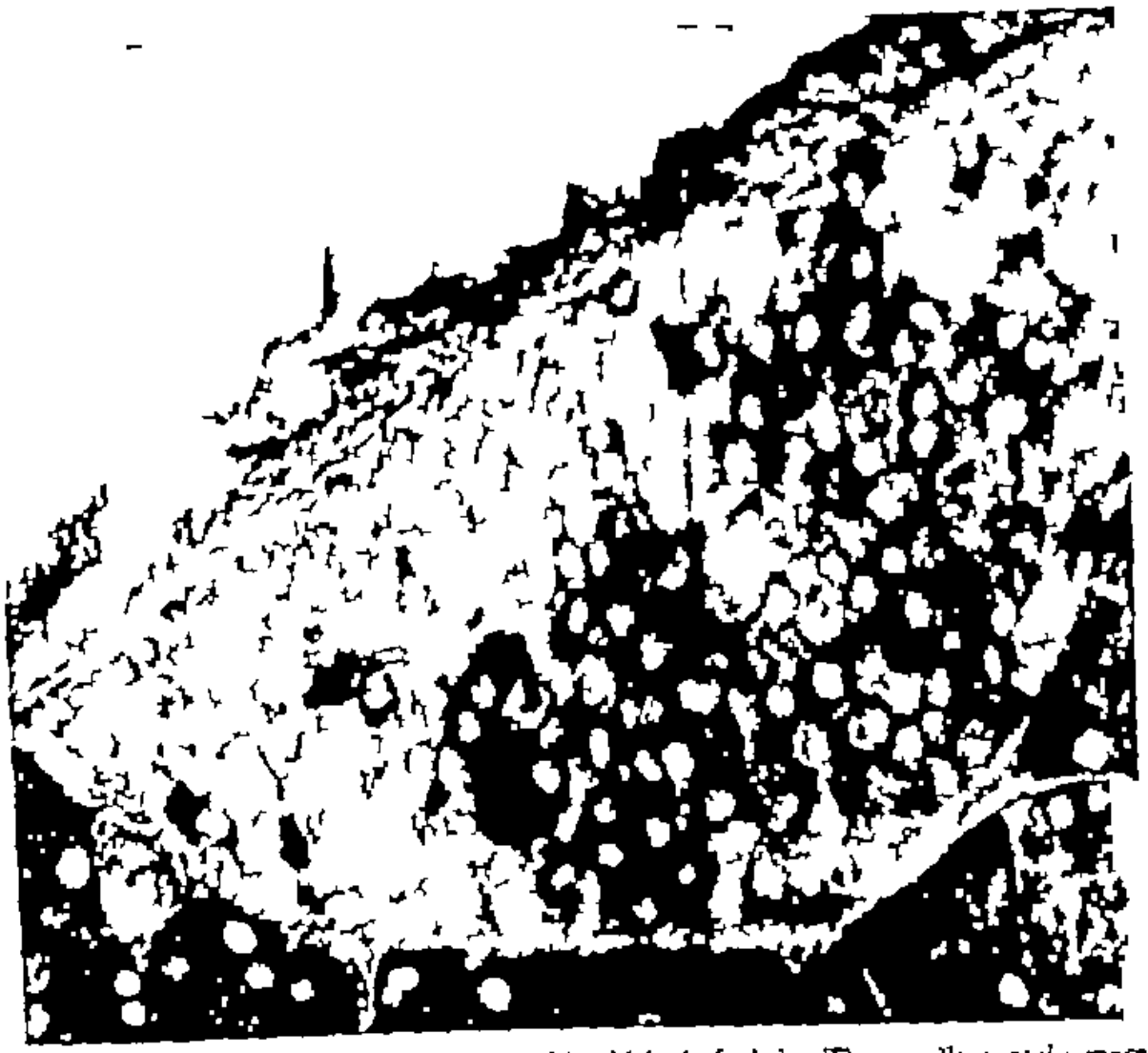


Fig. 3 b. The same islet after staining with aldehyde-fuchsin. The α-cells contain more radioactivity than the β-cells. Some α-cells are also more heavily labelled than other α-cells. (×380)

One hour after the injection of ^{3}H DOPA or ^{3}H 5-HTP some cells, mostly located at the periphery of the islets, contained a high activity (Fig. 2). The number of these cells varied between different islets. Subsequent aldehyde fuchsin staining showed that the cells belonged to the α-cell group. There were, however also α-cells with a lower amount of activity but also here the activity was considerable and seemed to exceed the activity in the β-cells to some extent (Fig. 3). In the exocrine pancreas more activity could be seen in the apical than in the basal parts of the cells. Also the excretory ducts contained a high activity.

Twenty-four hours after the injection of ^{3}H DOPA or ^{3}H 5-HTP a high activity usually still persisted in a few scattered cells at the periphery of the islets. The number of these cells, also belonging to the α-cell group varied considerably from islet to islet and were lacking in some islets. In the exocrine

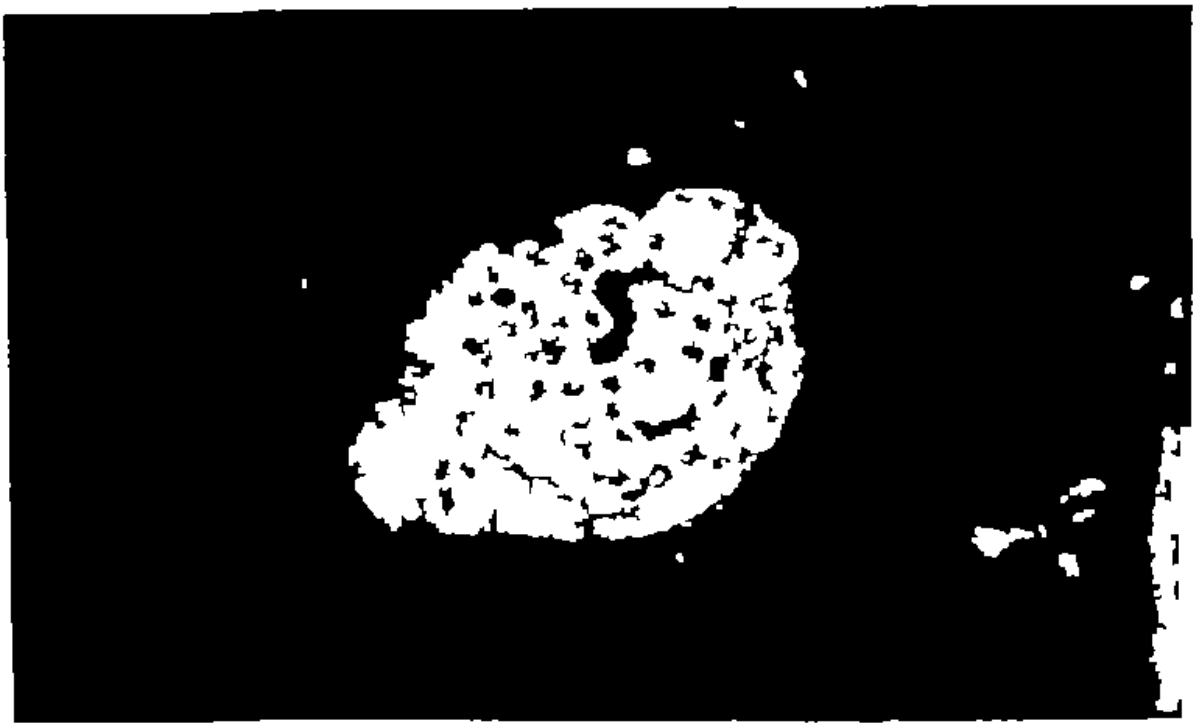


Fig. 4 Fluorescence photomicrograph of a pancreatic islet of mouse 30 minutes after an intravenous injection of DOPA. Fluorescence can be seen in all cells of the islet. Some peripherally located cells show little stronger fluorescence than the other cells. (×150)

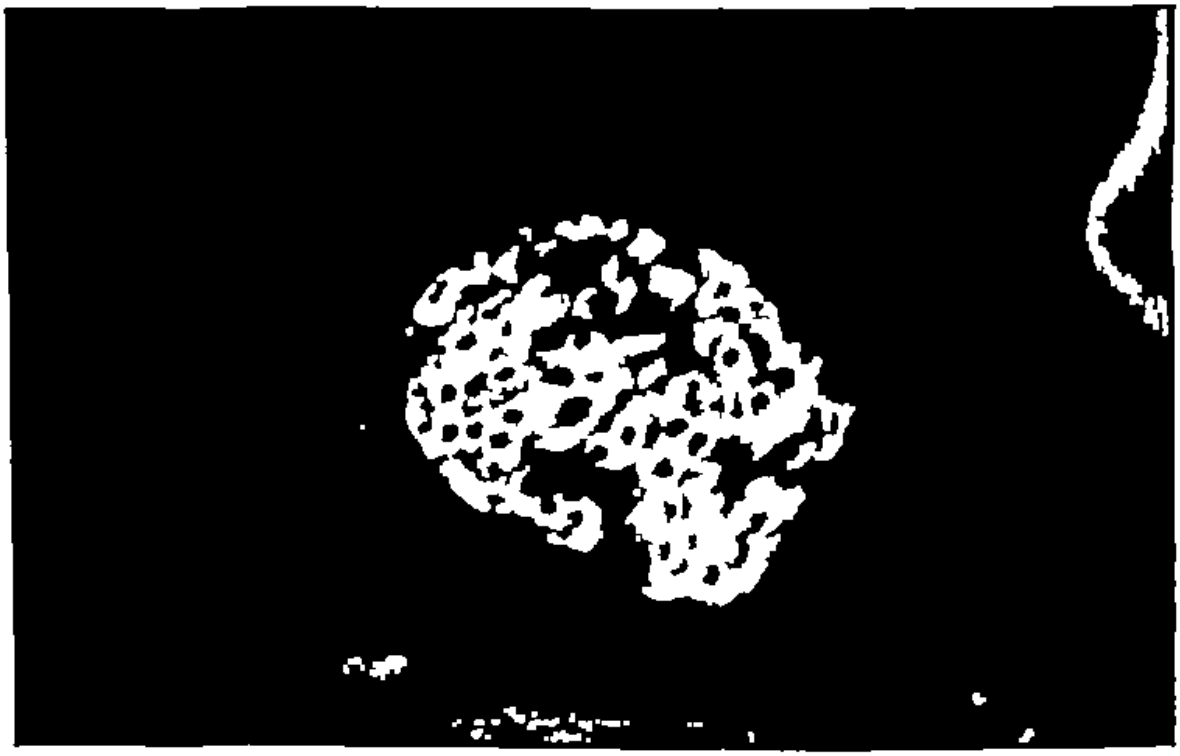


Fig. 5 Fluorescence photomicrograph of pancreatic islet of mouse 1 hour after an intravenous injection of DOPA. Some peripherally located cells show the strongest fluorescence. Some cells show lower and some even lower fluorescence. (×150)

pancreas activity could still be seen in the apical parts of acinar cells and in excretory ducts.

The fluorescence pictures obtained after injection of non-labelled DOPA and 5-HTP were also very similar to each other and showed the same general distribution patterns as seen in the autoradiograms (Fig 4 and 5) However the lower sensitivity of the fluorescence method, in comparison with the microautoradiographic method resulted in the disappearance of fluorescence 4 hours after the injection, while radioactivity could be observed in the islets 24 hours after the injection. The fluorescence pictures and the autoradiograms differed also, in so far as the gradation between cells with different label was more pronounced in the fluorescent pictures, where they gave the impression of an all-or nothing effect.

If the animals were pre treated with the monoamine oxidase inhibitor nialamide there was a longer persistence of fluorescence in the islets after the injection of DOPA or 5-HTP In addition there was no differentiation between peripherally located cells with high fluorescence and centrally located cells with lower fluorescence up to three hours after the injection (Fig 6) Four hours after the injection the picture was similar to a picture seen after two hours without pre treatment with nialamide.

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The combination of fluorescence microscopy and microautoradiography showed that the fluorescence obtained in the islets through the injection of one of the precursors (non-labelled) was in accordance with the radioactivity obtained through th injection of the other precursor (labelled) (Fig. 7) The result obtained was independent of whichever of the precursors was injected in la

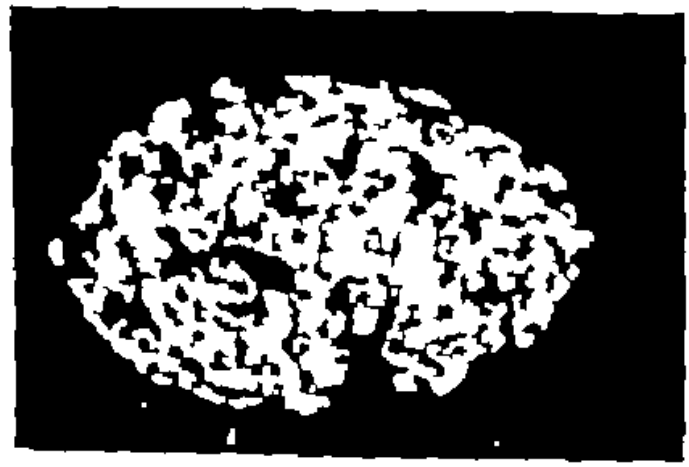


Fig. 6. Fluorescence photomicrograph of pancre tic islet 3 hours after an intravenous injection of 5-HTP int a mouse which has been pre-treated with the MAO-inhibitor lalamide Fluorescence can be seen in all cells of the islet. (×130)

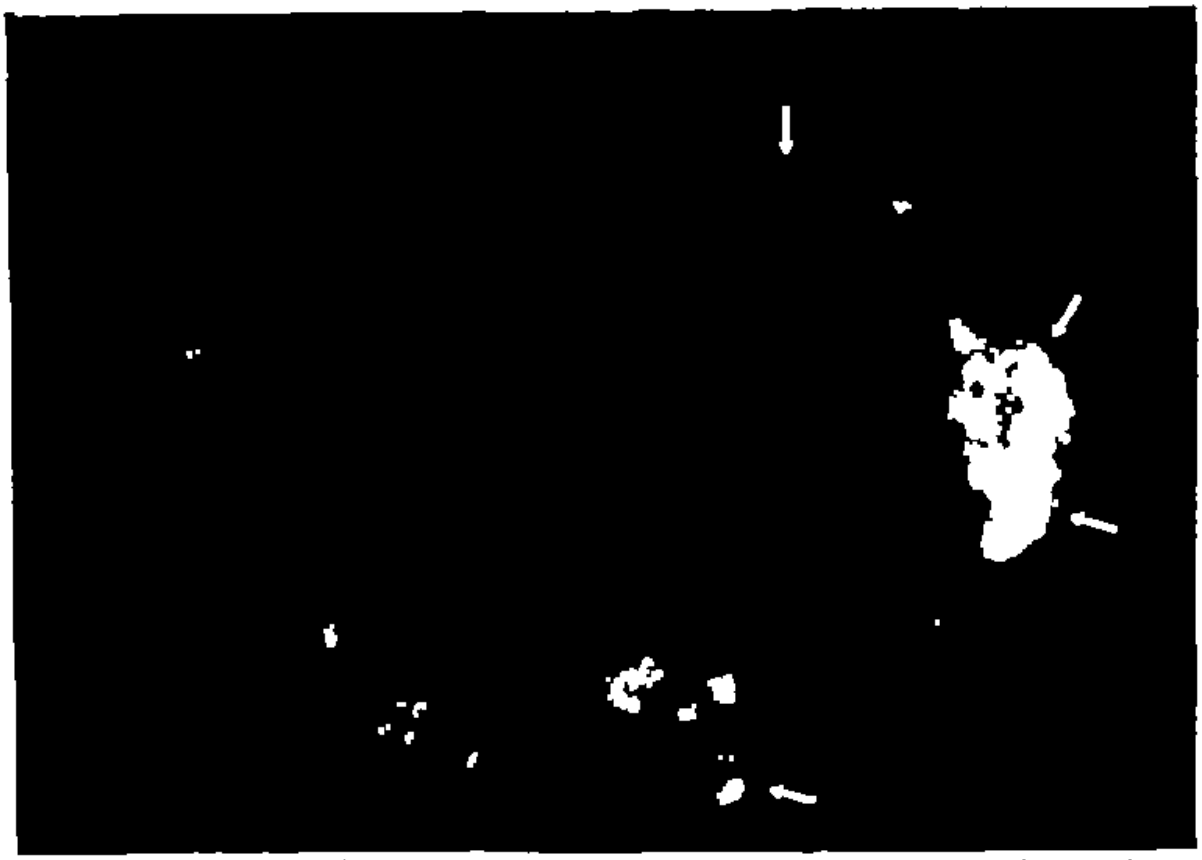


Fig. 7 a. Fluorescence photomicrograph of an islet of mouse 2 hours after an intravenous injection of non-labelled DOPA.



Fig. 7 b. Microautoradiogram of the same islet obtained through the simultaneous injection of ^{3}H 5-HTP Htx-eosin. Cells with the strongest fluorescence have also accumulated the highest radioactivity (arrow) (×350)

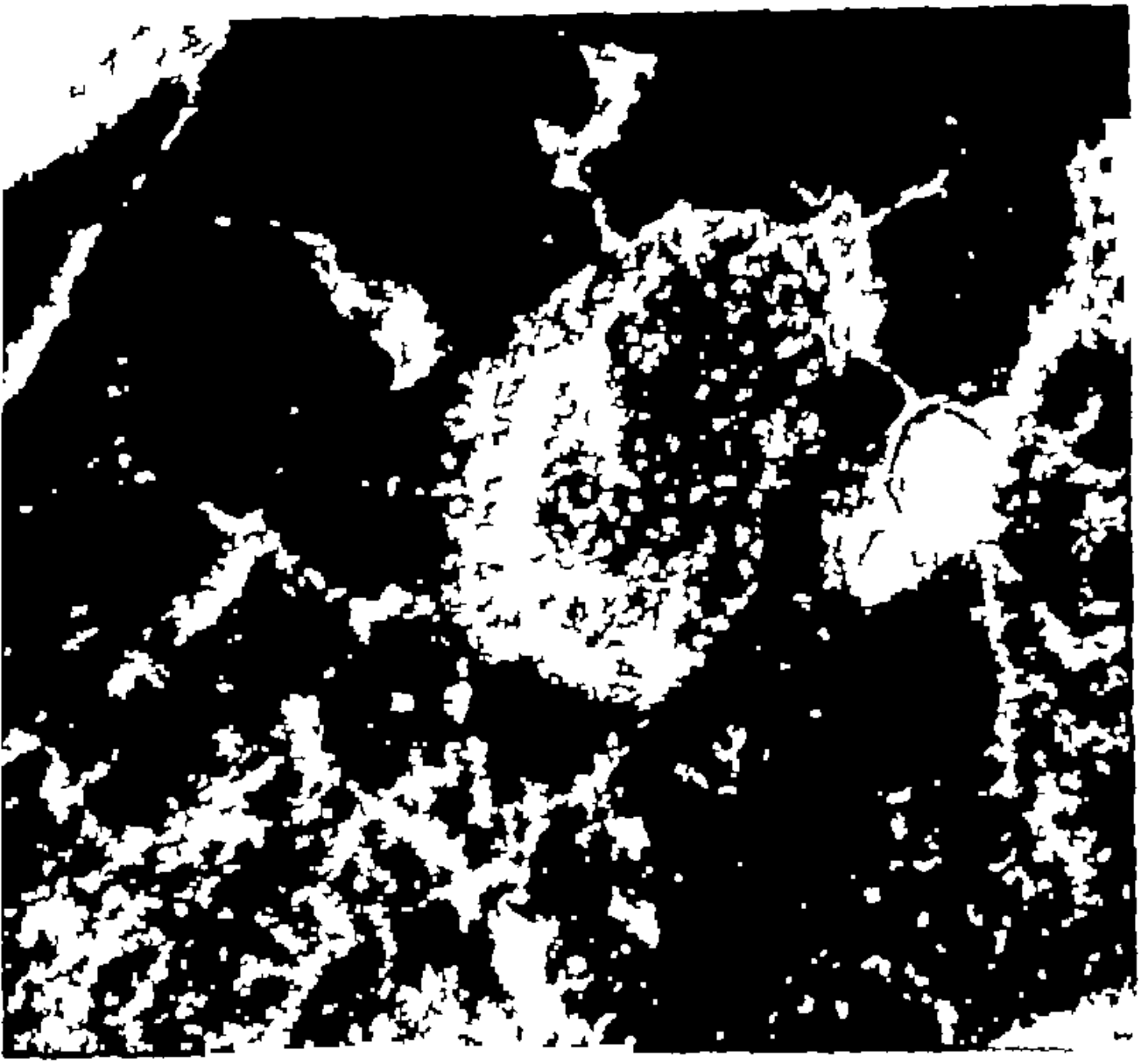


Fig. 8. MAO-activity in the pancreas of mouse. The strongest activity is seen in the exocrine pancreas. In the pancreatic islet most cells show a moderate activity. Some peripherally located cells show weak activity and some cells seem to entirely lack activity (×140)

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Monoamine oxidase histochemistry

The highest amount of formazan precipitates, indicating the highest monoamine oxidase activity was seen in the exocrine pancreas. In the islet the majority of cells showed a moderate activity. Other cells, localized more in the periphery of the islets, showed a weak activity and some cells, also peripherally localized, seemed to be entirely lacking activity (Fig. 8)

Discussion

The results obtained in the present investigation show that the localization of catechol- and indolamines formed in the pancreatic islets after the injection of DOPA and 5-HTP is similar

The accumulation of activity after the injection of the precursors is shown to appear very rapidly Thus accumulation is accompanied by a decarboxylation of the precursors (Cegrell 1968) The decarboxylation is a rapid process (Udenfriend *et al.* 1957) which takes place via the action of aromatic L-amino acid decarboxylase (Lovenberg *et al.* 1962 a) an enzyme common to the two precursors. The presence of this enzyme in the pancreas has been shown (Holz *et al* 1942, West 1958) Inhibition of the decarboxylase reduces the fluorescence induced in the islets after a DOPA injection (Cegrell 1968) This might indicate that the condition precedent for the retention of DOPA and 5-HTP in the pancreatic islets is the decarboxylation to the corresponding amine as will also be shown to be the case in the adrenal medulla (see Chapter 5) Reduced fluorescence after decarboxylase inhibition, when DOPA was injected into mice, in the parafollicular cells and in the enterochromaffin cell system has also been noted (Larsson *et al.* 1966, Håkanson *et al* 1967 a)

The monoamines persist in the pancreatic islets for a longer period after the inhibition of monoamine oxidase (MAO) This has been shown after the injection of DOPA (Cegrell 1968) and is now shown to be valid also for 5-HTP The differentiation between cells with high and low monoamine content in the islets also disappears after the MAO-inhibition. As has been pointed out (Cegrell 1968) there seems to be an inverse relation between the MAO activity in and the monoamine disappearance from the islets, since the highest MAO content is present in centrally located cells, probably the β-cells, while peripherally located cells either contain a low activity or none. The low MAO activity in peripherally located cells has been observed earlier in other species (Petkov 1965) A direct identification of α or β-cells with aldehyde fuchsin staining or silver impregnation methods on sections treated for MAO histochemistry was in the present investigation found to be impossible.

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CHAPTER 2

Uptake of DOPA and 5-HTP in the parafollicular cells of the thyroid of the mouse studied by autoradiography and combined autoradiography fluorescence microscopy

As was the case for the pancreatic islets, the parafollicular cells of the thyroid have been shown to contain monoamines in some species, while other species lack demonstrable amounts of monoamines. Thus serotonin has been found to be endogenously occurring in the parafollicular cells of goat and sheep (Falck *et al.* 1964 Falck and Owman 1968) As for the pancreatic islets, the injection of DOPA or 5-HTP leads to a formation of monoamines also in species where an endogenous monoamine storage cannot be demonstrated, which has been shown with autoradiography and fluorescence microscopy (Falck *et al.* 1964 Ritzén *et al.* 1965 Gershon and Ross 1966 a, Larsson *et al.* 1966, Pearse 1966 Owman and Sundler 1967 Ericson 1970)

In the present investigation the combined microautoradiographic—fluorescence microscopic technique has been used to investigate whether the cells which take up DOPA are identical with the cells which take up 5-HTP Some data from a separate microautoradiographic study are also presented.

Material and methods

The microautoradiographic method and the combined microautoradiographic—fluorescence microscopic method used have been described earlier (see Chapter 1) The number of animals, the substances injected and the survival times are given in Table 2.

Results

Microautoradiography

There seemed to be no difference between the microautoradiograms obtained after the ^{3}H DOPA- and H-5-HTP-injections.

High activity could be seen in the parafollicular cells already five minutes after the injection (Fig 9) and was still present after 24 hours. The activity was greater than the corresponding activity in the most active cells of the pancreatic islets at the corresponding time intervals.

The follicular cells contained a small amount of activity in comparison with the parafollicular cells. The activity which could be observed in them was as

TABLE II Thyroid.

Microautoradiography

No. of animals in each survival time	Treatment (substance and dose)	Survival time
3	H-DOPA, 0.5 mCi corresponding to 0.18 mg/kg	5 min, 1 h, 24 h
3	H-5-HTP 0.5 mCi corresponding to 2.16 mg/kg	5 min, 1 h, 24 h

Combined microautoradiography — fluorescence microscopy

No. of animals in each survival time	Treatment (substance and dose)	Survival time
2	H 5-HTP 0.5 mCi corresponding to 2.16 mg/kg + L-DOPA 100 g/kg	30 min, 2 h, 4 h
2	H DOPA, 0.5 mCi corresponding to 0.18 mg/kg + L 5-HTP 100 mg/kg	30 min 2 h, 4 h

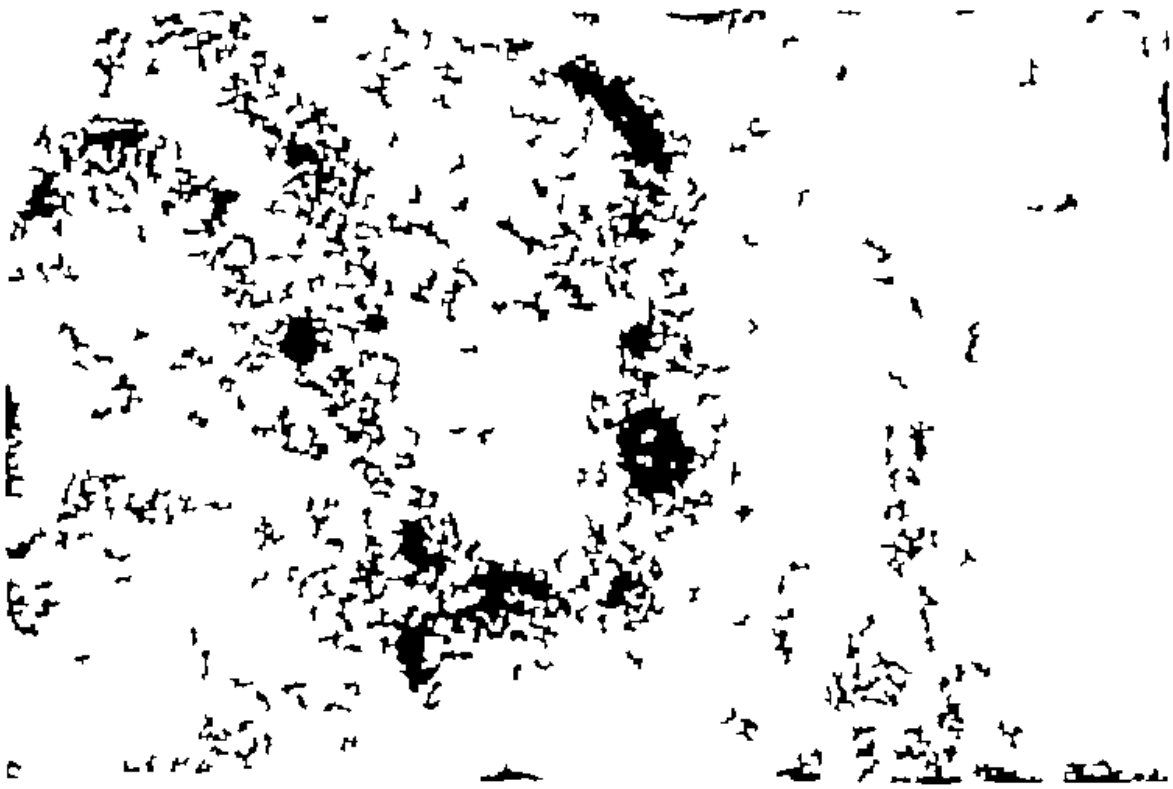


Fig. 9 Microautoradiogram of the thyroid of mouse 5 minutes after an intravenous injection of ^{3}H 5-HTP. A strong accumulation of radioactivity can be seen in the parafollicular cells. Htx-eosin (×360)

in the parafollicular cells restricted to the cytoplasm of the cells. The activity in the follicle cells was a little higher than the activity in the colloid.

Combined microautoradiography—fluorescence microscopy

The combination of microautoradiography and fluorescence microscopy showed that the localization of catechol- and indolamines corresponded well (Fig. 10). The strongest fluorescing cells contained the highest radioactivity and vice versa. As can be seen in Fig. 11 fluorescence is present in the parafollicular cells 4 hours after the injection, while at this time no fluorescence is present in the pancreatic islets.

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Formation of catechol- and indolamines takes place in the same parafollicular cells. The accumulation of activity occurs rapidly. Inhibition of the decarboxylase has been shown to reduce a precursor induced fluorescence in the parafollicular cells and inhibition of MAO has been shown to produce a longer persistence of the fluorescence (Larsson *et al.* 1966). These mechanisms have been discussed in Chapter 1.

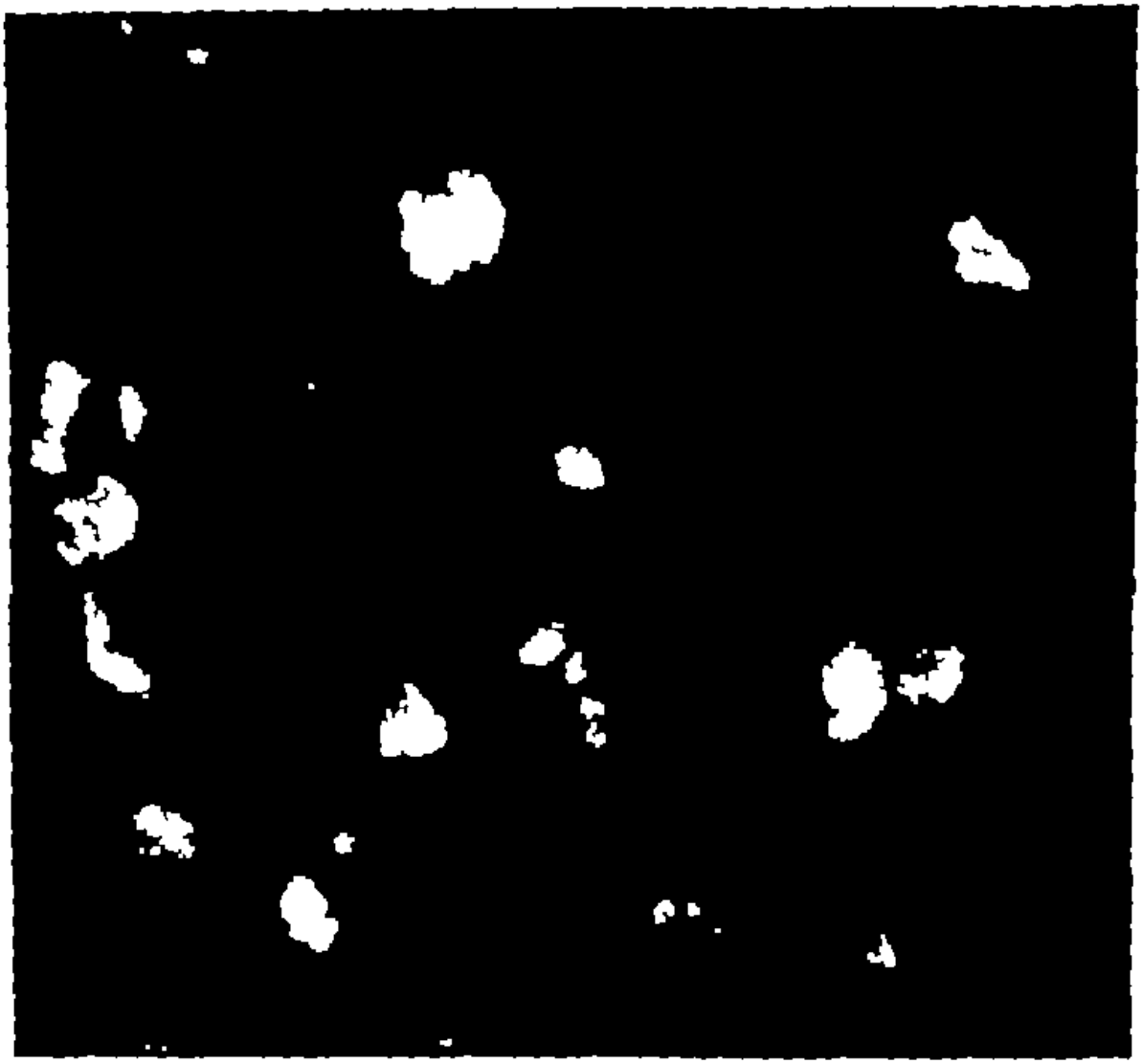


Fig. 10 a. Fluorescence photomicrograph of a section from the thyroid of a mouse 30 minutes after an intravenous injection of non-labelled DOPA.

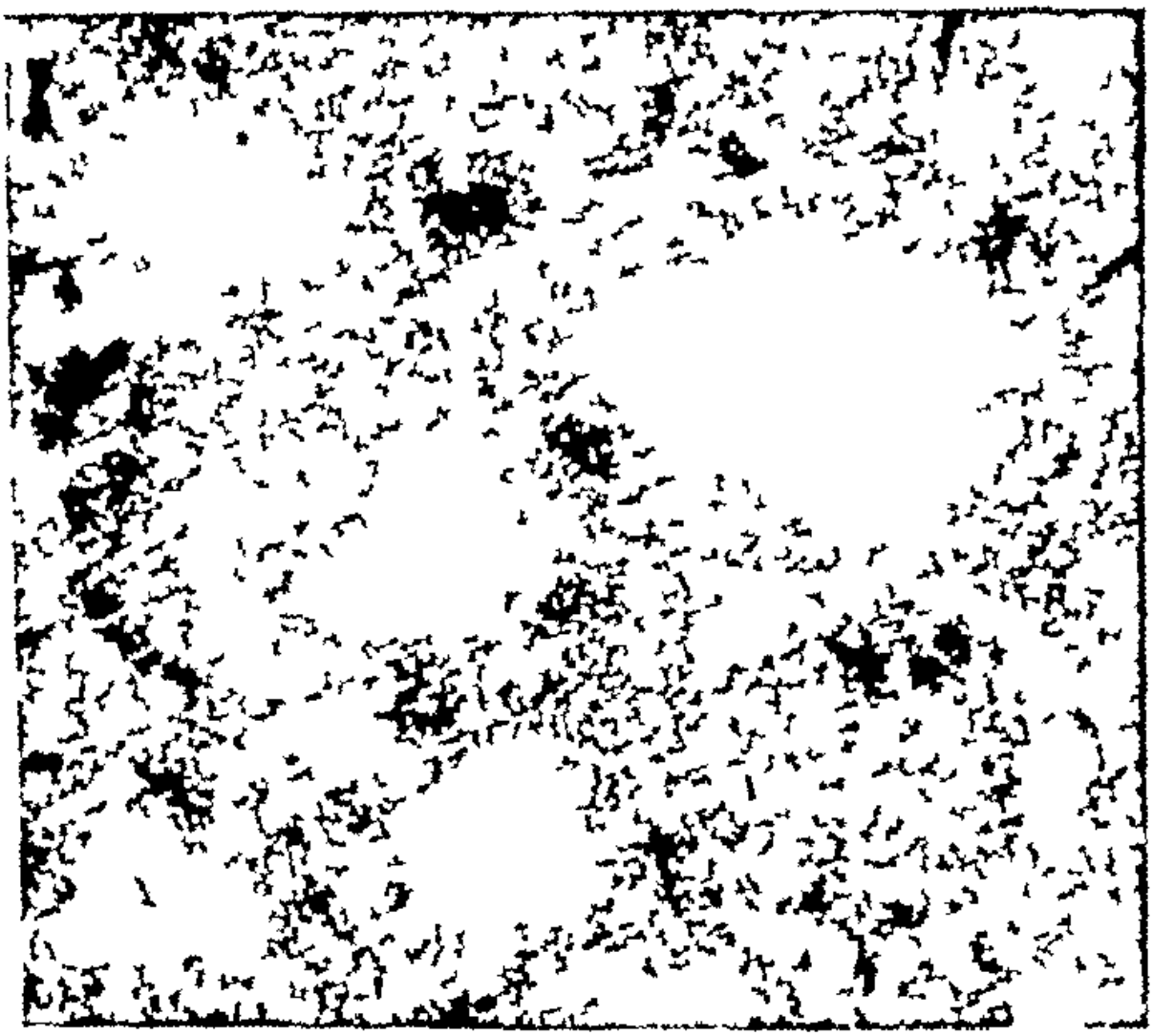


Fig. 10 b. Microautoradiogram of the same section obtained through the simultaneous injection of H 5-HTP Hex-roum. The fluorescence in the parafollicular cells is in accordance with the accumulation of silver grains (×410)



Fig 11 a. Fluorescence photomicrograph of section from the thyroid of mouse 4 hours after an intravenous injection of non-labelled DOPA.

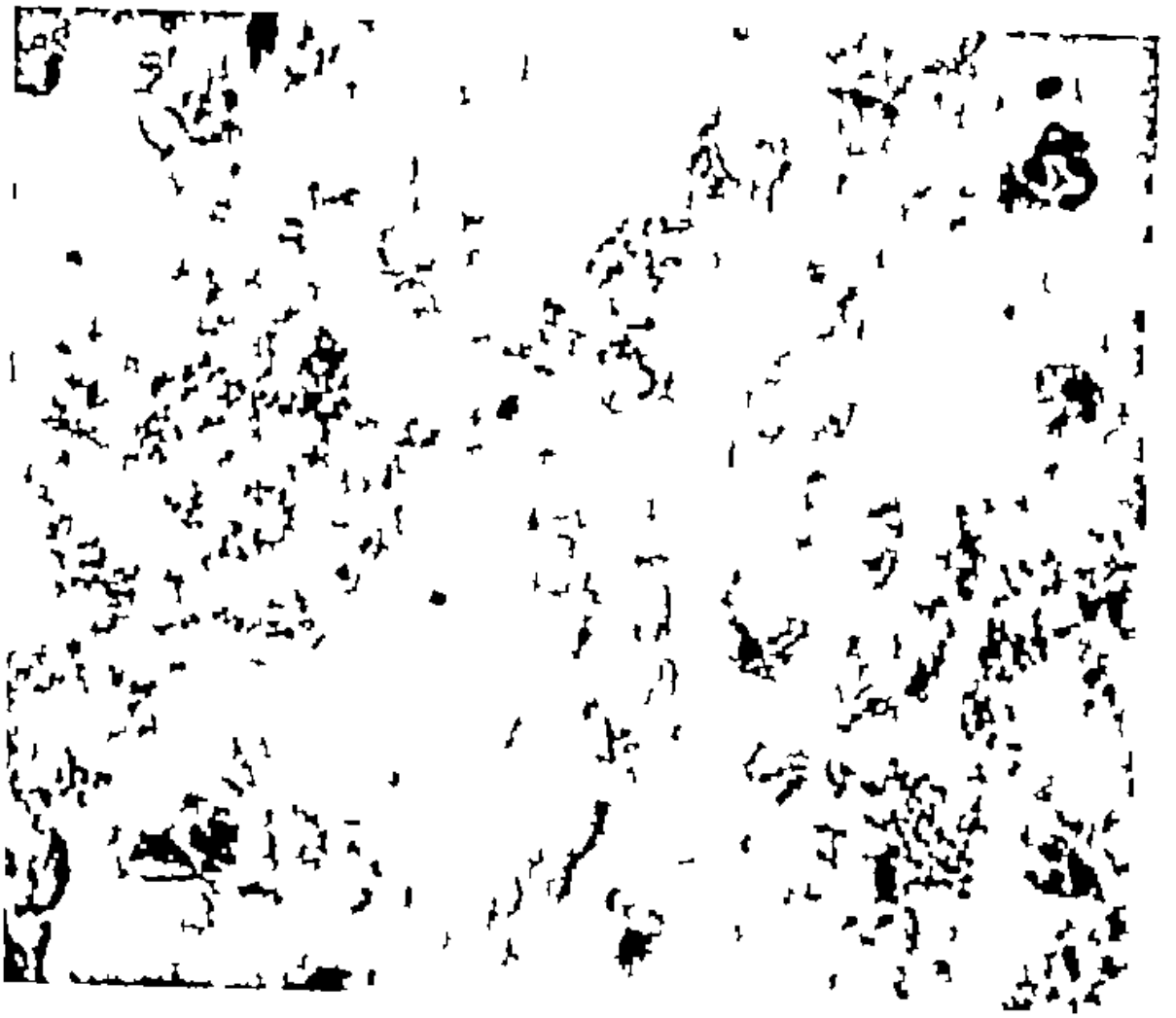


Fig. 11 b. Microautoradiogram of the same section obtained through the parallel injection of ^{3}H 5-HTP. Hix-exalin. Fluorescence and silver grains respectively are concentrated in the cytoplasm of the parafollicular cells and correspond with one another (×720)

CHAPTER 3

Uptake of DOPA and 5 HTP in the mucosa of the stomach of the mouse, studied by autoradiography fluorescence microscopy and combined autoradiography fluorescence microscopy

In the gastrointestinal tract the presence of serotonin has been known since long (Erspamer and Asero 1952) Most of this storage has been shown to be present in the enterochromaffin cells (see Pentulä 1966 Vialli 1966) In the cat and the rabbit dopamine containing enterochromaffin cells have been shown to be present (Håkanson *et al* 1970 a) In addition to these cells, other cells have been demonstrated, which lack endogenous demonstrable storage of monoamines, but possess the monoamine forming capability after injections of monoamine precursors (Håkanson *et al* 1967 a) These cells have recently been extensively studied with fluorescence methods (see Håkanson 1970) A third group of cells in the gastrointestinal mucosa capable of monoamine storage are the mast cells (see Enerbäck 1966 Håkanson *et al* 1970 a)

Apart from fluorescence methods, an uptake of monoamine precursors in the cells of the gastrointestinal mucosa has been shown with microautoradiography (Hammarström *et al.* 1966, Gershon and Ross 1966 a)

In the present investigation an attempt is made to establish in which of the three mentioned cell categories in the stomach of the mouse an uptake of DOPA and 5-HTP takes place and to investigate if the two precursors are taken up equally in the cells. For this purpose microautoradiography and combined microautoradiography—fluorescence microscopy has been employed. A mapping of the cells with endogenous fluorescence was found to be necessary for an evaluation of the results.

Material and methods

Ten untreated mice were used to study the cells with endogenous fluorescence Pieces of the stomach were taken along the smaller and the greater curvatures of the stomach and at varying distances from and parallel to these curvatures. The pieces were taken as to enable later studies of the forestomach, the fundic gland area and the pyloric gland area in the same section. The pieces were subjected to fluorescence microscopy as described earlier (see Chapter 1) Staining with toluidine blue in ethanol solution (Romeis 1948) was used

TABLE III. Stomach.

Microautoradiography

No. of animals at each survival time	Treatment (substance and dose)	Survival tim
3	H DOPA, 0.5 mCi corresponding to 0.18 mg/kg	5 mm. 1 h, 24 h
3	H-5-HTP 0.5 mCi corresponding to 2.16 mg/kg	5 min, 1 h, 24 h

Combined microautoradiography — fluorescence microscopy

No. of animals at each survival time	Treatment (substance and dose)	Survival time
2	H 5-HTP 0.5 mCi corresponding to 2.16 mg/kg + L-DOPA 100 mg/kg	2 h, 4 h
2	H DOPA, 0.5 mCi corresponding to 0.18 mg/kg + L-5-HTP 100 mg/kg	2 h, 4 h

to identify mast cells in the sections. By this staining mast cells show metachromasia.

The procedures used for the microautoradiographic and combined microautoradiographic—fluorescence microscopic studies have been described in Chapter 1. The number of animals used, the substances injected and the survival times are given in Table 3. In the microautoradiographic investigation pieces were taken from different parts of the stomach. In the combined microautoradiographic—fluorescence microscopic investigation pieces were taken from the fundic gland area of the stomach.

Results

Endogenous fluorescence

The fundic gland area adjacent to the forestomach contained a relatively high number of yellow fluorescing cells interspread among the epithelial cells. They were mostly located in the middle or in the superficial parts of the glands, but a few cells could also be seen basally in the glands (Fig. 12). They had their greatest extension along the greater curvature and became gradually limited to a narrower area towards the smaller curvature (Fig. 13). In a band



Fig. 12 Fluorescence photomicrograph of the stomach wall, f ndic gland area, from an untre ted mouse. Yellow fluorescing cells, probably mast cells, are seen. They are most numerous in the middle and the superficial parts of the glands. A few cells are also seen at the base of the glands and in the submucosa. The number of fluorescing cells are most numerous towards the forestomach (to the right in the picture) (×50)

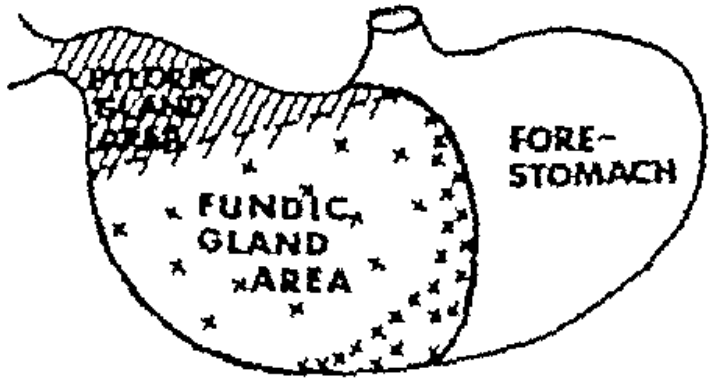


Fig. 13 Distribution of cells w th endogenous fluorescence in the mouse stomach mucosa × × × mast cells. //// enterochromaffin cells. The mast cells are most numerous ad jacent to the forestomach They ha ther greatest extension along the greater curvature and become gradually lighted t mos narrow area towards the smaller curvature. The enterochromaffin cells are presen in the pyloric gland area and in th gradual transition between th fundic gland rea and the pyloric gland area.



Fig. 14 a. Fluorescence photomicrograph of the stomach wall, of an untreated mouse, t the ridge dividing the forestomach (to the right) from the fundic gland area (to the left)

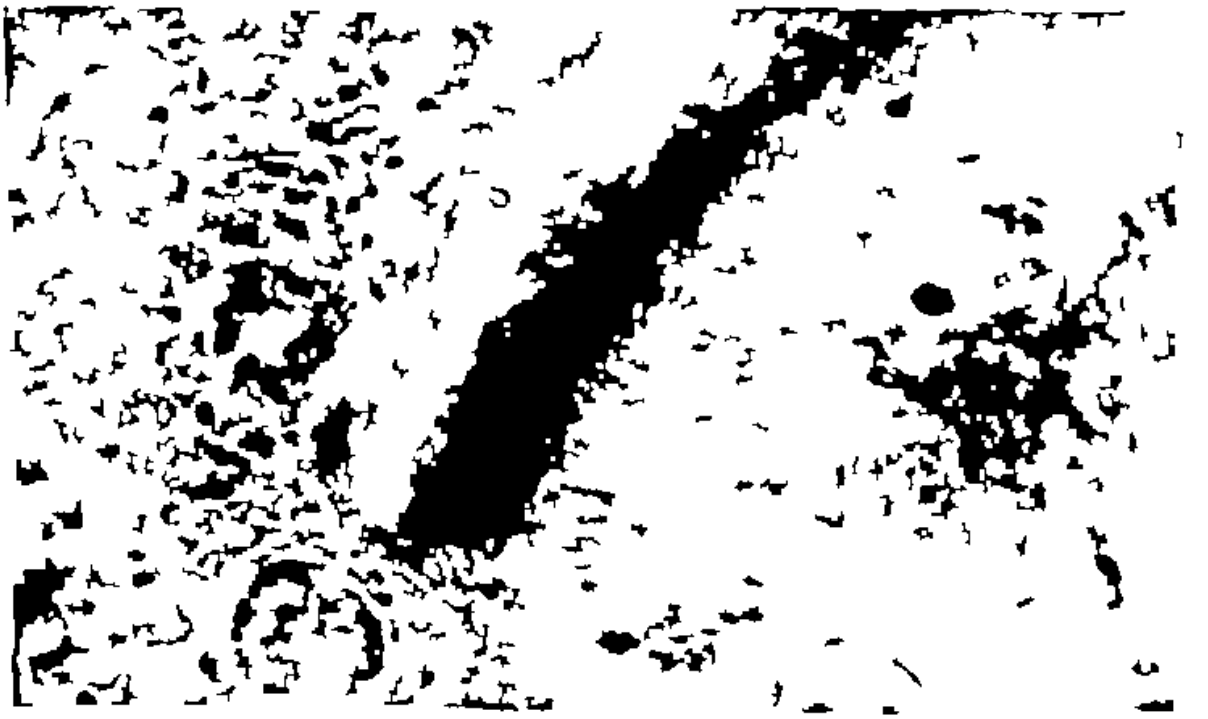


Fig. 14 b. The same section stained with toluidine blue for the identification of mast cells. The mast cells are stained metachromatically. A band of fluorescing cells can be seen in the fundic gland area parallel to the dividing ridge. In the forestomach fluorescing cells are seen in the submucosa. Most, but not all of the fluorescing cells in the fundic mucosa are stained metachromatically with toluidine blue. (×180)

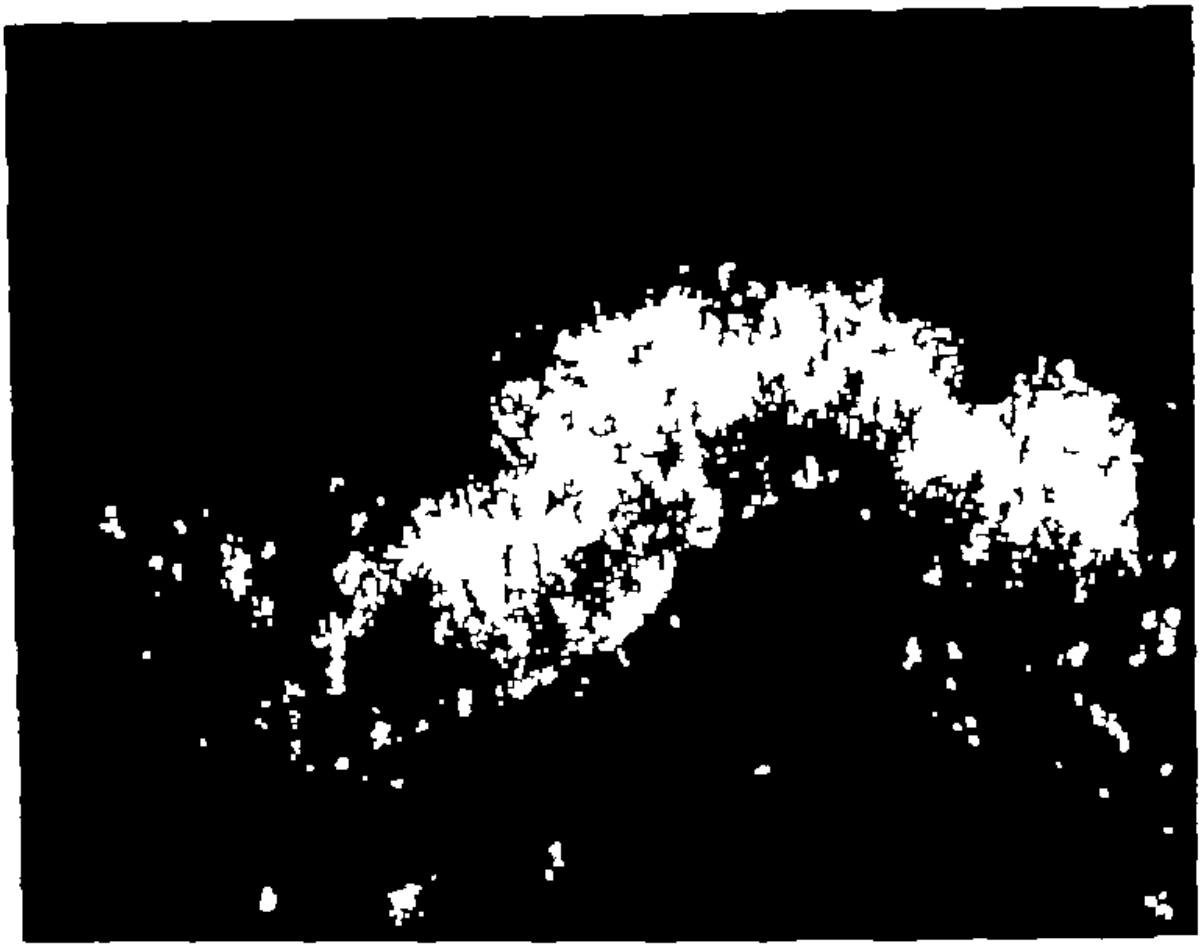


Fig. 12. Fluorescence photomicrograph of the stomach wall, fundic gland area, from an untreated mouse. Yellow fluorescing cells, probably mast cells, are seen. They are most numerous in the middle and the superficial parts of the glands. A few cells are also seen at the base of the glands and in the submucosa. The number of fluorescing cells are most numerous towards the forestomach (to the right in the picture) (×50)

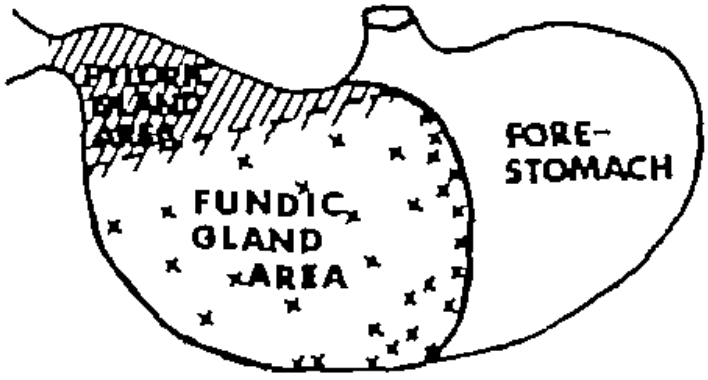


Fig. 13. Distribution of cells with endogenous fluorescence in the mouse stomach mucosa. × × × mast cells, //// enterochromaffin cells. The mast cells are most numerous adjacent to the forestomach. They have their greatest extension along the greater curvature and become gradually limited to a more narrow area towards the smaller curvature. The enterochromaffin cells are present in the pyloric gland area and in the gradual transition between the fundic gland area and the pyloric gland area.



Fig. 16. Microautoradiogram of the stomach wall, fundic gland area, 5 minutes after an intravenous injection of ^{3}H 5-HTP into mouse. The highest radioactivity is seen in basally located cells, probably enterochromaffin-like cells. Smaller and often more superficially located cells, probably mast cells, also contain high activity. The radioactivity in the gastric pits is higher than in the gastric glands. Hts-eosin. (×180)

Microautoradiography

The distribution pictures obtained after the injection of DOPA and 5-HTP were similar.

Five minutes after the injection there was a very high uptake in the whole mucosa and submucosa.

In the forestomach there was a high and even distribution of activity in the squamous epithelium and in the lamina propria. In the submucosa a very high activity could be seen in single mast cells.

In the fundic gland area the concentration of activity was higher in the gastric pits than in the fundic glands. Scattered among the fundic glands there were also several cells with a very high activity (Fig. 16). Their number was greatest at the base of the glands, but they were also found in the superficial parts of the glands. The basally located cells seemed to contain the highest activity. These cells often had a wedge shaped appearance. Identification of mast cells in the mucosa by metachromasia after toluidine blue staining was

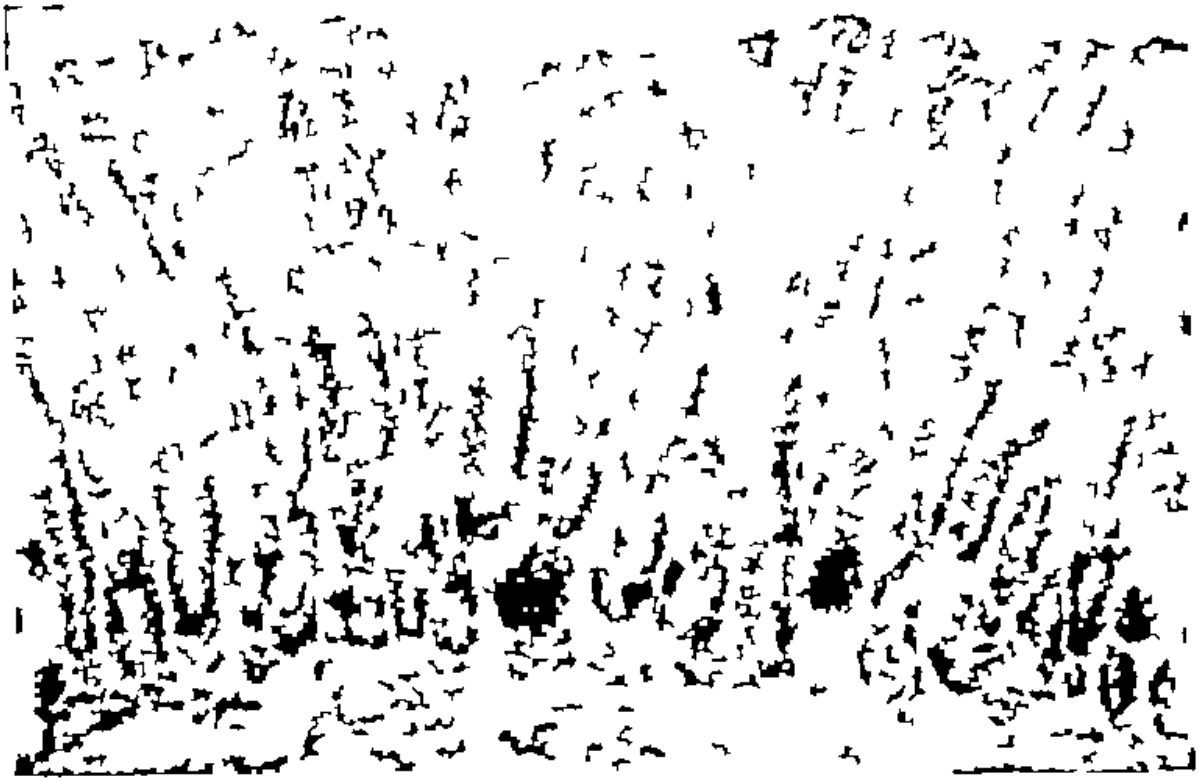


Fig. 17 Microa toradiogram of the stomach wall, pyloric gland area, 5 minutes after an intravenous injection of ^{3}H DOPA into a mouse. The highest activity is seen in basally located cells, probably enterochromaffin cells. High activity is also seen in gastric pits and pyloric glands. Htx-eosin. (×180)

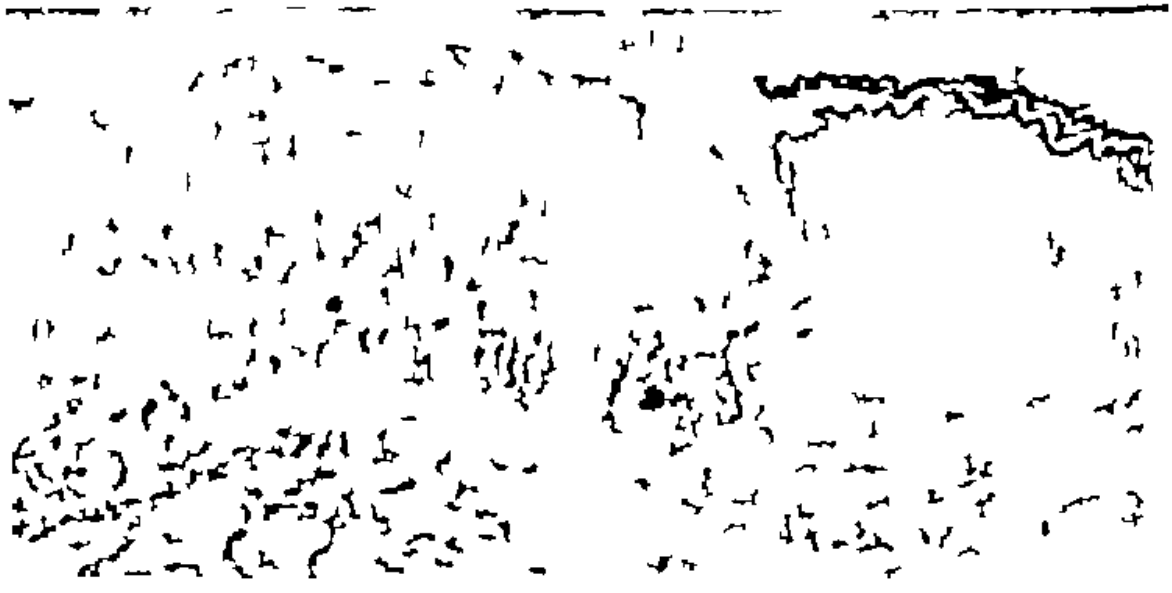


Fig 18. Microautoradiogram of the stomach wall, at the junction between the forestomach (to the right) and the fundic gland area (to the left) 1 hour after an intravenous injection of H 5-HTP int a mouse. Labelled mast cells are seen in the submucosa. In the fundic mucosa labelled cells can be observed both, at the base and more superficially Htx-eosin. (×60)

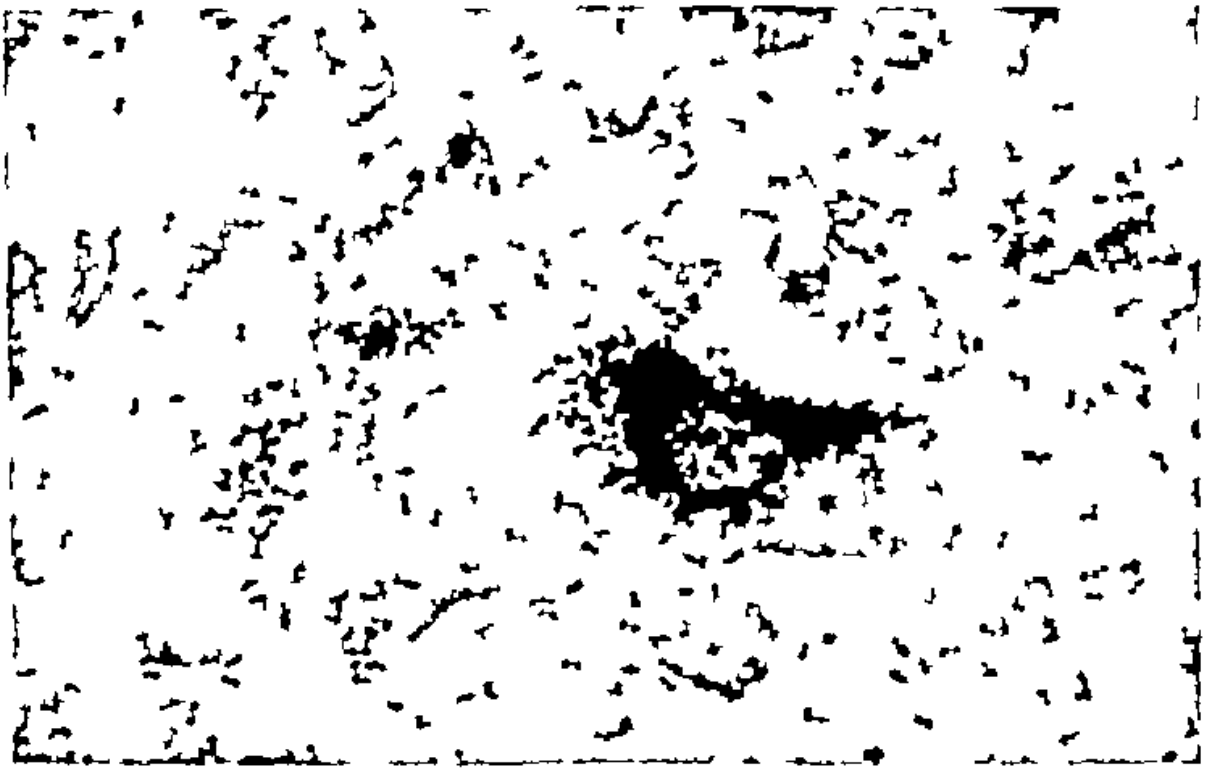


Fig. 19 Microautoradiogram from the fundic part of the stomach wall 1 hour after an intravenous injection of ^{3}H DOPA into a mouse. A strong accumulation of silver grains is seen in the cytoplasm of a wedge-shaped cell of enterochromaffin type. Toluidine blue. (×920)

not possible on sections covered with stripping film. Obviously the stripping film abolished the colour reaction.

In the mucosa of the pyloric gland area the activity was high 5 minutes after the injection with the same activity in gastric pits as in pyloric glands. In addition there were some, often wedge-shaped, cells with a basal location which had a very high activity (Fig. 17)

One hour after the injection the activity was low in the forestomach, in gastric pits in the fundic and the pyloric gland area and in fundic and pyloric glands. Intensely labelled cells could, however, be seen scattered in the fundic mucosa both basally and more superficially (Fig. 18). The more superficially located cells were considerably smaller than the basally located cells, which often showed a wedge-shaped appearance (Fig. 19). In the pyloric gland area highly labelled, [illegible] wedge-shaped cells with a basal location could be seen.

In the submucosa of [illegible] much scattered mast cells contained a high activity.

The picture seen 4 hours after the injection was similar to the one described after one [illegible] Fig. 20.

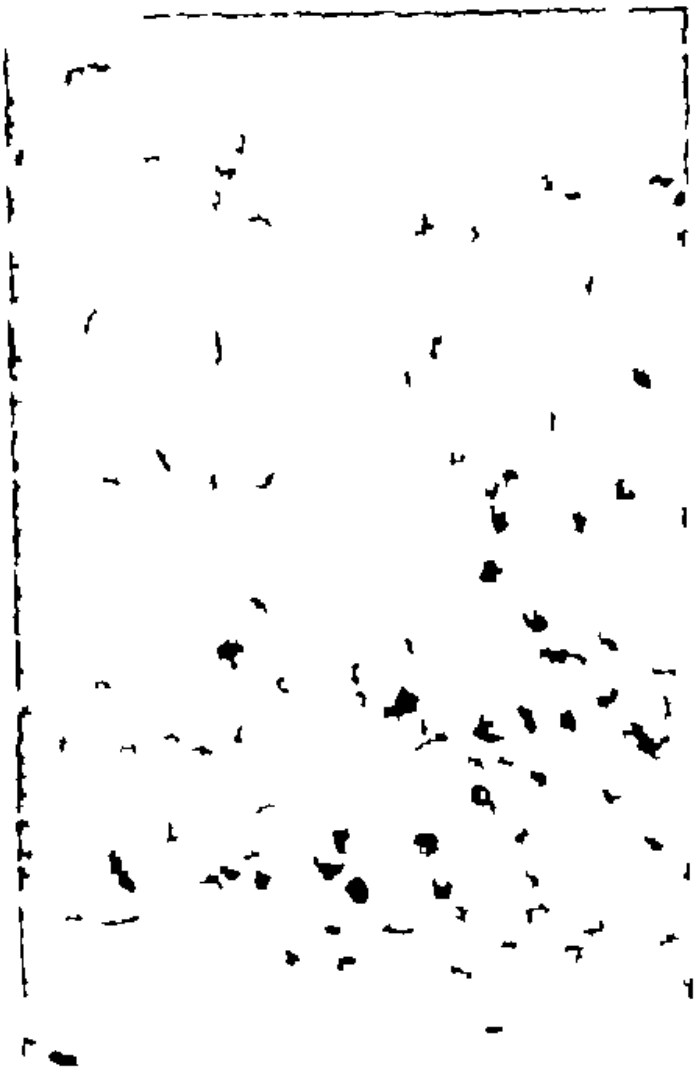


Fig. 20. Microautoradiogram of the stomach wall, fundic gland area, 24 hours after an intravenous injection of H 5-HTP into a mouse. Strongly labelled cells can still be seen in the mucosa and the submucosa. Htx-eosin. (×160)

In comparison with the cells in the pancreatic islets and the parafollicular cells, the cells in gastric mucosa were more heavily labelled at all the studied time intervals.

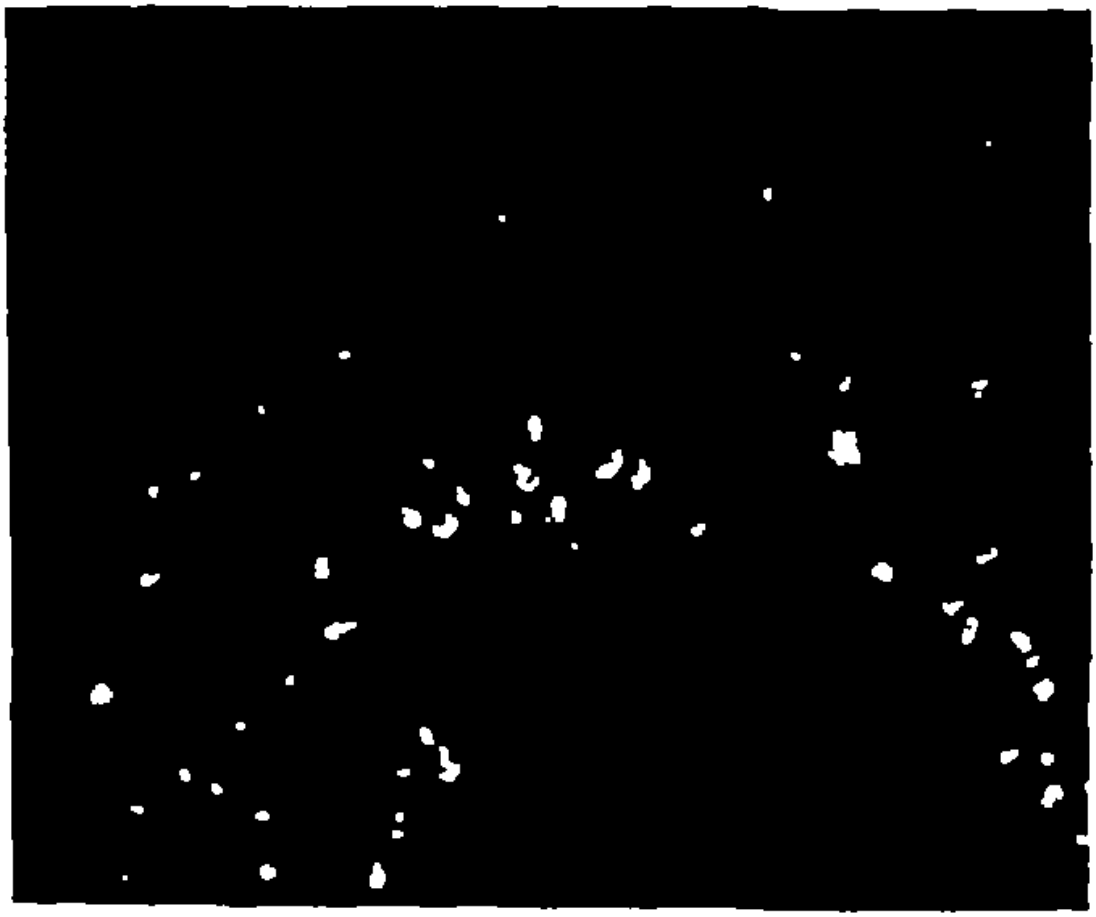
Combined microautoradiography—fluorescence microscopy

The combination was performed on pieces from the fundic gland area.

The highest fluorescence and radioactivity could be observed in cells with a basal location in the fundic glands. The correspondence between highly fluorescent and highly labelled cells was good (Fig. 21). Also in angular small cells located more superficially in the mucosa the correspondence between fluorescence and activity was good.

Fig. 21. Fluorescence photomicrograph of the stomach wall, fundic gland area, 2 hours after an intravenous injection of non-labelled DOPA into a mouse.

Fig. 21 b. Microautoradiogram of the same section obtained through the parallel injection of ^{3}H 5-HTP. Htx-eosin. Fluorescing cells and radioactively labelled cells correspond well both in the basal and superficial parts of the mucosa. (×150)





Discussion

The cells with endogenous fluorescence which have been described in the pyloric gland area belong to the system of enterochromaffin cells with endogenous 5-HT storage in the gastrointestinal tract. The fluorescing cells in the fundic mucosa probably represent a type of mast cells. These cells, which have been found in mice and some other species (see Håkanson 1970) show characteristics which differ them from other mast cells they are smaller than other mast cells in the dog and the rat they have been found not to be degranulated by compound 48/80 (Enerbäck 1966, Aures *et al.* 1968) They also show a weaker and more inconstant metachromasia after toluidine blue staining than other mast cells (Enerbäck 1966) The inability observed in the present investigation to stain all cells with endogenous fluorescence in the fundic gland area metachromatically with toluidine blue is probably due to this variance in staining properties.

In the present investigation these cells were shown to be very numerous in the fundic gland area, adjacent to the forestomach, especially along the greater curvature. Noteworthy is the band of cells in the mucosa adjacent to the ridge separating the fundic gland area from the forestomach. In the rat a band of argentaffin cells with the corresponding localization has been observed (Gregg 1960) It has not been established whether these cells are mast cells, but the mast cells in the submucosa of the rat have been shown to be stained by argentaffin techniques (Soule and Gregg 1965)

In the microautoradiographic investigation cells with a localization corresponding to both the enterochromaffin cells in the pyloric gland area and the mast cells in the fundic gland area, could be observed. It seems therefore probable that both these endogenously fluorescing cells are capable of monoamine formation after the precursor injections.

In addition to these cells strongly labelled cells were seen basally in the fundic mucosa. These cells have been studied with fluorescence methods (Håkanson *et al.* 1967 a, see also Håkanson 1970) They have been termed enterochromaffin like cells by these authors. In the mouse and the rat they have been shown to contain histamine. The substance produced by them has been proposed to be the gastric antipernicious principle (the intrinsic factor) (Håkanson *et al.* 1969 a, 1970 b)

The combination of microautoradiography and fluorescence microscopy in the fundic gland area also show that the fluorescence and the radioactivity obtained after the precursor injections are in agreement both in the basally located cells, probably corresponding to the enterochromaffin-like cells and in the more superficially located cells probably representing mast cells.

The monoaminergic mechanisms in these cells seem to be similar to the

cells in pancreatic islets and the parafollicular cells the accumulation of activity appears rapidly in the enterochromaffin-like cells decarboxylase inhibition reduces and MAO inhibition prolongs the fluorescence (Håkanson *et al.* 1967 a) These principal mechanisms have been discussed in Chapter 1

CHAPTER 4

Electron microscope autoradiography of the mouse fundic mucosa after the injection of tritiated L-DOPA

In the electron microscope the cells of the enterochromaffin cell system show several characteristics common to endocrine cells. One of the characteristics is the specific granules present in the cytoplasm of the cells. Several types of enterochromaffin cells with different granules have been described lately (e.g. Forssmann *et al.* 1969 Vasallo *et al* 1969 Capella *et al* 1969) The different parts of the gastrointestinal tract have also in these studies been shown to contain more than one of the enterochromaffin cell types.

The fact that these cells have ultrastructural characteristics common to endocrine cells, together with their monoamine storing capability have led to the mentioned assumptions that the cells of the enterochromaffin type might be endocrine cells producing the gastrointestinal hormones.

For the study of the enterochromaffin cell system in the light microscope, silver impregnation methods have been extensively used. These methods are based on the ability of the cells to reduce ammoniacal silver salts. It has been proposed that 5-HT is the reducing agent in the cells (Erspamer and Asero 1952) Several silver impregnation methods exist (see Pearse 1960) Differences in staining properties by these techniques have led to assumptions that the cells might belong to several different categories (see Håkanson 1970) It is, however impossible to correlate the different cell types seen in the electron microscope with the cells identified in the light microscope by the silver impregnation methods. When the uptake of the monoamine precursors is studied with microautoradiography or fluorescence microscopy as hitherto has been the case, it is impossible to say which of the cell types seen in the electron microscope are capable of the precursor accumulation. In the present investigation therefore an attempt is made, by means of electron microscope autoradiography to establish in which cells a monoamine accumulation takes place after the injection of DOPA.

The fundic gland area of the mouse stomach has been chosen for the present investigation. This area contains cells with the monoamine precursor accumulating capability (see Chapter 3)

Material and methods

Two male albino mice (NMRI-strain) weighing about 20 g were injected through a tail vein with 1.5 mCi ring 2 5-6- H-labelled L-3(3,4-dihydroxy phenyl-alanine (^{3}H DOPA) spec. act. 28 Ci/mM (The Radiochemical Centre Amersham, England) The dose corresponded to 0.54 mg L-DOPA/kg body weight.

The mice were sacrificed two hours after the injection by cervical dislocation and small pieces of the stomach, from the part of the fundic gland area shown to contain only few cells with endogenous fluorescence (see Chapter 3) were rapidly removed and immersed in a cold solution of 2.5 % glutaraldehyde in a Tyrode medium, pH 7 4 After 2 hours of fixation the glutaraldehyde fixed specimens were rinsed briefly in Tyrodes solution and transferred to a cold isotonic solution of osmiumtetroxide buffered to pH 7 4 for an additional fixation period of 2 hours. After dehydration in ethanol the tissue pieces were embedded in Epon.

The procedure for electron microscopic autoradiography was in principle the same as described by Maunsbach (1966) 400—600 A thick sections were cut on a LKB Ultrotome. The sections were picked up on Formvar-coated molybdenum grids (Ernest F Fullam, Inc., Schenectady U.S.A.) Ilford L 4 emulsion was applied to the grids by means of a wireloop. During the exposure time of 8 weeks the grids were stored in a refrigator at +4 C. The preparations were developed for 1 minute in Kodak D 19 and fixed for 4 minutes in Kodak Rapid Fix.

The specimens were studied in a Siemens Elmiskop I at an accelerating voltage of 80 kV with the double condensor and a 50 μ objective aperture Micrographs were taken at magnifications ranging from about 5 000 to 10.000 times.

Controls

Control sections from the fundic mucosa of 2 mice not injected with H DOPA were taken and handled for autoradiography the same way as described for the ^{3}H DOPA-injected animals.

Results

On the electron microscope autoradiographs a strong accumulation of silver grains could be seen over cells of enterochromaffin type which on basis of the different appearance of the granules, could be said to be of at least two different types. Silver grains over zymogen cells or over parietal cells were few



Fig. 12. Fluorescence photomicrograph of the tomach wall, fundic gland area, from an untreated mouse. Yellow fluorescing cells, probably mast cells, are seen. They are most numerous in the middle and the superficial parts of the glands. A few cells are also seen t the base of the glands and in the submucosa. The number of fluorescing cells are most numerous towards th forestomach (t the right in the picture) (×50)

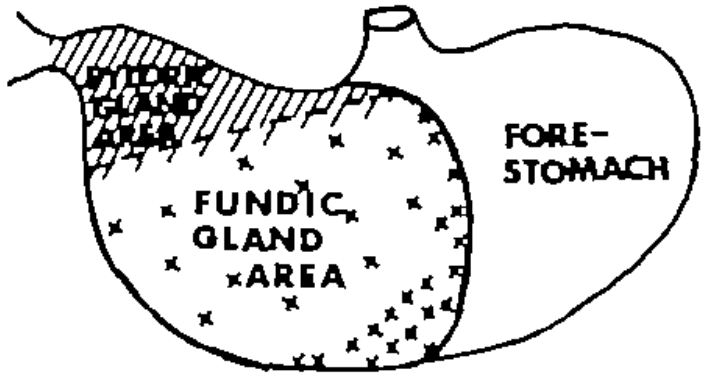


Fig. 13 Distribution of cells w th endogenous fluorescence in the mouse stomach mucosa. × × × mast cells, //// enterochromaffin cells. The mast cells are most numerous adjacent to the forestomach. They have their greatest extension along the greater curvature and become gradually limited to mor narrow area towards the smaller curvature. The enterochromaffin cells a present in the pyloric gland area and in th gradual transition between the fundic gland area and the pyloric gland area.

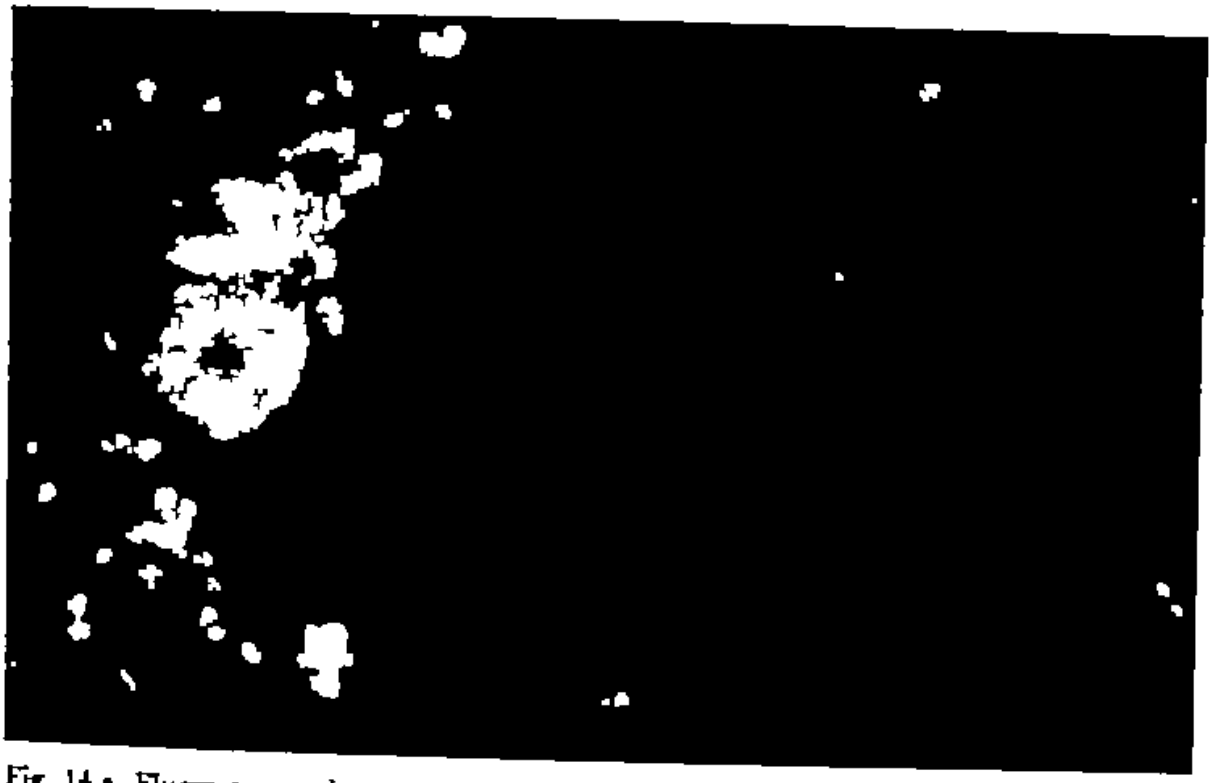


Fig. 14 a. Fluorescence photomicrograph of the stomach wall, of an untreated mouse, at the ridge dividing the forestomach (to the right) from the fundic gland area (to th left)



Fig. 14 b. The same section stained with toluidine blue for the identification of mast cells The mast cells are stained metachromatically A band of fluorescing cells can be seen in the fundic gland area parallel t the dividing ridge. I the forestomach fluorescing cells are seen in the submucosa. Most, but not all of the fluorescing cells in the fundic mucosa are stained metachromatically with toluidine blue. (× 180)



Fig 1 Fluorescence photomicrograph of the stomach wall, fundic gland area, from an untreated mouse Yellow fluorescing cells, probably mast cells, are seen. They are most numerous in the middle and the superficial parts of the glands. A few cells are also seen at the base of the glands and in the submucosa. The number of fluorescing cells are most numerous towards the forestomach (to the right in the picture) (×50)

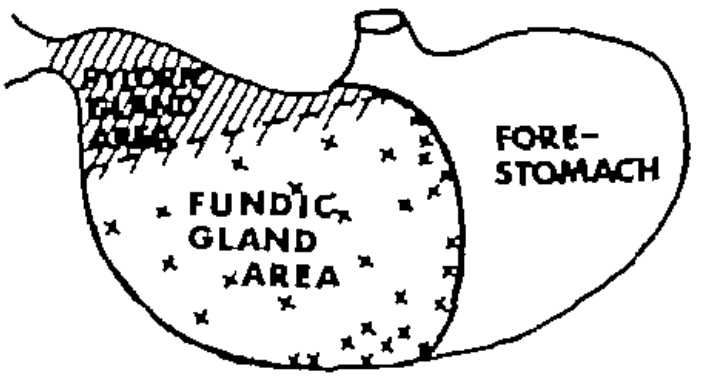


Fig. 13. Distribution of cells with endogenous fluorescence in the mouse stomach mucosa. × × × mast cells, //// enterochromaffin cells. The mast cells are most numerous adjacent to the forestomach. They have their greatest extension along the greater curvature and become gradually limited to more narrow areas towards the smaller curvature. The enterochromaffin cells are present in the pyloric gland area and in the gradual transition between the fundic gland area and the pyloric gland area.



Fig. 14 a. Fluorescence photomicrograph of the stomach wall, of an untreated mouse at the ridge dividing the forestomach (to the right) from the fundic gland area (to the left)

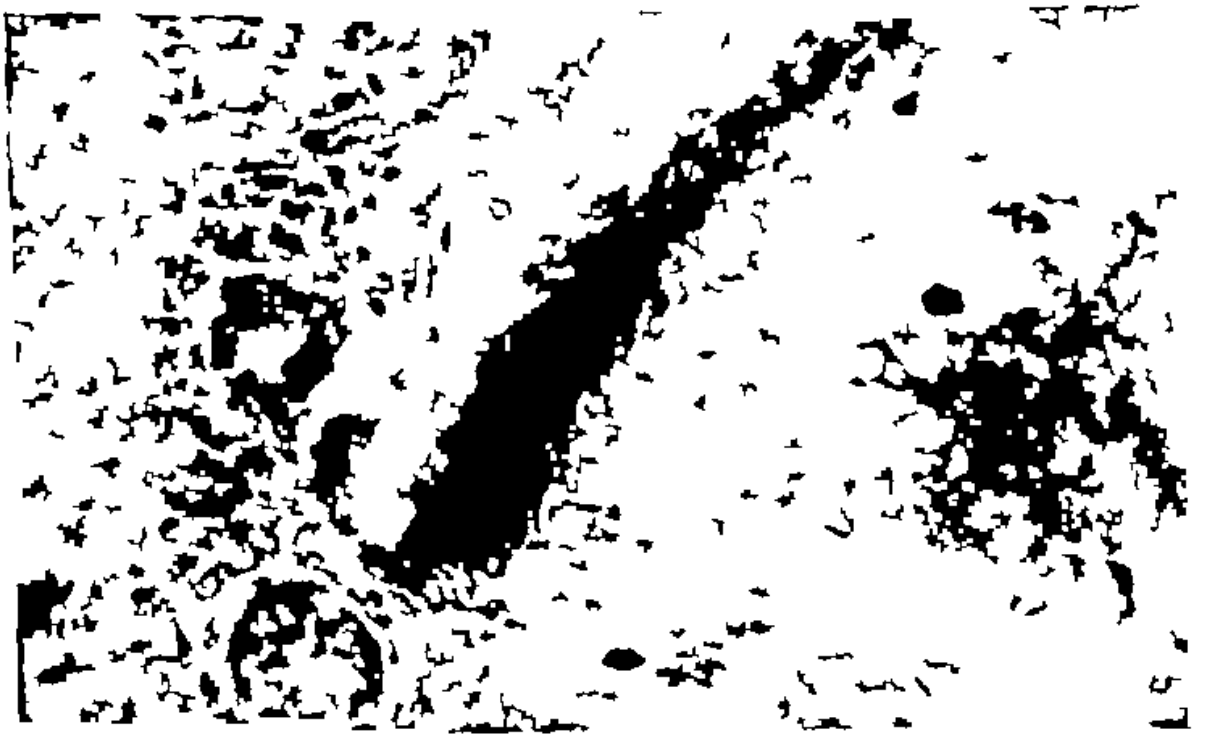


Fig. 14 b. The same section stained with toluidine blue for the identification of mast cells. The mast cells are stained metachromatically. A band of fluorescing cells can be seen in the fundic gland area parallel to the dividing ridge. In the forestomach fluorescing cells are seen in the submucosa. Most, but not all of the fluorescing cells in the fundic mucosa are stained metachromatically with toluidine blue. (×180)

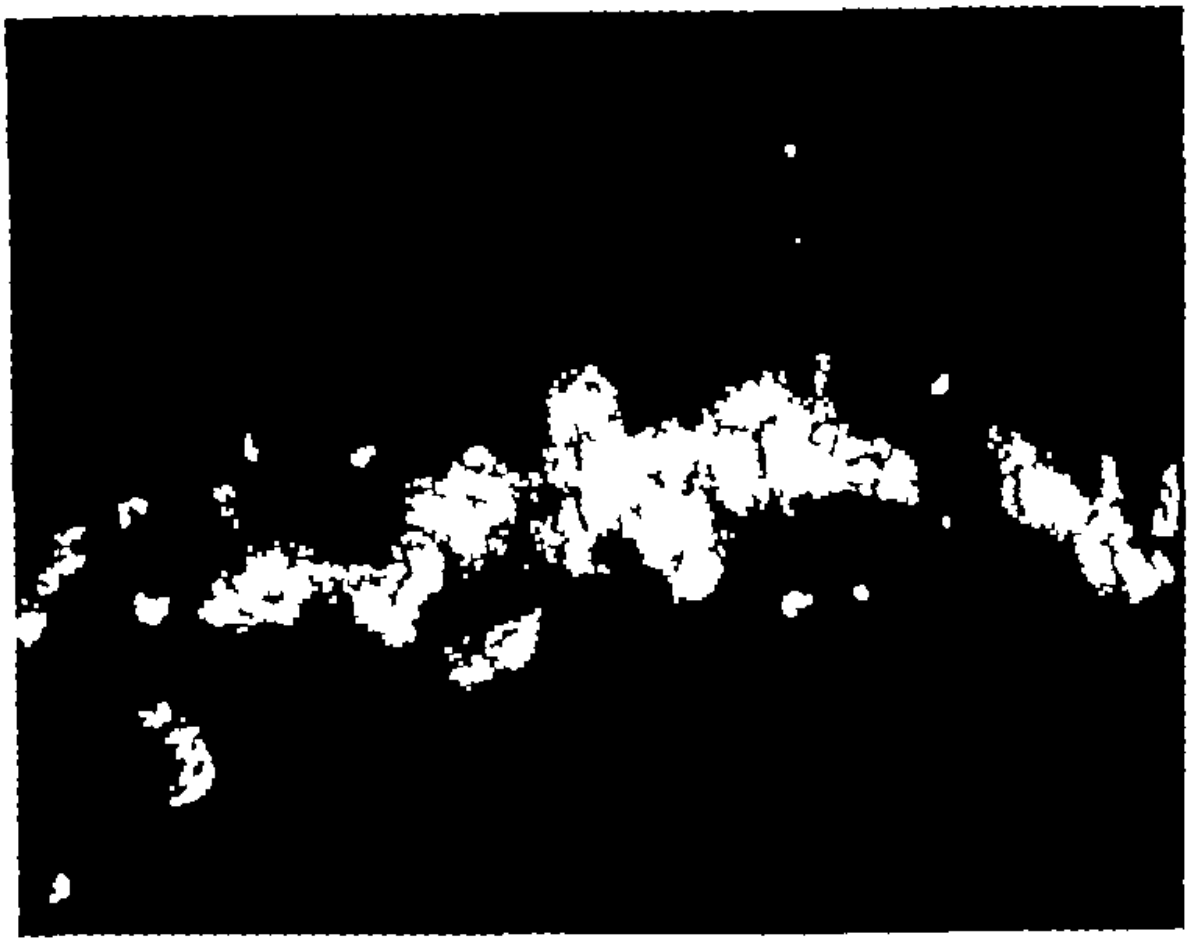


Fig. 15. Fluorescence photomicrograph of the stomach wall, pyloric gland area, of an untreated mouse. Fluorescing enterochromaffin cells can be seen in the basal half of the mucosa. (× 150)

of the mucosa seen immediately adjacent and parallel to the dividing ridge separating the forestomach from the fundic gland area, especially many yellow fluorescing cells could be seen (Fig. 14). When the sections were successively stained with toluidine blue, most of these cells showed a distinct metachromasia characteristic for mast cells. Some of the cells were however not stained with toluidine blue.

A great part of the fundic gland area was devoid of fluorescing cells or contained only a few fluorescing cells (see Fig. 13).

In the gradual transition between the fundic gland area and the pyloric gland area yellow fluorescing enterochromaffin cells could be observed. In the pyloric gland area they became rather numerous. They were restricted to the basal half of the mucosa, they were large and often flask-shaped and they did not show metachromasia after toluidine blue staining (Fig. 15).

In addition to the described cells yellow fluorescing mast cells (identified through their metachromasia) could be seen in the submucosa and in the lamina propria in all parts of the stomach.



Fig. 16. Microautoradiogram of the stomach wall, fundic gland area, 5 min tes after an intravenous injection of H-5-HTP into a mouse. The highest radioactivity is seen in basally located cells, probably enterochromaffin-like cells. Smaller and often more superficially located cells, probably mast cells, also contain high activity The radioactivity in the gastric pits is higher than in the gastric glands. Hts-eosin. (×180)

Microautoradiography

The distribution pictures obtained after the injection of DOPA and 5-HTP were similar

F've minutes after the injection there was a very high uptake in the whole mucosa and submucosa.

In the forestomach there was a high and even distribution of activity in the squamous epithelium and in the lamina propria. In the submucosa a very high activity could be seen in single mast cells.

In the fundic gland area the concentration of activity was higher in the gastric pits than in the fundic glands. Scattered among the fundic glands there were also several cells with a very high activity (Fig. 16) Their number was greatest at th base of the glands, but they were also found in the superficial parts of the glands. The basally located cells seemed to contain the highest activity These cells often had a wedge-shaped appearance. Identification of mast cells in th mucosa by metachromasia after toluidine blue staining was



Fig. 17 Microa toradiogram of the stomach wall, pyloric gland area, 5 minutes after an intra enous injection of ^{3}H DOPA into mouse. The highest activity is seen in basally located cells probably enterochromaffin cells. High activity is also seen i gastric pits and pyloric glands. Htx-eosin. (×180)

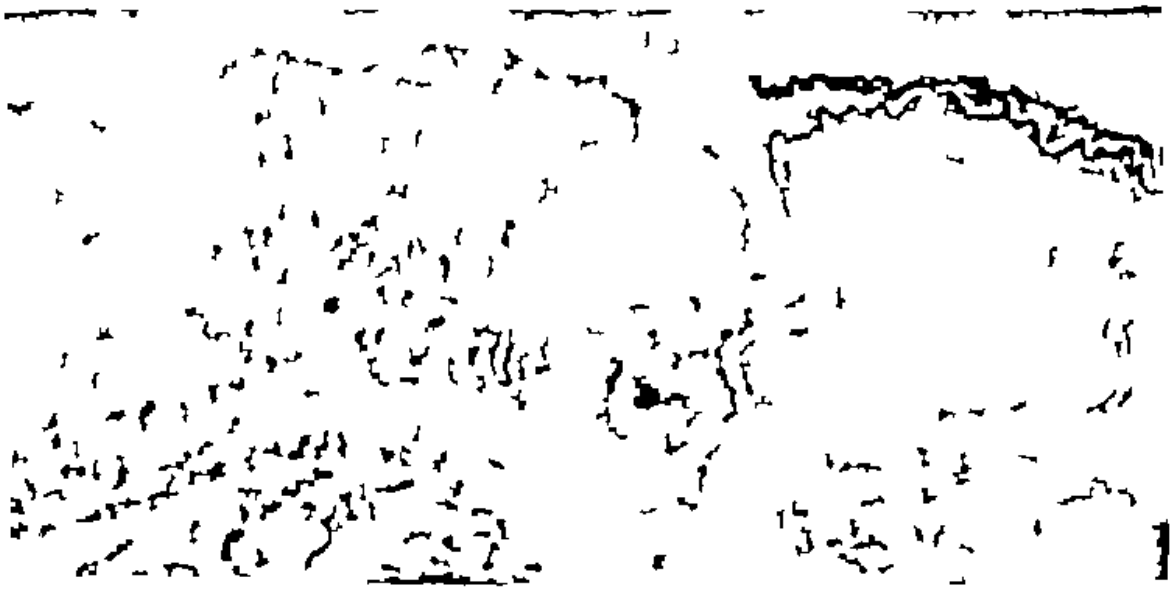


Fig. 18. Microautoradiogram of the stomach wall, at the junction between the forestomach (to the right) and the fundic gland area (to the left) 1 hour after an intravenous injection of H 5-HTP into a mouse. Labelled mast cells are seen in the submucosa In the fundic mucosa labelled cells can be observed both, at the base and more superficially Htx-eosin. (×60)

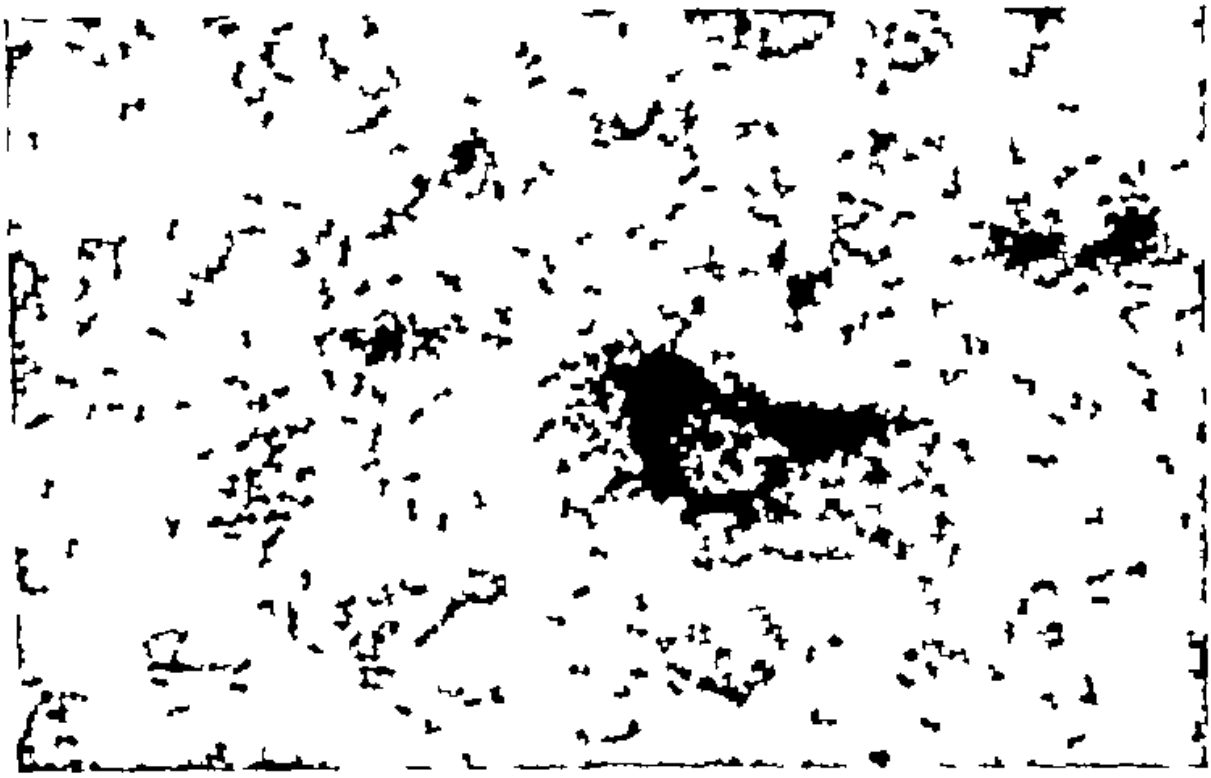


Fig. 19 Microautoradiogram from the fundic part of the stomach wall 1 hour after an intravenous injection of ^{3}H DOPA into mouse. A strong accumulation of silver grains is seen in the cytoplasm of a wedge-shaped cell of enterochromaffin type. Toluidine blue. (×920)

not possible on sections covered with stripping film. Obviously the stripping film abolished the colour reaction.

In the mucosa of the pyloric gland area the activity was high 5 minutes after the injection with the same activity in gastric pits as in pyloric glands. In addition there were some, often wedge-shaped, cells with a basal location which had a very high activity (Fig. 17)

One hour after the injection the activity was low in the forestomach, in gastric pits in the fundic and the pyloric gland area and in fundic and pyloric glands. Intensively labelled cells could, however be seen scattered in the fundic mucosa both basally and more superficially (Fig. 18) The more superficially located cells were considerably smaller than the basally located cells, which often showed a wedge-shaped appearance (Fig. 19) In the pyloric gland area highly labelled often wedge shaped cells with a basal location could be seen.

In the submucosa of the stomach scattered mast cells also contained a high activity

The picture seen 24 hours after the injection was similar to the one described after one hour (Fig 20)

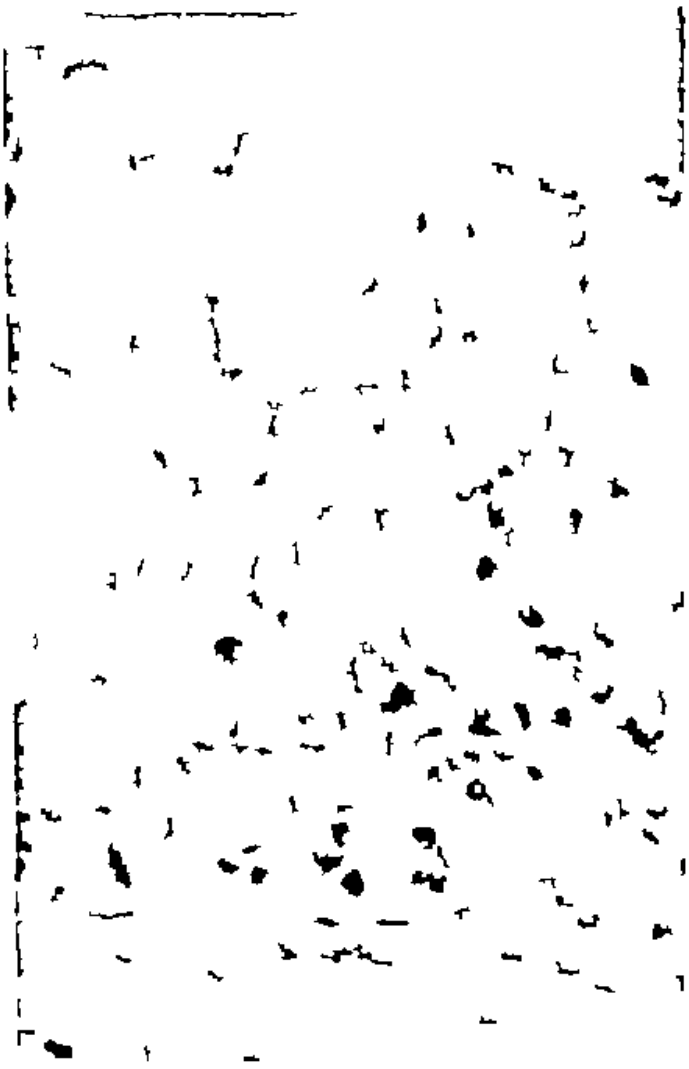


Fig. 20. Microautoradiogram of the stomach wall, fundic gland area, 24 hours after an intravenous injection of H 5-HTP into mouse. Strongly labelled cells can still be seen in the mucosa and the submucosa. Hx-eosin. (×160)

In comparison with the cells in the pancreatic islets and the parafollicular cells, the cells in gastric mucosa were more heavily labelled at all the studied time intervals.

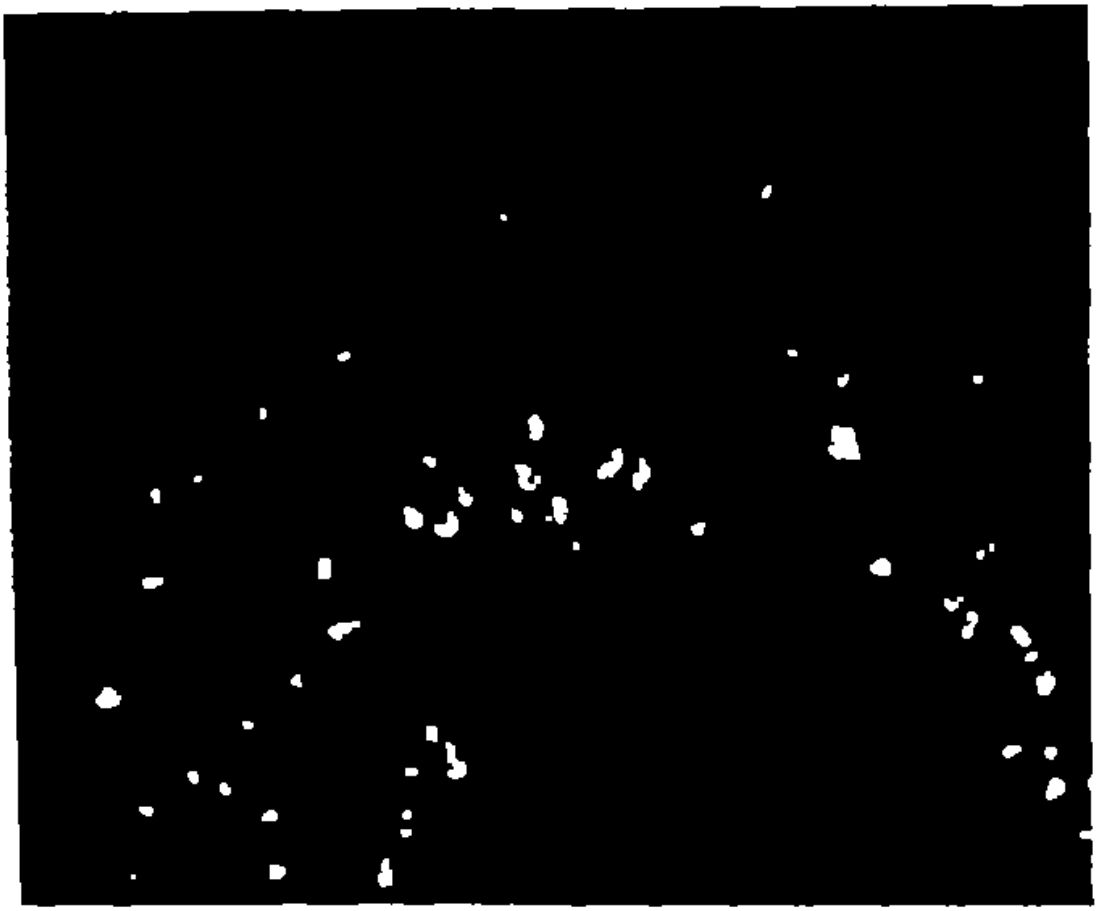
Combined microaut radiography—fluorescence microscopy

The combination was performed on pieces from the fundic gland area.

The highest fluorescence and radioactivity could be observed in cells with a basal location in the fundic glands. The correspondence between highly fluorescent and highly labelled cells was good (Fig. 21). Also in singular small cells located more superficially in the mucosa the correspondence between fluorescence and activity was good.

Fig. 1. Fluorescence photomicrograph of the stomach wall, fundic gland area, hours after an intravenous injection of non-labelled DOPA into mouse.

Fig. 21 b. Microautoradiogram of the same section obtained through the parallel injection of ^{3}H 5-HTP. Hx-eosin. Fluorescing cells and radioactively labelled cells correspond well both in the basal and superficial parts of ×1 0





Discussion

The cells with endogenous fluorescence which have been described in the pyloric gland area belong to the system of enterochromaffin cells with endogenous 5-HT storage in the gastrointestinal tract. The fluorescing cells in the fundic mucosa probably represent a type of mast cells. These cells, which have been found in mice and some other species (see Håkanson 1970) show characteristics which differ them from other mast cells they are smaller than other mast cells in the dog and the rat they have been found not to be degranulated by compound 48/80 (Enerbäck 1966, Aures *et al.* 1968) They also show a weaker and more inconstant metachromasia after toluidine blue staining than other mast cells (Enerback 1966) The inability observed in the present investigation to stain all cells with endogenous fluorescence in the fundic gland area metachromatically with toluidine blue is probably due to this variance in staining properties.

In the present investigation these cells were shown to be very numerous in the fundic gland area, adjacent to the forestomach, especially along the greater curvature. Noteworthy is the band of cells in the mucosa adjacent to the ridge separating the fundic gland area from the forestomach. In the rat a band of argentaffin cells with the corresponding localization has been observed (Gregg 1966) It has not been established whether these cells are mast cells, but the mast cells in the submucosa of the rat have been shown to be stained by argentaffin techniques (Soule and Gregg 1965)

In the microautoradiographic investigation cells with a localization corresponding to both the enterochromaffin cells in the pyloric gland area and the mast cells in the fundic gland area, could be observed. It seems therefore probable that both these endogenously fluorescing cells are capable of monoamine formation after the precursor injections.

In addition to these cells strongly labelled cells were seen basally in the fundic mucosa. These cells have been studied with fluorescence methods (Håkanson *et al* 1967 a, see also Håkanson 1970) They have been termed enterochromaffin like cells by these authors. In the mouse and the rat they have been shown to contain histamine. The substance produced by them has been proposed to be the gastric antipernicious principle (the intrinsic factor) (Håkanson *et al* 1969 a, 1970 b)

The combination of microautoradiography and fluorescence microscopy in the fundic gland area also show that the fluorescence and the radioactivity obtained after the precursor injections are in agreement both in the basally located cells, probably corresponding to the enterochromaffin-like cells and in the more superficially located cells, probably representing mast cells.

The monoaminergic mechanisms in these cells seem to be similar to the

cells in pancreatic islets and the parafollicular cells the accumulation of activity appears rapidly in the enterochromaffin-like cells decarboxylase inhibition reduces and MAO inhibition prolongs the fluorescence (Håkanson *et al.* 1967 a) These principal mechanisms have been discussed in Chapter 1

CHAPTER 4

Electron microscope autoradiography of the mouse fundic mucosa after the injection of tritiated L-DOPA

In the electron microscope the cells of the enterochromaffin cell system show several characteristics common to endocrine cells. One of the characteristics is the specific granules present in the cytoplasm of the cells. Several types of enterochromaffin cells with different granules have been described lately (e.g. Forssmann *et al.* 1969 Vassallo *et al.* 1969 Capella *et al.* 1969) The different parts of the gastrointestinal tract have also in these studies been shown to contain more than one of the enterochromaffin cell types.

The fact that these cells have ultrastructural characteristics common to endocrine cells, together with their monoamine storing capability have led to the mentioned assumptions that the cells of the enterochromaffin type might be endocrine cells producing the gastrointestinal hormones.

For the study of the enterochromaffin cell system in the light microscope, silver impregnation methods have been extensively used. These methods are based on the ability of the cells to reduce ammoniacalic silver salts. It has been proposed that 5-HT is the reducing agent in the cells (Erspamer and Asero 1952) Several silver impregnation methods exist (see Pearse 1960) Differences in staining properties by these techniques have led to assumptions that the cells might belong to several different categories (see Håkanson 1970) It is, however impossible to correlate the different cell types seen in the electron microscope with the cells identified in the light microscope by the silver impregnation methods. When the uptake of the monoamine precursors is studied with microautoradiography or fluorescence microscopy as hitherto has been the case it is impossible to say which of the cell types seen in the electron microscope are capable of the precursor accumulation. In the present investigation therefore an attempt is made, by means of electron microscope autoradiography to establish in which cells a monoamine accumulation takes place after the injection of DOPA.

The fundic gland area of the mouse stomach has been chosen for the present investigation. This area contains cells with the monoamine precursor accumulating capability (see Chapter 3)

Material and methods

Two male albino mice (NMRI-strain) weighing about 20 g were injected through a tail vein with 1.5 mCi ring ? 5-6- H labelled L-3(3 4-dihydroxy phenyl-alanine (H DOPA) spec. act. 28 Ci/mM (The Radiochemical Centre, Amersham, England) The dose corresponded to 0.54 mg L-DOPA/kg body weight.

The mice were sacrificed two hours after the injection by cervical dislocation and small pieces of the stomach, from the part of the fundic gland area shown to contain only few cells with endogenous fluorescence (see Chapter 3) were rapidly removed and immersed in a cold solution of 2.5 % glutaraldehyde in a Tyrode medium, pH 7 4 After 2 hours of fixation the glutaraldehyde fi ed specimens were rinsed briefly in Tyrodes solution and transferred to a cold isotonic solution of osmiumtetroxide buffered to pH 7 4 for an additional fixa tion period of 2 hours. After dehydration in ethanol the tissue pieces were embedded in Epon.

The procedure for electron microscopic autoradiography was in principle the same as described by Maunsbach (1966) 400—600 A thick sections were cut on a LKB Ultrotome. The sections were picked up on Formvar-coated molybdenium grids (Ernest F Fullam, Inc. Schenectady U.S.A.) Ilford L 4 emulsion was applied to the grids by means of a wireloop. During the exposure time of 8 weeks the grids were stored in a refrigator at +4° C. The prepara tions were developed for 1 minute in Kodak D 19 and fixed for 4 minutes in Kodak Rapid Fix.

The specimens were studied in a Siemens Elmskop I at an accelerating voltage of 80 kV with the double condensor and a 50 μ objective aperture. Micrographs were taken at magnifications ranging from bout 5.000 to 10 000 times.

Controls

Control sections from the fundic mucosa of 2 mice not injected with ^{3}H DOPA were taken and handled for autoradiography the same way as described for the H DOPA injected animals.

Results

On the electron microscope autoradiographs a strong accumulation of sil r grains could be seen over cells of enterochromaffin type which on basis of the different appearance of the granules, could be said to be of t least two dif ferent types. Silver grains over zymogen cells or over parietal cells were few



and seemed to be without any preferential localization. The first enterochromaffin cell type (Fig 22 23 and 26) over which an accumulation of silver grains could be seen contained granules of an almost uniformly round shape. The granules had a homogenous appearance with a rather high electron opacity The diametre varied from 150 to 250 mμ The observed cells of this type had a basal localization in the epithelium of the deeper parts of the gastric glands without any contact with the gland lumen. Within the cells the granules were localized especially in the basal region. The distribution of silver grains showed a good concurrence with the distribution of the granules. Silver grains with localization other than over the specific granules were few

The second enterochromaffin cell type (Fig 24 25 and 27) over which an accumulation of sil ver grains could be seen, had granules of a completely dif ferent appearance. Some of them were small (about 150 mμ) containing a round or irregular osmophilic body surrounded by a clear space. Other granules were considerably larger (300—600 mμ) and seemed to lack content or contained only a small osmophilic body These cells also had a basal location, mostly in the deeper parts of the gastric glands, and appeared to lack con tact with the gland lumen. The sil ver grains in these cells were seen in association with the granules, and then in association with both types of granules.

Other cells of enterochromaffin type, labelled or non-labelled than the two described could not be observed.

Specific accumulation of silver grains over special cells or cell-structures could not be seen in any of the sections from the control mice.

Discussion

The present study clearly demonstrates a very selective localization of th label in cells of enterochromaffin type Signs of diffusion or displacement of the activity have not been observed. The same selective localization of th label in cells of this type has also been observed in light microscope in vestigations (see Chapter 3) Gershon and Ross (1966 b) demonstrated that 5-HT which is formed in tissues after the injection of 5-HTP is very firmly bound to the tissues while this is not the case for the 5-HTP or the 5-HT metabolites. It has also been shown that the binding of amines in tissues exists after the applica-

Fig. 22. Electron microscopic toradiogr ph of an enterochromaffin cell of the first described type lying on the basement membrane (bm) of fundic gland of the stomach of mouse. The mouse was injected intr enously with 11 DOPA 2 hours before it was killed. Silver grains are seen predominantly over the round dense granules in the cytoplasm. Zymogen granules (zg) and endoplasmatic reticulum (er) of adjacent chief cells (×15.000)

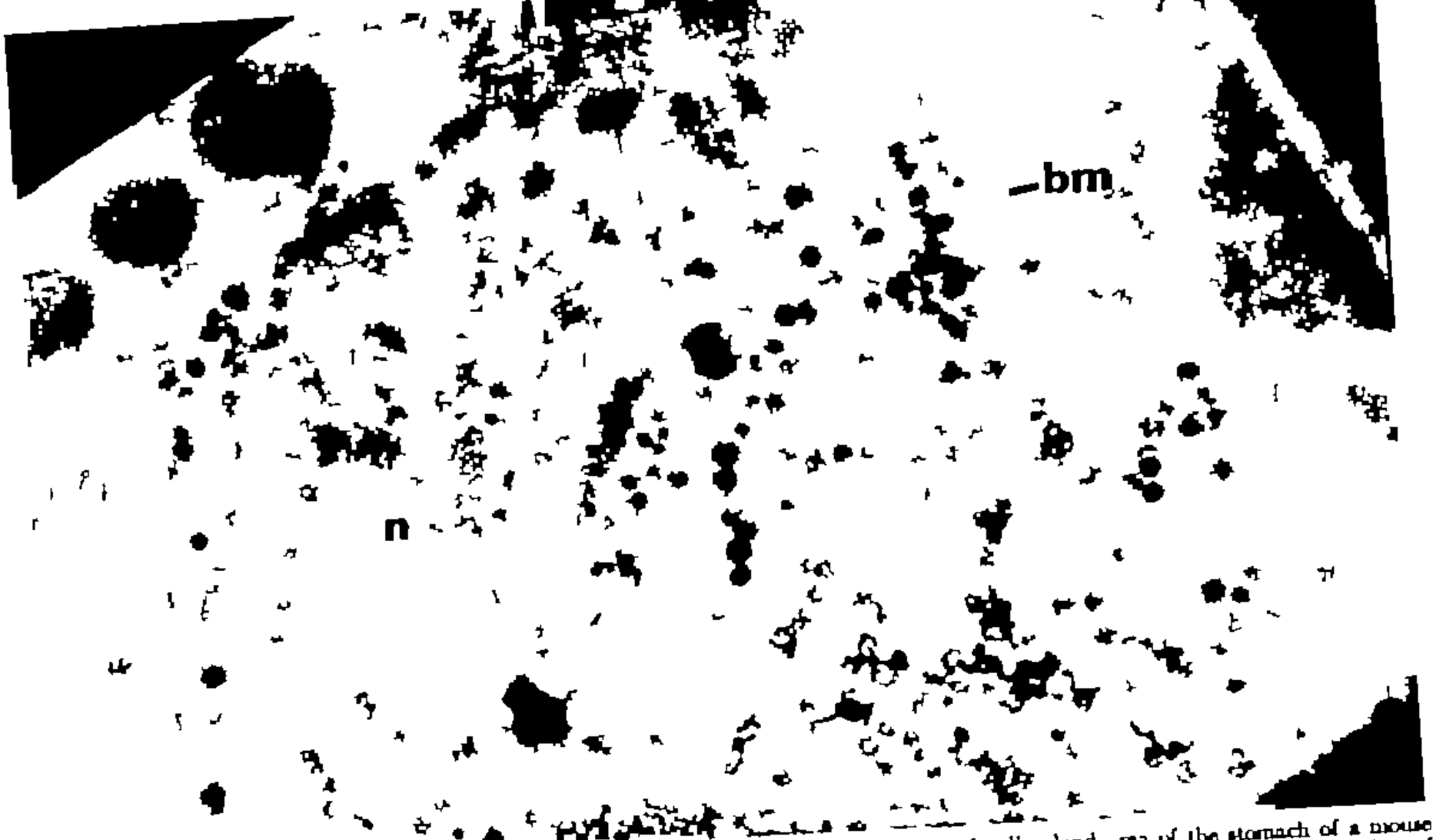


Fig. 23. Electron microscopic autoradiograph of a cell of enterochromaffin type from the fundic gland area of the stomach of a mouse 2 hours after an intravenous injection of H-DOPA. The enterochromaffin cell is of the same type as in Fig. 22. The distribution of the specific granules and the silver grains correspond well. The nucleus (n) shows a narrow chromatin border. Basement membrane (bm); zymogen granule (zg). (×16,000)

tion of chemical fixatives, and that the combination of glutaraldehyde and OsO_4 in such a case is the most effective fixative (Gershon and Ross 1966 b, Hökfelt and Jonsson 1968)

As mentioned in Chapter 3 it has also been shown that the products obtained in the enterochromaffin cell types after the injection of the monoamine precursors are the primary amines (see Håkanson 1970)

The application of an electron microscopic technique, as has been the case in the present investigation, requires a binding to the tissues of the amines which are formed after the injection of their precursors. Such a binding thus occurs, either directly *in vivo* or in connection with the chemical fixation. In an earlier electron microscope autoradiographic investigation the localization of 5-HT in the parafollicular cells after the injection of 5-HTP into mice has been studied in a similar way (Ericson 1970)

In the present investigation a monoamine accumulation after injection of DOPA has been observed in two enterochromaffin cell types with completely different granules in the fundic mucosa of the mouse.

One of these cell types has round granules with a homogenous appearance and a rather high electron opacity These cells have a resemblance to cells described by Helander (1961 1967) in the fundic mucosa of the mouse, and called argyrophil cells by him. Forssmann *et al.* (1969) describe a similar cell in the rat fundic mucosa and call them intestinal A cells. In both these investigations the similarities between these cells and the α-cells of the pancreatic islets are pointed out. This cell type might also be identical with the cholinesterase-rich cell type described by Carvalheira *et al.* (1968) Similar cells have also been described in the stomach of the dog, cat, guinea-pig, rabbit and in man (Solcia and Sampietro 1965 Forssmann *et al.* 1968, Orci *et al.* 1968 Vassallo *et al* 1969 Capella *et al* 1969) Forssmann *et al* (1969) describe besides the A-cells another cell type in the rat fundic mucosa with a very similar morphology called D-cells, which might correspond to the D-cells of the pancreatic islets. This cell has, however not been described in the mouse and the morphological differences between the granules of these two cell types are too small to be observed in the present investigation.

The second cell type over which silver grains could be observed had very characteristic granules. They were partly small containing a round or irregular osmophilic body partly big without or with only a small osmophilic body Similar cells have earlier been described in the fundic mucosa of the rat (Forssmann *et al* 1969) rabbit (Capella *et al* 1969) and cat (Vassallo *et al* 1969) This cell type shows similarities with cells in the sympathetic trunk (Siegrist *et al* 1966) They have by some authors been called enterochromaffin-like cells (Vassallo *et al.* 1969 Capella *et al.* 1969) It has been pointed out by


ch
p
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n

these authors that the cells at least to some extent might correspond to the cells which by Håkanson *et al.* (1967 a) have been called enterochromaffin like cells.

As mentioned in the results other enterochromaffin cell types besides the two which have been described, could not be found in the fundic mucosa of the mouse in the present investigation. There was, however an accumulation of silver grains over both cell types found. The classical enterochromaffin cell with endogenous 5-HT content, is lacking almost totally in big parts of the fundic mucosa of the mouse (see Chapter 3)

It is not definitively proven whether these cells are also capable of monoamine precursor accumulation, but the localization of the cells accumulating the precursors in the pyloric gland area in the microautoradiographic investigation in Chapter 3 was similar to the localization of the enterochromaffin cells with endogenous fluorescence. Perhaps all the cells of enterochromaffin type possess this capability

Several other cells with endocrine functions, such as the different cells of th islets of Langerhans in the pancreas and the parafollicular cells of the thyroid have, as mentioned, the same capability Each of these cell types has distinctive electron-microscopic features, and is the producer of a specific polypeptide hormone. It seems therefore natural that each of the different enterochromaffin cell-types in the gastrointestinal tract might have a specific product, which then might be of polypeptide hormone nature. As has been suggested (see Aures *et al.* 1970) th polypeptide hormones produced in these cells might be the different gastrointestinal hormones as for example gastrin, secretin and pancreozymin-cholecystokinin. The presence of gastrin in cells of enterochromaffin type in the pyloric gland area of man and pig has actually been demonstrated with an immunofluorescent technique (Mc Guigan 1968) These results have been confirmed in a recent work (Bussolati and Pearse 1970)

As at least two enterochromaffin cell types exist in the mouse fundic mucosa it seems probable that these cells ha ve at least tv o different products.

The first cell type described in this work might be the producer of the intestinal glucagon (Unger *et al* 1966) which, considering the similarities with the α-cells of the pancreatic islets, has been proposed to be the function of the corresponding cell in the rat (Forssmann *et al* 1969) It has been surmised that the gastric antipernicious principle (the intrinsic factor) might be produced by the so called enterochromaffin-like cells in the mouse, rat, hamster

Fig. 4 Electron microscopic autoradiograph showing tw enterochromaffin cells of the second described type in the fundic mucosa of the stomach, two hours after an intravenous injection of H-DOPA into mouse. The silver grains hav selectiv localization over the cytoplasm of the enterochromaffin cells. Nucleus ( ) of an enterochromaffin cell parietal cell (p) chief cells (ch) (×12.000)


ch
p
ch
n

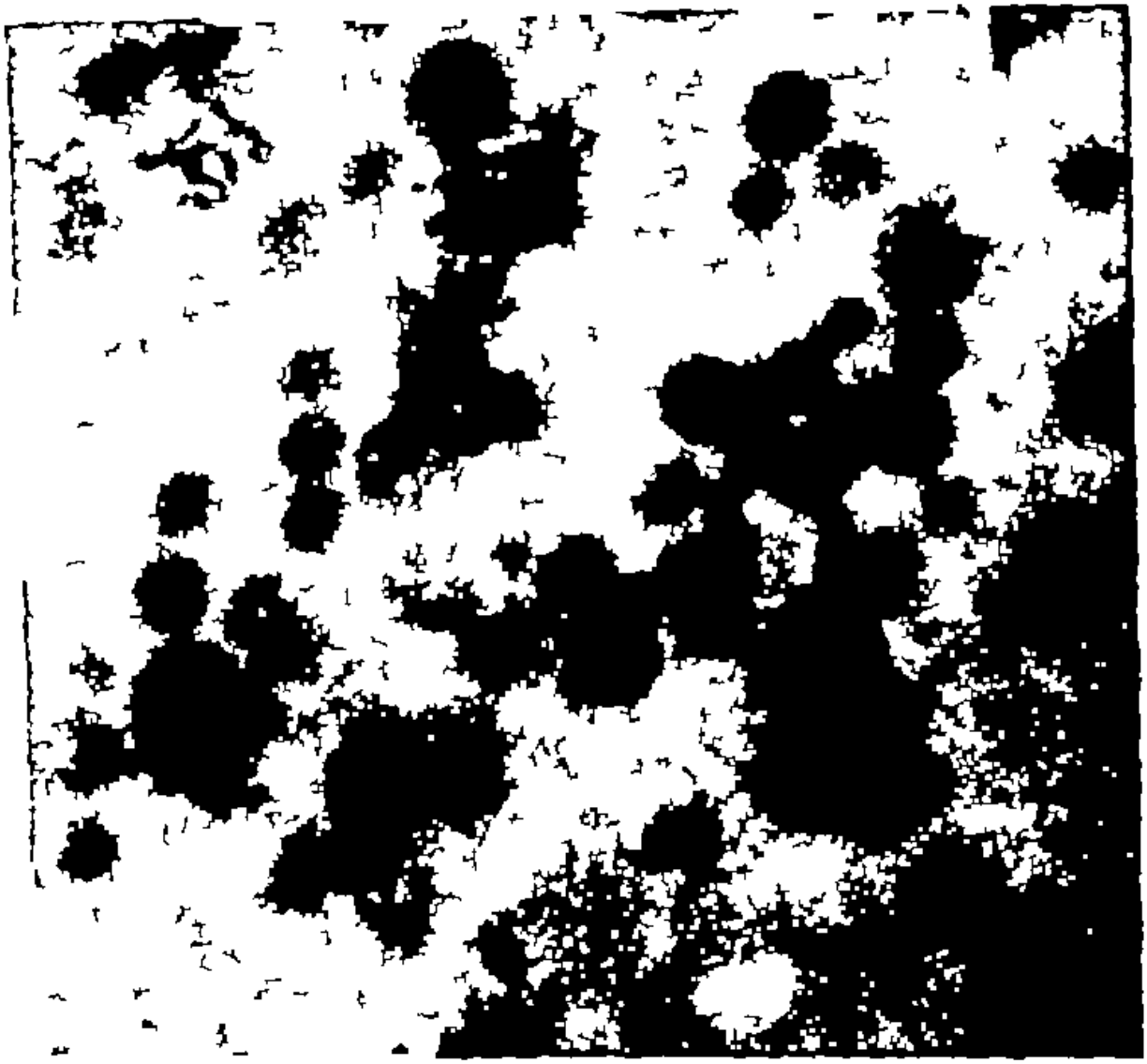


Fig. 26. Electron microscopic autoradiograph showing detail of the first enterochromaffin cell type. The silver grains are seen in association with the dense granules of mainly round shape. (×47 000)

and guinea-pig (see Håkanson *et al.* 1970 b). This might then take place in the second cell type which has been described in the present investigation. This cell, like the other described cell type has however not been observed to have any contact with the gland lumen in the present investigation or in most earlier investigations, where the corresponding cells have been observed (Helander 1962, Forssmann *et al.* 1969, Vassallo *et al.* 1969, Capella *et al.* 1969). From the morphological point of view it therefore does not seem probable that the products of these cells are secreted into the gland lumen. Håkanson and Owman (1967) have also shown that histamine is present in most, but not all of the enterochromaffin-like cells in the mouse and the rat. This can be ex-

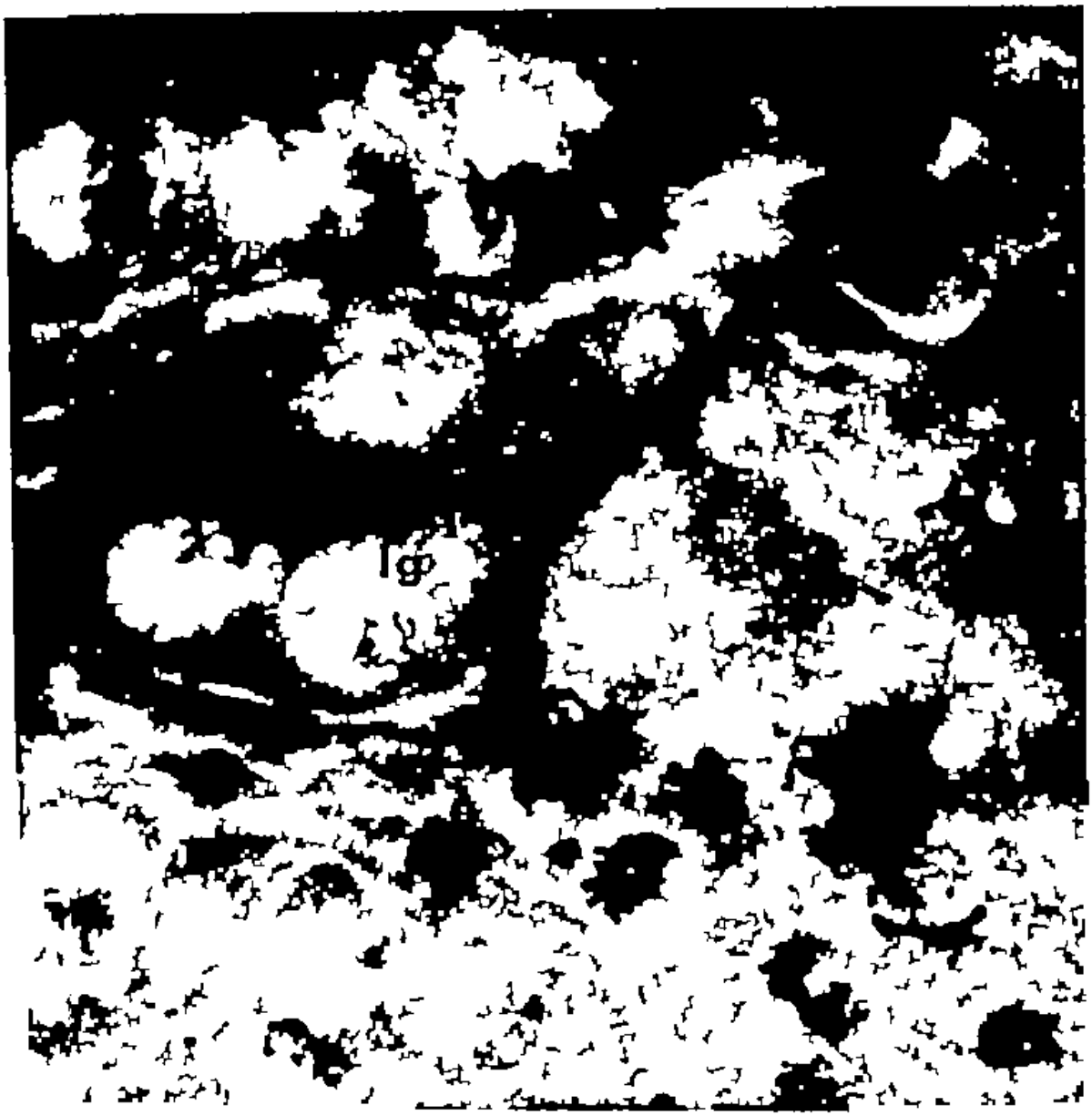


Fig. 27 Electron microscopic autoradiograph showing a detail of th second enterochromaffin cell type. The silver grains can be seen in association with both types of granules. Small granules (sg) large granules (lg) (×47.000)

plained by the presence of histamine in one of the two enterochromaffin cell types observed in the present work, but not in the other

Beside the fact that there are two different enterochromaffin cell types in the fundic mucosa of the mouse capable of monoamine formation from injected DOPA, it is also apparent from th present investigation that the monoamines are localized predominantly to the specific granules of the cells. A localization of 5-HT in the specific granules in the parafollicular cells of the thyroid has

recently been demonstrated in an electron microscope autoradiographic investigation where 5-HTP was injected into mice (Ericson 1970)

In some species 5-HT or dopamine is present endogenously in the pancreatic islets and the parafollicular cells. Such an endogenous monoamine storage has also been demonstrated with a cytochemical electron microscope method to be in accordance with the specific granules in one investigation on guinea-pig pancreatic islets (Jaim-Etcheverry and Zieher 1968 a) and in one investigation on sheep parafollicular cells (Jaim-Etcheverry and Zieher 1968 b) The specific granules of the endocrine cells are considered to be the storage sites for the hormones produced in the cells. If then the enterochromaffin cells are the producers of polypeptides, it is probable that these products are also stored in the specific granules of the cells. The possible participation of the monoamines in the synthesis, or release of the substances produced in the cells, is underlined by the common localization in the same granules. For the different enterochromaffin cell types, however such a function remains to be proved.

The effect of monoamines in the release of insulin is discussed in Chapter 8.

CHAPTER 5

Distribution of DOPA 1 ^{14}C (carboxyl ^{14}C-labelled DOPA) in relation to DOPA 2 ^{14}C in mice before and after inhibition of DOPA-decarboxylase, studied by whole body autoradiography

If amino acids are injected into animals, the highest uptake takes place in organs with a rapid protein synthesis such as the pancreas, the gastrointestinal mucosa, the bone marrow and the lymphatic tissues (see Hansson 1959) The uptake of DOPA by various organs seems to be similar to that of other aromatic amino acids (Guroff and Udenfriend 1962) After the injection of DOPA an initial accumulation also occurs in organs characterized by a rapid protein synthesis (Rosell *et al* 1963) The DOPA, however rapidly leaves the protein forming organs indicating that it is not incorporated into the proteins (Rosell *et al.* 1963)

In addition it is generally accepted that DOPA constitutes a precursor in the biosynthesis of catecholamines (see Blaschko 1959) Such a catecholamine formation also occurs in various organs after injection of DOPA (e g Gey and Pletscher 1964) The first step is the decarboxylation of DOPA via the action of aromatic L-amino acid decarboxylase.

If the uptake of DOPA and/or the catecholamines formed from DOPA is studied by autoradiographical methods, it must therefore be taken into account that the activity observed can either represent the DOPA itself or the formed catecholamines. A third alternative is that the activity can be due to formed metabolites of DOPA or catecholamines.

In the present investigation a whole-body autoradiographic study has been performed in mice using ^{14}C-DOPA labelled either in the carboxyl-group (C-1-group) or in the C-2-group (Fig 28) On the autoradiograms obtained from mice injected with carboxyl labelled DOPA, the catecholamines and the catecholamine metabolites are not visualized since the labelled carboxyl group is split off by the decarboxylation. The so formed $^{14}CO_2$ is then to the greatest extent exhaled (see Hansson *et al.* 1964) In mice injected with DOPA labelled in the C-^{2}-group the amino acid as well as the formed catecholamines and the metabolites are visible. This investigation has been performed on untreated mice and on mice pre treated with the DOPA-decarboxylase inhibitor Ro 4-1602 (Roche)

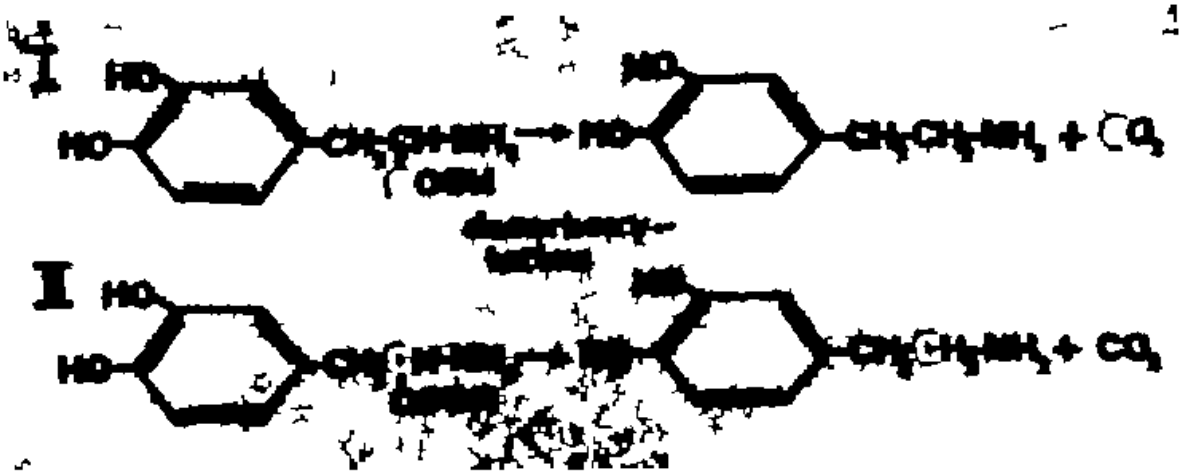


Fig. 28. When the carboxyl-labelled DOPA is decarboxylated the so formed catechol amines become non-labelled and therefore invisible in the autoradiograms (reaction I). When the DOPA labelled in the C-2-group is decarboxylated, the so formed catechol-amines become labelled and can therefore be seen in the autoradiograms (reaction II). The CO_2 formed in the reactions is exhaled. The white letters indicate the site of labelling.

Material and methods

The labelled substances DL-3(3,4-dihydroxyphenyl) alanine-1-^{11}C (DOPA 1-^{11}C) spec. act. 12.5 mCi/mM and DL-3(3,4-dihydroxyphenyl)alanine-2-^{11}C, spec. act. 52 mCi/mM were obtained from The Radiochemical Centre, Amersham, England. The DOPA-decarboxylase inhibitor Ro 4-4602 (2,3,4-trihydroxybenzyl hydrazine) was obtained from Hoffman-La Roche, Switzerland. The substances were dissolved in physiological saline and 0.2 ml was injected into the mice. In the case of intravenous injection which was performed into a tail vein, the injection time was about 10 seconds.

White mice of the NMRI-strain were used as experimental animals. The weight of the mice was about 20 g.

Four series of mice were injected.

1. 6 male and 3 female mice were injected intravenously with 5 μCi DOPA 1-^{11}C corresponding to 4.0 mg/kg body weight. The male mice were sacrificed at 1 minute, 5 minutes, 30 minutes, 1 hour, 4 hours and 8 hours after the injection. The female mice were sacrificed at 30 minutes, 1 hour and 2 hours after the injection.

2. 6 male and 3 female mice were injected intravenously with 5 μCi DOPA 1-^{11}C, corresponding to 1.0 mg/kg body weight. The male mice were sacrificed at 1 minute, 5 minutes, 30 minutes, 1 hour, 4 hours and 8 hours after the injection. The female mice were sacrificed at 30 minutes, 1 hour and 2 hours after the injection.

3 2 male mice were injected intraperitoneally with Ro 4-4602, dose 200 mg/kg body weight. Thirty minutes later they were injected intravenously with 5 μ Ci DOPA 1 ^{1} C (4.0 mg/kg) and killed 5 minutes and 30 minutes after the second injection.

4 5 mice were injected intraperitoneally with Ro 4-4602 dose 200 mg/kg body weight. Thirty minutes later they were injected intravenously with 5 μ Ci DOPA 2 ^{14}C (1 0 mg/kg) and killed 5 minutes, 30 minutes, 1 hour 2 hours and 4 hours after the second injection. The mice killed 30 minutes and ? hours after the DOPA injection were females, the others were males.

After the survival periods stated above, the animals were anaesthetized with ether and then immersed into a mixture of hexane and solid CO_2 (-78 C) The mice killed after a survival period of 1 minute were killed by direct immersion in the hexane-CO_2 mixture. They were then embedded in a mixture of carboxymethyl cellulose and water Sagittal sections (20 μ) of the whole body were cut and dried at -15 C according to Ullberg s autoradiographic technique, by which each section is attached onto tape (No. 810 Minnesota Mining and Manufacturing Co) (Ullberg 1954 1958) The tape mounted sections were allowed to reach room temperature in an air tight box. Autoradiograms were made by apposition of the sections against x ray films (Structurix, Gevaert) During the exposure time of 2 weeks to 3 months, the sections were stored in a refrigerator at -15 C. After the exposure, the sections were separated from the films. The films were then developed in Gevaert G 230 and fixed in Gevaert G 305 A.

Results

The ^{14}C-DOPA was rapidly taken up from the blood by various tissues. The distribution pictures from the mice not pre treated with, and those pretreated with the DOPA-decarboxylase inhibitor will be described separately

DOPA injected into mice not pre-treated with the decarboxylase inhibitor Ro 4-4602 (series 1 and 2 und r Meth ds)

Adrenal. One minute after the injection, there was a strong accumulation of activity in the adrenal medulla of the DOPA 2 ^{4}C-injected animal. In the animal injected with DOPA 1 ^{4}C the activity was low and even considerably lower than in the adrenal cortex (Fig 29 and 30)

The high activity in the adrenal medulla could be observed at all survival periods in animals injected with DOPA ? ^{14}C. In animals injected with DOPA 1 ^{1} C, the acti ity was low during the observation period (see Fig 31) On some autoradiograms, however small spots with a slightly higher activity



Fig. 29 a. Autoradiogram of mouse 1 minute after an intravenous injection of DOPA-2 ^{14}C.



Fig. 29 b. Autoradiogram of mouse 1 minute after an intravenous injection of DOPA-1 ^{14}C. Note the high accumulation of radioactivity (light areas) in the adrenal medulla, liver and intestinal mucosa in the DOPA-2 ^{14}C-injected animal, but not present in the DOPA-1 ^{14}C-injected animal.

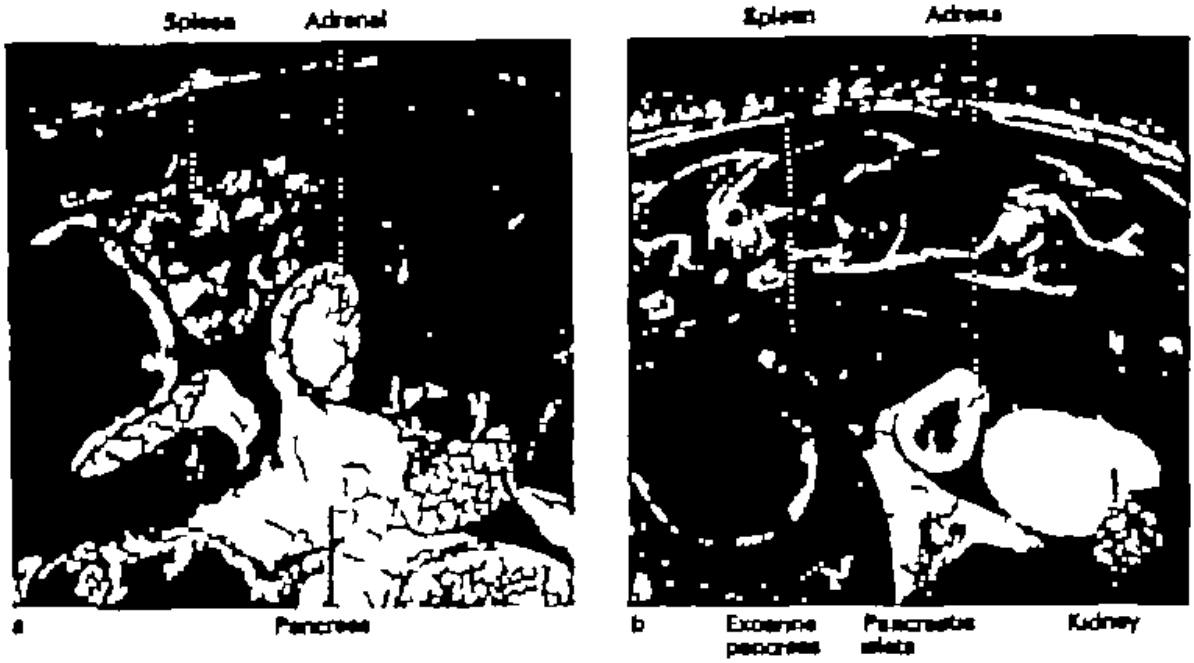


Fig. 30 a and b. Details of Fig. 29 a and b respectively. In the DOPA 2 ^{14}C-injected animal there is a high accumulation of radioactivity (light area) in the adrenal medulla (Fig. 30 a). In the DOPA-1 ^{14}C-injected animal there is no accumulation of radioactivity in the adrenal medulla (Fig. 30 b). In the exocrine pancreas there is a strong accumulation of radioactivity in both animals. In the animal injected with DOPA 1 ^{14}C there is no accumulation of radioactivity in the pancreatic islets.

could be observed in the adrenal medulla in animals injected with DOPA 1 ^{14}C (Fig. 32). These spots were preferentially localized in the periphery of the medulla. In the adrenal cortex, the activity was relatively high shortly after the injection of both DOPA 2 ^{14}C and DOPA 1 ^{14}C. Then the activity diminished rapidly.

Thyroid. A persevering activity could be seen in the thyroid after the DOPA 2 ^{14}C-injections, while following the DOPA 1 ^{14}C-injections no activity could be observed in the thyroid, not even shortly after the injection.

Pancreatic islets. The same as for the thyroid applied to the pancreatic islets (see Fig. 30 b).

Exocrine pancreas. One minute after the injection, there was a strong accumulation of activity in the pancreas (Fig. 29). This could then be observed up to 4 hours after the injection of both DOPA 2 ^{14}C and DOPA 1 ^{14}C.

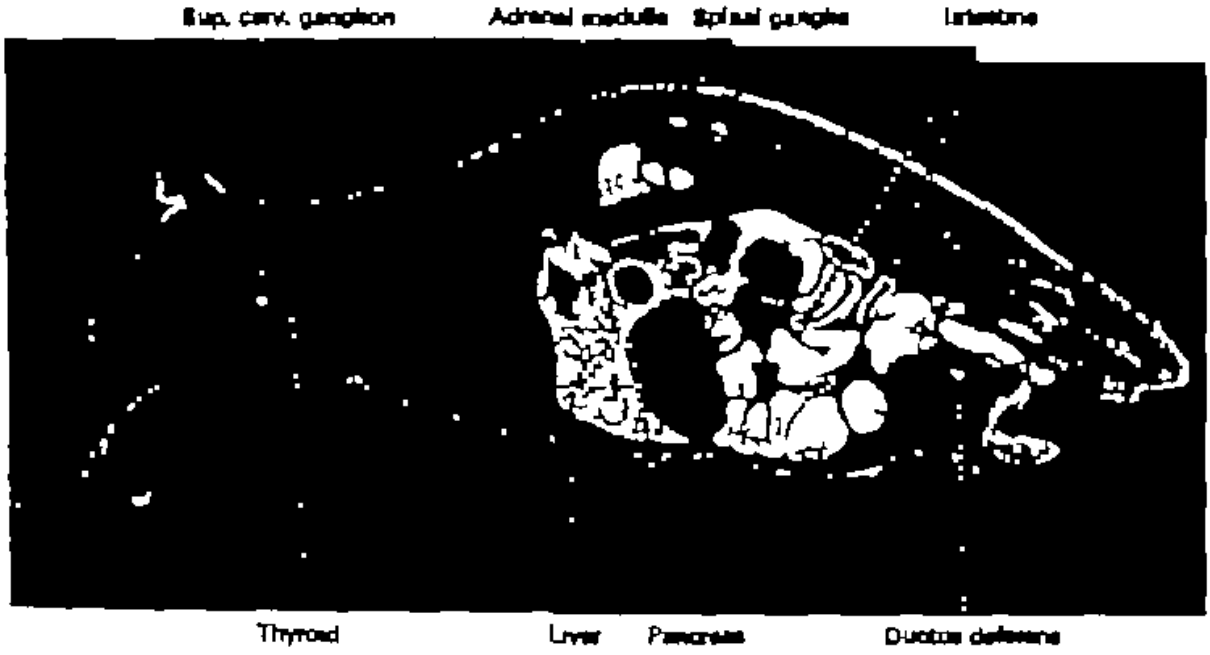
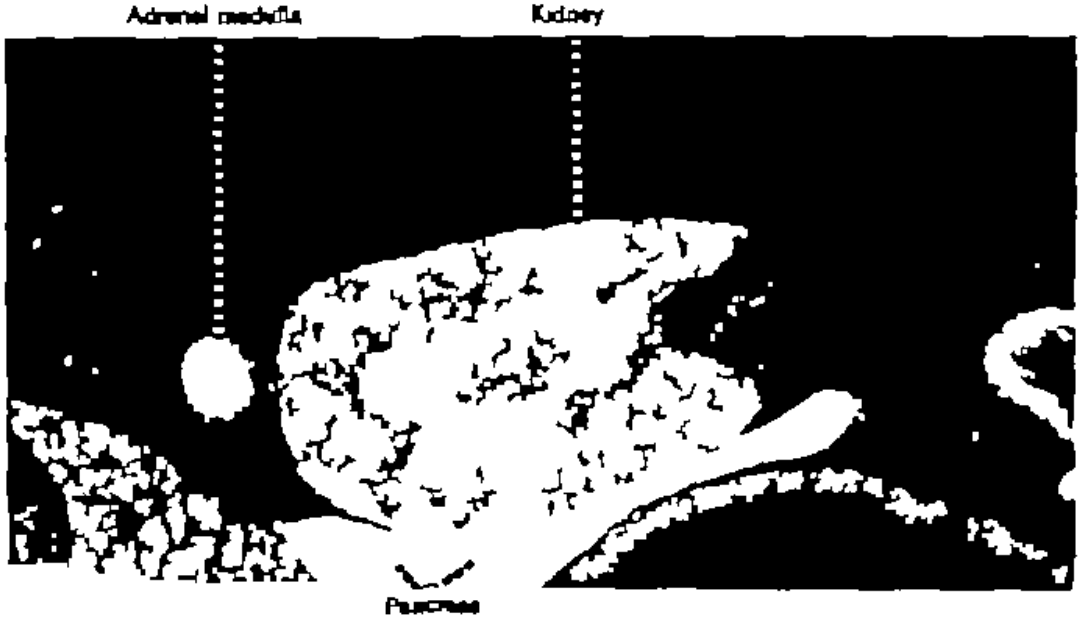


Fig. 31 a. Autoradiogram of a mouse 30 minutes after an intravenous injection of DOPA-2 ^{14}C.



Fig. 31 b. A toradiogram of mo er aft an intra enous injection of DOP V- 1-^{14}C. Note the high accu f uscular ity (light areas) in the adrenal med lla, liver and intestine in the D P\ 2 ^{14}C-injected animal, but not in the DOPA I ^{14}C-injected animal I the D(^{14}C-injected animal there is higher accumulation of radioactivity in sup er ganglion than in the spinal ganglia. In the DOPA I ^{14}C-injected animal there is some accumulation of radioacti y equal in the sup. cerv ganglion and in the spinal ganglia.



b

F g. 32. Details of
injections of) DOI
there is strong acc
32) In the DOPA i
the medulla which hav
the activity is low (Fig.
spinal ganglion in the pict

Liver The activity in the liver in the animal injected with DOPA 2 ^{1}C, was one minute after the injection almost as high as the activity in the blood. In the animal injected with DOPA 1 ^{14}C, the activity was considerably lower in the liver than in the blood at the one minute interval (Fig 29) Also at later time intervals the activity in the liver was higher in relation to the blood in animals injected with DOPA 2 ^{14}C than in animals injected with DOPA 1 ^{1}C (Fig 31)

Alimentary tract. As in the liver the intestinal mucosa and the mucosa of the glandular part of the stomach contained a higher activity in relation to the blood in the animal injected with DOPA $^{?}$ C than in the one injected with DOPA 1 ^{14}C, at the one minute interval (Fig 29)

Also longer lapses of time still produced the same situation, as has been described for the li er (Fig 31) The intestinal lumen contained a higher activity at longer survival periods after DOPA 2 ^{4}C-injections, than in animals injected with DOPA 1 ^{14}C

The mucous membranes of the mouth, the oesophagus and the forestomach contained a relatively high activity shortly after the injection of both DOPA 2 C and DOPA 1 ^{14}C. This activity then diminished rather quickly

The uptake of both DOPA $^{?}$ C and DOPA 1 ^{14}C in the salivary glands was high shortly after the injection and then diminished successively

Peripheral nervous system. An accumulation of activity could be seen in spinal and in sympathetic ganglia both after DOPA $^{?}$ ^{14}C- and DOPA 1 ^{14}C-injec tions (Fig. 31) In the DOPA 1 ^{14}C-injected animals the acti ity was about equal in the sympathetic and the spinal ganglia, while in the DOPA C-injected animals, the activity in the sympathetic ganglia was higher than in the spinal ganglia.

C ntral nervous system The injected DOPA passed the blood brain barrier slowly (see Fig 29 and 31) The slight acti ty which could be observed, as mainly localized in the grey matter The pictures seen after DOPA 2 ^{1}C- and DOPA 1 ^{14}C-injections were similar In the choroid plexus the activity was rather high both after DOPA C- and DOPA 1 ^{1} injections. The same applied to the hypophysis.

U genital rgans. The kidneys contained a high activity in animals injected both with DOPA 2 ^{4}C and DOPA 1 ^{1}C. High acti ty could also be seen in the urinary bladder

In the testicles the interstitial cells showed a moderate activity both after

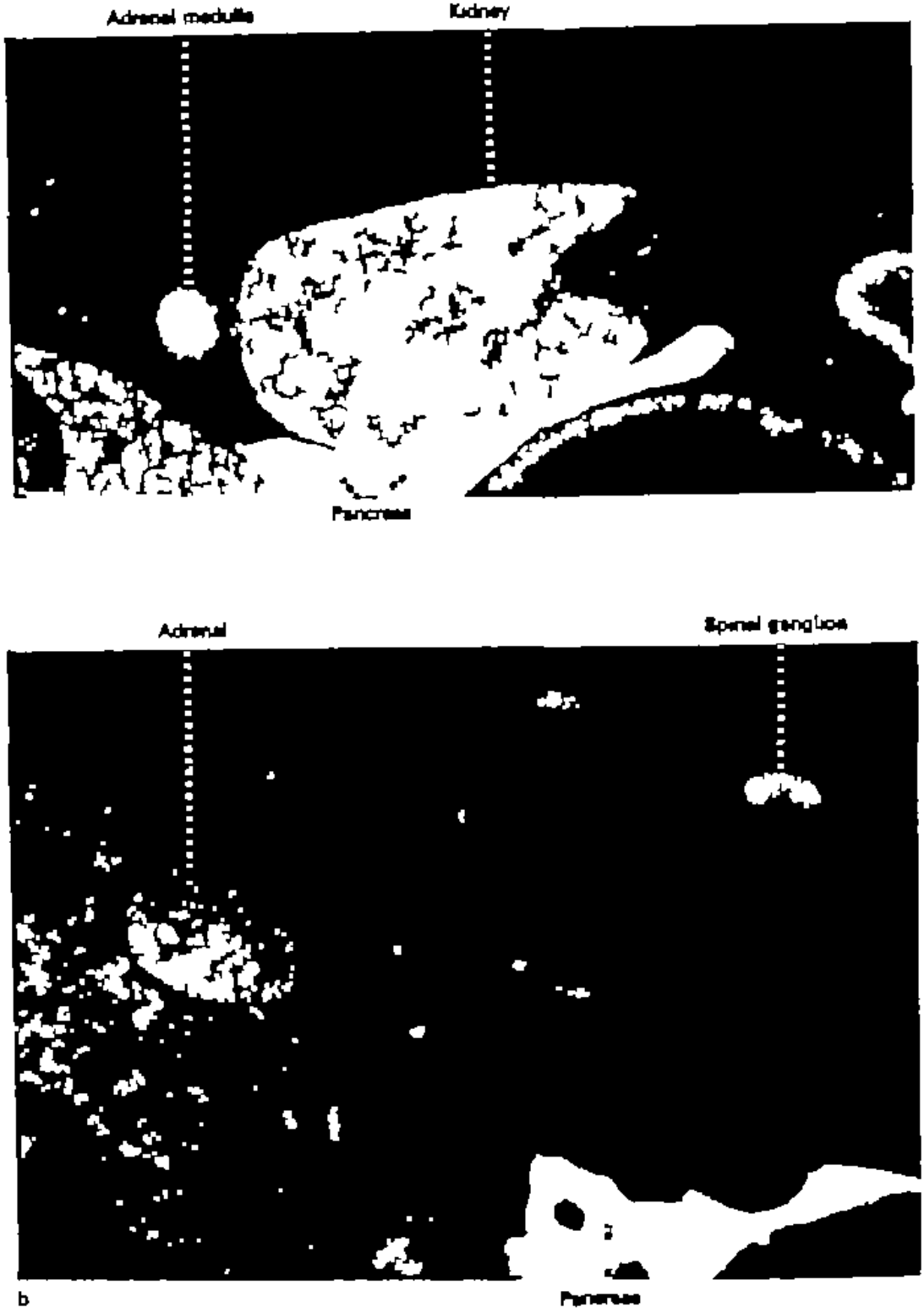


Fig. 32. Details of whole-body autoradiograms from mice 30 min tes after intravenous injections of) DOPA-2 ^{4}C and b) DOPA 1 C. In the DOPA C-injected animal there is strong accumulation of radioactivity (light area) in the adrenal medulla (Fig. 32) In the DOPA 1 ^{4}C-injected animal there are small spots in the periphery of the med lla which hav slightly higher activity than the rest of the medulla, where the activity is low (Fig. 32 b) The activity in these spots is about the same as in the spinal ganglion in the picture.

Liver The activity in the liver in the animal injected with DOPA 2 ^{14}C, was one minute after the injection almost as high as the activity in the blood. In the animal injected with DOPA 1 ^{14}C, the activity was considerably lower in the liver than in the blood at the one minute interval (Fig 29) Also at later time intervals the activity in the liver was higher in relation to the blood in animals injected with DOPA 2 ^{14}C, than in animals injected with DOPA 1 ^{14}C (Fig. 31)

Alimentary tract. As in the liver the intestinal mucosa and the mucosa of the glandular part of the stomach contained a higher activity in relation to the blood in the animal injected with DOPA 2 ^{14}C, than in the one injected with DOPA 1 ^{14}C, at the one minute interval (Fig 29)

Also longer lapses of time still produced the same situation, as has been described for the liver (Fig. 31) The intestinal lumen contained a higher activity at longer survival periods after DOPA 2 ^{14}C-injections, than in animals injected with DOPA 1 ^{14}C.

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Peripheral nervous system An accumulation of activity could be seen in spinal and in sympathetic ganglia both after DOPA 2 ^{14}C- and DOPA 1 ^{14}C-injections (Fig. 31) In the DOPA 1 ^{14}C-injected animals the activity was about equal in the sympathetic and the spinal ganglia, while in the DOPA 2 ^{14}C-injected animals, the activity in the sympathetic ganglia was higher than in the spinal ganglia.

Central nervous system. The injected DOPA passed the blood-brain barrier slowly (see Fig 29 and 31) The slight activity which could be observed, was mainly localized in the grey matter The pictures seen after DOPA 2 ^{14}C- and DOPA 1 ^{14}C-injections were similar In the choroid plexus the activity was rather high both after DOPA 2- ^{14}C- and DOPA 1 ^{14}C injections. The same applied to the hypophysis

Urogenital organs. The kidneys contained a high activity in animals injected both with DOPA 2 ^{14}C and DOPA 1 ^{14}C. High activity could also be seen in the urinary bladder

In the testicles the interstitial cells showed a moderate activity both after

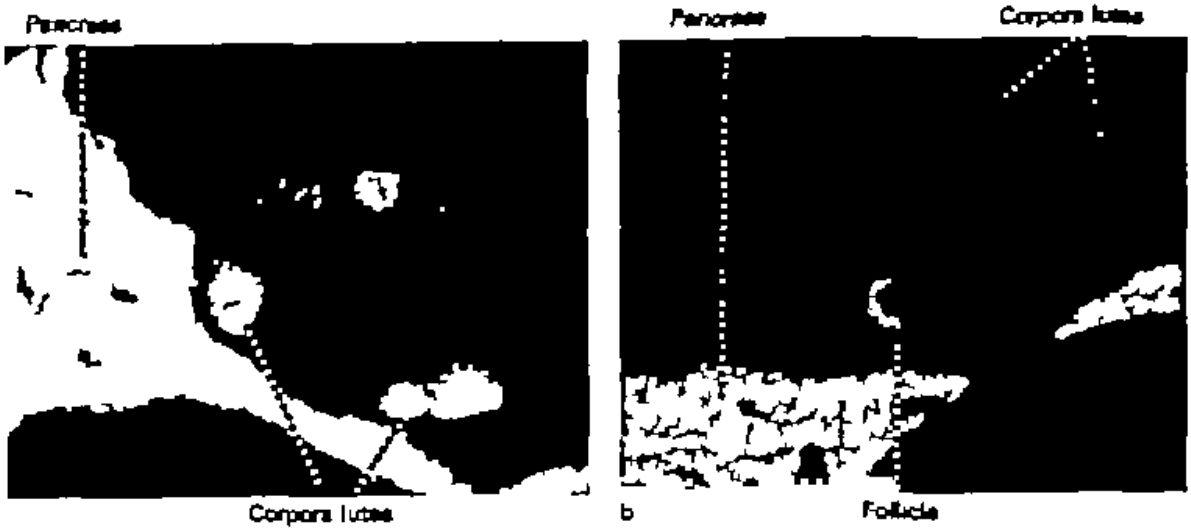


Fig. 33 Details of whol -body autoradiograms showing the accumulation of radioactivity in the ovaries of mice, a. 30 minutes and b. 2 hours after intravenous injections of DOPA 1 ^{14}C. Note the high accumulation of radioactivity (light areas) in the follicle walls and corpora lutea.

DOPA 2 ^{14}C- and DOPA 1 ^{14}C-injections, the activity being highest at the short survival periods. A part of caput epididymidis showed a high uptake of activity both after DOPA 2 ^{14}C- and DOPA 1 ^{14}C-injections. The activity persisted up to 4 hours after the injection. Ductus deferens accumulated a rather high activity after the injection of DOPA 2 ^{14}C. After the injection of DOPA 1 ^{14}C the activity there was low.

A relatively high uptake of activity could also be seen in the ovary especially in the follicle walls and corpora lutea (Fig. 33). This activity was seen up to 2 hours after the injection of both DOPA 2 ^{14}C and DOPA 1 ^{14}C. During this observation period there was also considerable activity in the oviduct and in the uterus.

Respiratory organs In the lungs, the activity was high both after DOPA 2 ^{14}C- and DOPA 1 ^{14}C-injections shortly after the injection. The activity then successively diminished. The nasal mucosa showed a rather high activity during the observation period after both DOPA 2 ^{14}C- and DOPA 1 ^{14}C-injections.

Circulatory organs There was a relatively high uptake of activity in the myocardium shortly after injection of both DOPA 2 ^{14}C and DOPA 1 ^{14}C. The activity then diminished rather rapidly.

The bone marrow initially accumulated a rather high activity both after DOPA 1 ^{14}C- and DOPA 2 ^{14}C-injections.

In the spleen the white pulp showed a rather high uptake shortly after the injection of either DOPA 2 ^{14}C or DOPA 1 ^{14}C (Fig 30) At short time intervals the red pulp contained a higher activity in relation to the white pulp in animals injected with DOPA 2 ^{14}C, than with DOPA 1 ^{14}C. At long time intervals the activity was low in the entire spleen both after DOPA 2 ^{14}C- and DOPA 1 ^{14}C-injections.

The thymus did not accumulate any appreciable amount of activity

Lens Initially there was no accumulation in the lens, but from 30 minutes up to 4 hours the activity was considerable. The activity was highest in the lens capsule and the pictures from DOPA 2 ^{14}C- and DOPA 1 ^{14}C-injected animals were similar

Skin. There was a relatively high accumulation of activity in the connective tissue of the dermis, in the corneal layer and in hair follicles and hair The activity could be observed both after DOPA 2 ^{14}C- and DOPA 1 ^{14}C-injections. It appeared rapidly and persisted throughout the observation period.

Animals pre-treated with the decarboxylase inhibitor R 4-4602 (series 3 and 4 under Methods)

At the observed survival intervals the distribution pictures after the DOPA 1 ^{14}C-injections were similar to the pictures seen after the DOPA 2 ^{14}C-injections (see Fig 34 and 35) These pictures were similar to those obtained after injection of DOPA 1 ^{14}C into untreated animals.

The activity in the blood diminished rapidly after the injection.

The highest accumulation of activity could, during the whole observation period, be seen in the pancreas.

A high activity could also be seen in the kidney In the urinary bladder in part of the caput epididymidis and in follicle walls and corpora lutea of the ovary Also in the skin and the nasal mucosa the activity was rather high.

The salivary glands, the intestinal mucosa and the mucosa of the glandula part of the stomach accumulated a moderate activity From 30 minutes to 4 hours the activity in the lens, especially in the capsule, was rather high.

In the adrenal medulla there was low accumulation of activity up to 2 hours after the injection (Fig 35) However small spots with a slightly higher activity preferentially in the periphery of the medulla, could be observed Four hours after the injection of DOPA ^{14}C a high activity could be seen in the adrenal medulla (Fig 36)

In the thyroid and the pancreatic islets there was a low accumulation of activity

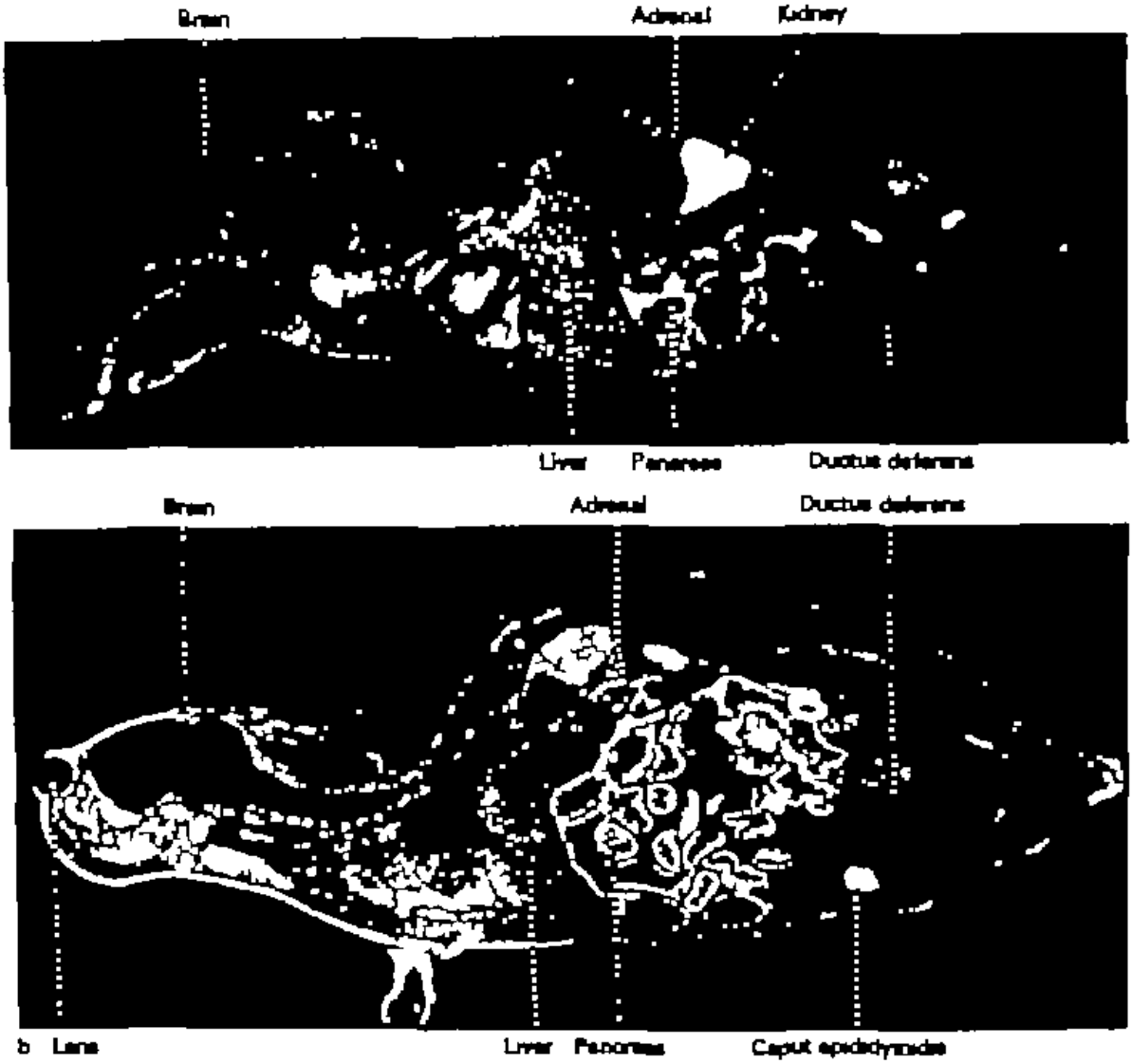


Fig. 34 a. Autoradiogram of mouse 5 minutes after an intra enous injection of DOPA 2 ^{14}C.

b. Autoradiogram of mouse 5 min tes after an intravenous injection of DOPA 1 ^{14}C. The animals have been pre-treated with th DOPA-decarboxylase Inhibitor Ro 4 4602. Th distribution pictures are similar Note the high accumulation of radioactivity (light areas) in the pancreas and in the caput epididymidis. There is no accumulation of adioactivity in the adrenal medulla.

In the liver the lung, the bone marrow and the spleen, the distribution patterns were similar to each other The activity was never high in these organs, but exceeded the activity in the blood to some extent up to 30 minutes after the injection.

In the central nervous system the activity was low during the observation period with exception of the choroid plexus and the hypophysis, which contained a rather high activity

In the peripheral nervous system there was a moderate activity both in sympathetic and spinal ganglia.

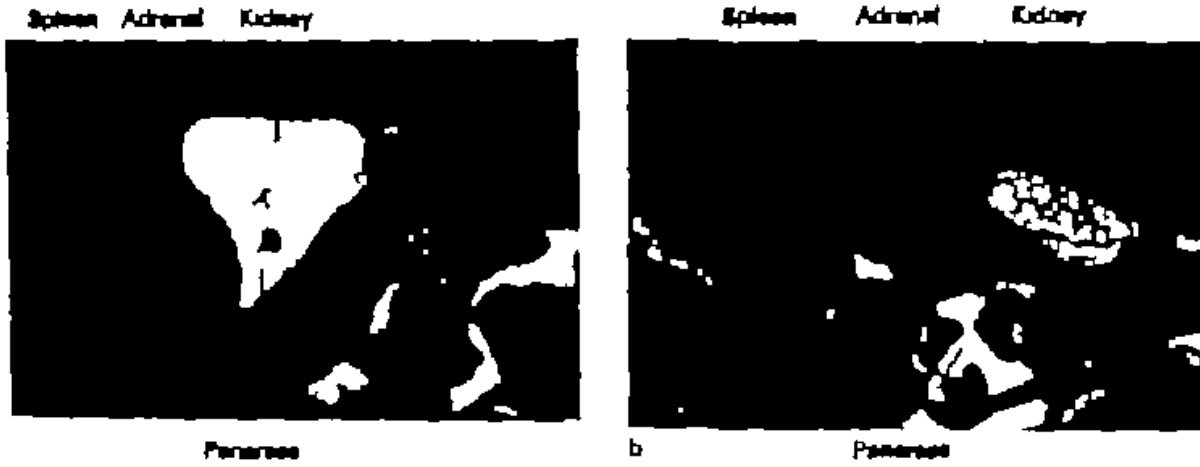


Fig. 35 a and b. Details of Fig. 34 a and b respectively. There is no accumulation of radioactivity (light areas) in the adrenal medulla in the animal injected with DOPA-2-^{14}C (a) or with DOPA 1-^{14}C (b)

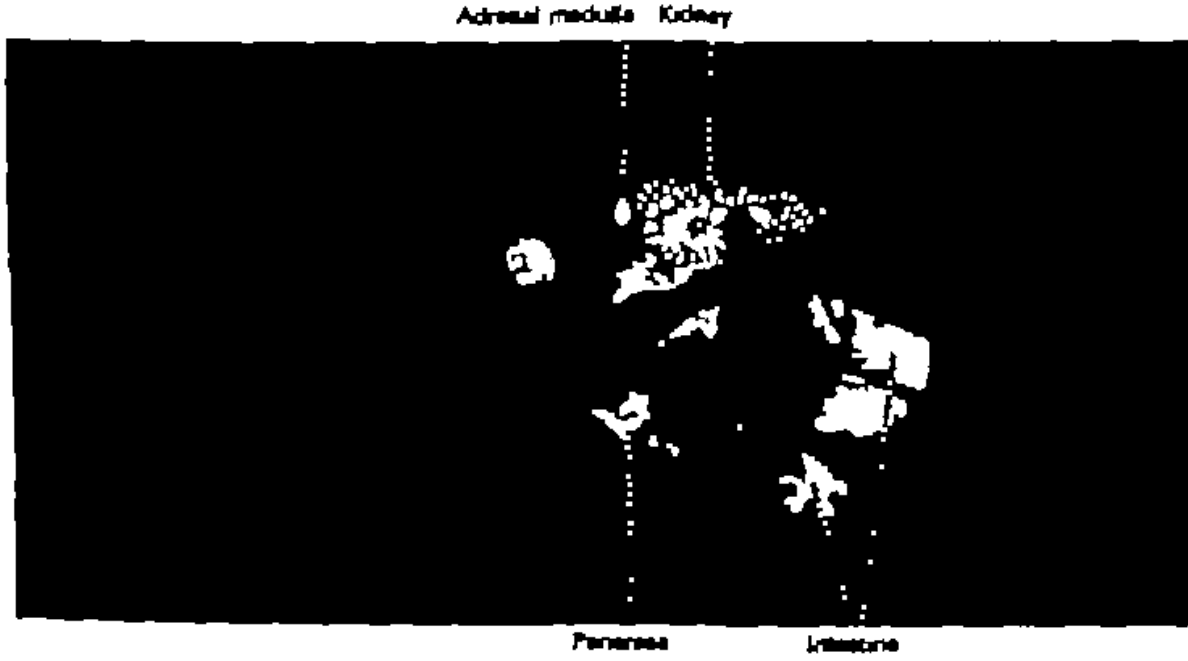


Fig. 36. Autoradiogram of a mouse 4 hours after an intravenous injection of DOPA-2-^{14}C. The animal was pre-treated with the DOPA-decarboxylase inhibitor Ro4-4602 30 minutes before the DOPA-2-^{14}C-injection. Radioactivity (light area) can be seen in the adrenal medulla. The DOPA-decarboxylase-inhibiting effect of Ro4-4602 has probably disappeared at this time interval.

Discussion

Some general mechanisms will be discussed first, followed by a discussion of the distribution patterns obtained in some organs.

The injection of DOPA rapidly leads to a formation of large amounts of catecholamines, especially dopamine in various tissues (Weiss and Rossi 1963

Gey and Pletscher 1964 Wurtman *et al* 1970) It is therefore obvious that a decarboxylation is of great importance in the metabolism of exogenous DOPA.

The second major pathway in the metabolism of exogenous DOPA seems to be an O-methylation via the action of the enzyme catechol O-methyl transferase (COMT) (Gey and Pletscher 1964 Wurtman *et al.* 1970) 4-hydroxy 3-methoxy-phenylalanine (methoxy-DOPA) is formed by this process.

The metabolism of the catecholamines formed from DOPA takes place via two different ways. The first way is an oxidative deamination through the action of the enzyme monoamine oxidase (MAO) (e.g. Zeller 1962) The second way is an O-methylation through the action of COMT (e.g Axelrod 1966) MAO is in its action limited to amines (see Arvy 1963) while COMT O-methylates all catechols, regardless of the substituent on the aromatic nucleus (Axelrod 1966)

The oxidative deamination and the O-methylation are inactivating processes, since the physiological activities of the products are weak and the products also leave the body rapidly (Kopin 1964 Axelrod 1966) It has recently been reported that methoxy-DOPA might be an exception from this rule since it seems to stay in the pancreas for rather a long time (Bartholini *et al.* 1970)

Other pathways of metabolism for DOPA or catecholamines except those mentioned seem to be of minor importance. Thus after the injection of DOPA into mice, the major compounds found in the body have been reported to be DOPA methoxy DOPA catecholamines and catecholamine metabolites (Wurtman *et al* 1970)

The radioactivity seen in the autoradiograms after the injection of DOPA 1 C in the present investigation should therefore largely represent DOPA and methoxy DOPA. When catecholamines are formed from DOPA 1 ^{14}C the labelled C is split off and $^{14}CO_2$ is formed. This is then to the greatest extent exhaled (Hansson and Clark 1962, Hansson *et al.* 1964) The catecholamines and the catecholamine-metabolites are rendered autoradiographically visible In mice injected with DOPA 2 C, DOPA and methoxy-DOPA as well as catecholamines and catecholamine metabolites are visible. The proportion of these components varies of course considerably in different organs. In organs such as the liver the intestine and the kidneys, the proportion of metabolites is great while in organs such as the adrenals and the pancreas, the proportion of respectively catecholamines and DOPA is great (Rosell *et al* 1963 Gey and Pletscher 1964)

When DOPA is injected into mice, where the DOPA-decarboxylase has been inhibited an autoradiographic condition similar to the one seen after injection of DOPA 1 C, is obtained inasmuch as no catecholamines are formed and the radioactivity therefore does neither represent catecholamines nor cate-

cholamine metabolites. The precedent condition being that the decarboxylase inhibitor used is effective. Ro 4-4602, which has been used in the present investigation, has been shown to be very effective *in vivo* (Pletscher and Gey 1963 Hanuson *et al.* 1964) Evidence for the effectiveness of the decarboxylase inhibitor in the present investigation is the fact that the autoradiograms obtained after the injection of DOPA 1 ^{14}C and DOPA 2 ^{3}C into animals, in whom the decarboxylase has been inhibited, are similar

The possibility exists that the decarboxylase inhibitor in itself might interfere with the uptake of DOPA in various organs. Such an interference has, however only been achieved with other aromatic amino acids (see Gey and Pletscher 1964) Interference with decarboxylase inhibitors such as α-methyl-DOPA (Schanberg 1963) or Ro 4-4602, has not been observed and there are no indications that this is the case in the present investigation (see below)

It should be pointed out that DL-DOPA was used in the present investigation. The D-forms of aromatic amino acids appear to enter different tissues less than the L-forms (Guroff and Udenfriend 1962, Yoshida *et al.* 1963) In addition the catecholamine formation only takes place from L-DOPA.

The distribution patterns obtained in some organs will be discussed separately below

Adrenal

The autoradiograms illustrate the rapidity by which the decarboxylation takes place, and by which the so formed catecholamines are accumulated in the adrenal medulla after the injection of DOPA. The catecholamine formation in the adrenal medulla, which is obtained after the injection of DOPA, has been studied with autoradiographic methods by several authors (Rosell *et al.* 1963 Hempel 1963 Elfvin *et al.* 1966, Moppert 1966, Hempel and Kraft 1968) L-amino acid decarboxylase has been shown in the adrenal medulla of several species (Langemann 1951 Westermann 1956)

The autoradiograms from animals in whom the decarboxylase was inhibited, show that DOPA in itself is not accumulated in the adrenal medulla. Hempel and Männl (1966, 1967) similarily observed that ^{3}H DOPA formed from ^{3}H tyrosine in the adrenal medulla of the cat and bird, could not be stored there *when the decarboxylase was inhibited.* The catecholamines formed in the adrenal medulla have a very long half life (Burack and Draskóczy 1964 Hempel and Kraft 1968) In the adrenal medulla the catecholamines are stored in the chromaffin granules of the cytoplasm. It has been proposed that the amines are bound there in a nondiffusible form as a complex with ATP and protein (Hillarp 1958) but the exact mechanism for this is not known (Smith 1968) Obviously the catechol-configuration is not in itself of chief

importance for the storage, as has been proposed by Weiner and Jardetzky (1964). Four hours after the injection of DOPA 2 ^{14}C into a Ro 4-4602 -pre-treated animal activity could be seen in the adrenal medulla. At this point the decarboxylase inhibating effect of Ro 4-4602 has probably disappeared, since this effect has been shown to be of a rather short duration (Hansson *et al* 1964). The spots with a slightly higher activity observed preferentially in the periphery of the medulla in mice injected with DOPA 1 ^{14}C and in mice in whom the decarboxylase had been inhibited, might be activity in ganglion cells, which have been shown to be present in the medulla (Coupland 1965). The activity there is similar to the activity observed in other ganglion cells under the same conditions (see below).

The activity observed in *the thyroid* after injection of DOPA 2 ^{14}C into animals in whom the decarboxylase was not inhibited probably represents activity in the parafollicular cells. As in *the pancreatic islets* there was no accumulation of radioactivity in the thyroid in animals where the decarboxylase was inhibited or after the injection of DOPA 1 ^{14}C into untreated animals. As in the adrenal medulla decarboxylation is obviously necessary for retention of radioactivity in the pancreatic islets and in the parafollicular cells. This has been discussed earlier (Chapter 1 and 7).

Exocrine pancreas

Injected amino acids are strongly accumulated in protein forming organs, such as the pancreas (see Hansson 1959). DOPA is also initially accumulated in these organs, but because it is not incorporated into proteins, it is rapidly cleared from such organs (Rosell et al 1963). In the present investigation an accumulation in the pancreas could be observed independent of whether the animals were pre-treated with Ro 4-4602, or whether the DOPA was labelled in C1 or in C2 position.

Using a fluorescence method for monoamine demonstration Alm *et al.* (1969) noted that after a DOPA injection, when the decarboxylase had been inhibited, the fluorescence over zymogen granules diminished, while the DOPA fluorescence in the rest of the cell persisted. They concluded that catecholamines were formed in the zymogen granules and that this process was blocked by the decarboxylase inhibition.

As has been mentioned methoxy DOPA seems to stay in the pancreas for a rather long time (Bartholini *et al* 1970).

The liver and the intestinal mucosa are characterized by a rapid protein synthesis and therefore an initial accumulation of DOPA might be expected here. These organs, however also possess a high DOPA-decarboxylase activity (see Erspamer 1961). From the present investigation it is also obvious that

the high accumulation of radioactivity seen shortly after the injection of DOPA 2 ^{14}C to a large extent represent the decarboxylated product, since after DOPA 1 ^{14} injections, the activity is considerably lower in these organs than after the injections of DOPA 2 ^{14}C. At longer time intervals a higher activity is seen in the intestinal lumen after the injection of DOPA 2 ^{14}C, than after DOPA 1 ^{14}C. This activity therefore also represents the decarboxylated products.

Nervous system

In ganglia a moderate accumulation of radioactivity could be seen after the injection of DOPA 1 ^{14}C and when the decarboxylase was inhibited. After the injection of DOPA 2 ^{14}C into untreated animals, a stronger accumulation could be observed in sympathetic ganglia than in spinal ganglia, suggesting a catecholamine formation in the sympathetic ganglia. Small dopamine containing cells (SIF-cells) have been demonstrated in sympathetic ganglia (Björklund *et al.* 1970). The presence of DOPA-decarboxylase in the sympathetic ganglia has also been shown (see Roch-Ramel 1968). Adrenergic nerves have been demonstrated both in autonomic and in spinal ganglia (Hamberger *et al.* 1965 b, Owman and Santini 1966). The amount of catecholamines in ganglia has been correlated to the number of neurons in the ganglia (Norberg and Sjöqvist 1966). The low radioactivity in the brain demonstrates the unique property of the brain with respect to amino acid penetration observed earlier (Guroff and Udenfriend 1962, Udenfriend and Zaltzman-Nirenberg 1963, Gey and Pletscher 1964). The injection of DOPA has been shown to give a catecholamine formation in the walls of cerebral vessels (Bertler *et al.* 1966).

Sexual glands

High activity of both DOPA 2 ^{14}C and DOPA 1 ^{14}C could be seen in ovarian structures, such as the follicle walls and corpora lutea in females and in parts of the caput epididymidis in males. In these structures a high enzyme activity (and hereby protein synthesis) seems to be present. The stratum granulosum is suggested to be the source of the ovarian estrogen (Bjersing and Carstensen 1964) and the corpus luteum is generally accepted as the source of ovarian progesteron (see Appelgren 1967). In the epididymis there are variations of different hydroxysteroid dehydrogenases in different zones (Baillie *et al.* 1966). A high accumulation of 4-iodophenylalanine has been observed in part of the epididymis (Ullberg and Bloomquist 1968). The pancreas is the only additional organ which accumulates any higher concentration of this amino acid analogue, which, like DOPA, is not incorporated into proteins. Normano

(1967) and Kormano and Penttilä (1968) using fluorescence methods, observed fluorescence in the epididymis after the injection of DOPA and dopamine into rats. The accumulation of DOPA in these structures does of course not exclude the possibility that in addition catecholamines are formed. This seems to be the case in ductus deferens, where a higher accumulation of activity could be seen after the injection of DOPA 2 ^{14}C, than after the injection of DOPA 1 ^{14}C. Ductus deferens has been shown to contain a dense network of adrenergic nerve terminals (see Sjöstrand 1965) where *in vitro* a catecholamine formation from DOPA has been shown to occur (Hamberger 1967). After the injection of ^{1} C-5-HTP radioactivity presumably due to serotonin has been shown by autoradiographic techniques to accumulate in the sympathetic neurons of the rat ductus deferens (Taxi and Droz 1966).

The lens accumulated DOPA slowly. This may be put in relation to the slow accumulation of amino acids seen in the lens under *in vitro* conditions (see Brasil and Kern 1968).

The skin accumulated much radioactivity both after DOPA 1 ^{1} C- and DOPA ^{2} ^{14}C-injections. Such an accumulation has been observed earlier with DOPA and 5-HTP (Gey and Pletscher 1964, Ritzén *et al.* 1965). It has been suggested that this accumulation of DOPA might be related to special functions of the skin such as melanin formation, which however has been lost in albino animals (Gey and Pletscher 1964).

The rate limiting step in the endogenous formation of catecholamines in tissues, seems to be the hydroxylation of tyrosine to DOPA (see Bloom and Glarman 1968). Once DOPA is formed it is rapidly converted to catecholamines. DOPA is endogenously present only in very small amounts in tissues (Anton and Sayre 1964, Hempel and Mlinnl 1967). In certain tumours such as phaeochromocytom, neuroblastom and melanom the endogenous DOPA concentration is higher (von Studnitz *et al.* 1963, Cabana *et al.* 1964, Falck *et al.* 1969).

CHAPTER 6

Histochemical demonstration of monoamine oxidase (MAO) and its relation to the *in vitro* uptake of serotonin ^{14}C, studied in whole-body sections of mice

Monoamine oxidase (MAO) catalyzes the oxidative deamination of monoamines in the body. It is widely distributed in the animal kingdom and probably involved in the *in vivo* inactivation of a number of biologically active monoamines of physiological importance, although its precise function has yet to be defined (see Arvy 1963, Gorkin 1966, Collins *et al.* 1970).

To investigate the distribution and activity of MAO several histochemical methods, and quantitative methods based on the disappearance of a substrate, or the formation of deaminated products from a substrate, have been used (*e.g.* Koelle and Valk 1954, Glenner *et al.* 1957, Wernbach *et al.* 1960, Lovenberg *et al.* 1962 b). Lately radiochemical methods for MAO-determination have been developed. (Otsuka *et al.* 1964, Consolo *et al.* 1968, Fellman *et al.* 1969).

Of the histochemical methods for MAO-demonstration the method described by Glenner *et al.* (1957) has been most extensively used. This method is based on a reduction of tetrazolium salts to coloured formazan deposits.

Tissues are incubated in a buffer containing a tetrazolium salt and a suitable substrate for MAO such as tryptamine or serotonin. In connection with the oxidation of the substrate the tetrazolium salt is reduced to form the formazan deposits in the tissues. The mechanism by which tetrazolium salts are reduced in the MAO reaction has been described by Wernbach *et al.* (1957).

In the present investigation whole-body sections from mice have been used for the histochemical demonstration of MAO. In this way it is possible to compare the relative concentration of MAO-activity in the same section in different tissues treated in exactly the same way.

In addition the uptake of ^{14}C-serotonin has been studied in whole-body sections from mice incubated in a buffer under conditions similar to those used in the MAO-investigation.

Material and methods

Whole-body freeze sections 10 μ thick from NMRI mice, were taken on tape (nr 688, Minnesota Mining and Manufacturing Co, USA) according to the

method described earlier (page 54) The sections were dipped in acetone at −10 C for about 15 seconds before they were allowed to reach room temperature in an air tight box. 5 male mice and 5 female mice (one of which was pregnant) were used for the MAO determination. For the experiments with serotonin-^{14}C 2 male mice were used.

The MAO activity was determined according to the method described by Glenner *et al.* (1957) The sections were incubated for one hour at 37 C in a 0 1 M phosphate buffer solution pH 7 6 Tryptamine hydrochloride* (0 05 M) or serotonin creatinine sulphate* (0 05 M) were used as substrates. Nitro-blue tetrazolium* was used as tetrazolium salt. The incubation solution had the following composition.

Tryptamine hydrochloride or Serotonin creatinine sulphate	0 05 M
Sodium sulphate	4 mg
Nitro-blue tetrazolium	5 mg
0 1 M phosphate buffer pH 7 6	5 ml
Distilled water	15 ml

After the incubation the sections were briefly washed in running water They were then fixed in buffered 4 % formaldehyde solution for 24 hours and embedded in glycerol-gelatine. Control sections were incubated without substrate or were pre incubated with the MAO inhibitor nialamide (0 01 M) (Pfizer) for 15 minutes. In addition sections from mice injected i.p. with nialamide 500 mg/kg body weight, 4 hours before they were killed, were used as controls.

In the experiments with serotonin ^{1} C, sections were incubated in a solution with the same composition as described earlier though substituting serotonin-C (5-hydroxytryptamine 2 ^{14}C-binoxalate, spec. act. 18 mCi/mM (68 μCi/mg) New England Nuclear) for tryptamine hydrochloride or serotonin creatinine sulphate as substrate. 2.5 μCi serotonin- C (corresponding to 37 μg 5-hydroxytryptamine ^{2} ^{14}C-binoxalate) was added to each 10 ml of incubation medium The sections were incubated for one hour at 37 C. They were then rinsed for 5 minutes in water and thereafter air-dried. Autoradiograms were made by apposition of the sections against x ray films (Structurix, Gevaert) The exposure time was about 3 weeks. Also in these experiments some sections were pre-incubated fo 15 minutes with nialamide (0.01 M) before the serotonin-^{1} C- ncubation.

Sigma Chemical Company Missouri, USA.

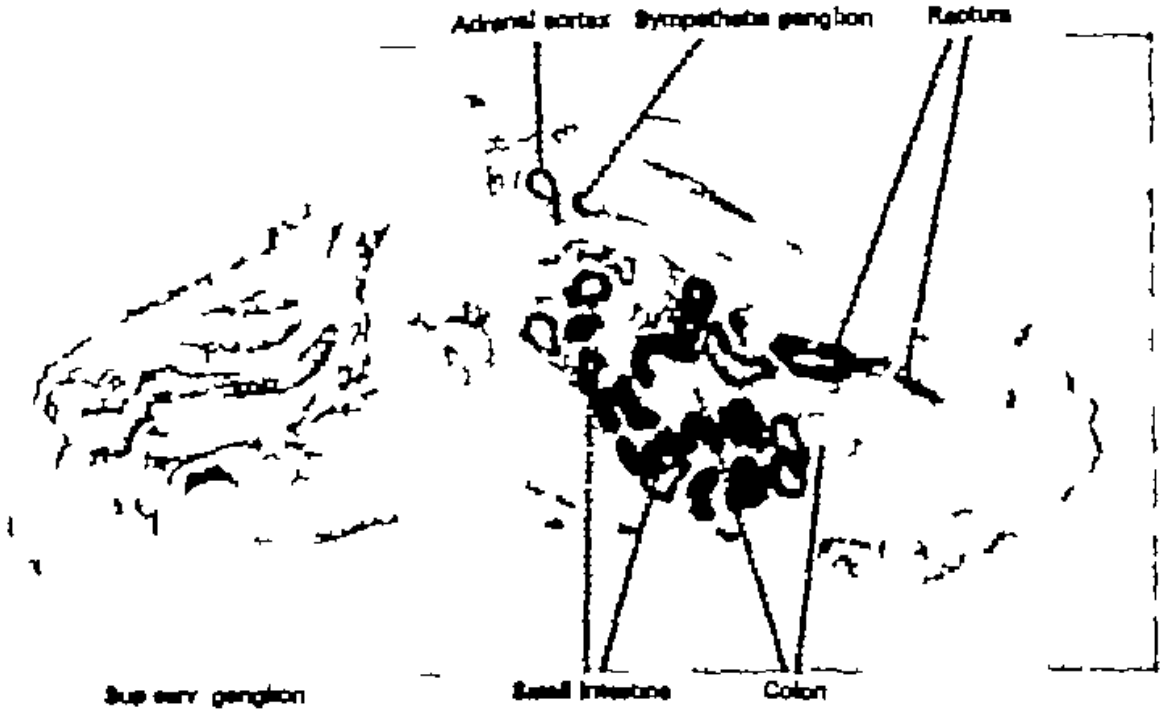


Fig. 37 Whole-body section of a mouse showing the MAO-activity The highest enzyme activity (dark areas) is present in sympathetic ganglia, in the mucosa of the small intestine and rectum, and in the adrenal cortex.

Results

MAO histochemistry

The results obtained with tryptamine hydrochloride and serotonin creatinine sulphate as substrates were similar

The most prominent formazan deposits were seen in sympathetic ganglia followed by the mucosa of small intestine and rectum (Fig 37 and 38) Prominent formazan deposits were also seen in the adrenal cortex (Fig. 37) In other organs except the mentioned and in the fetal tissues the formazan deposits were considerably less prominent.

A rather high enzyme activity could be seen in the liver in the kidneys, in the pancreas, in brown fat and in different parts of the central nervous system. A more detailed description of the enzyme activity in some organs will be presented below

In the sympathetic ganglia most cells showed a strong activity (Fig 39) In spinal ganglia the enzyme activity was low

In the mucosa of the small intestine the activity was most intense at the striated border of the villous epithelium. The epithelial cells of the crypts showed less activity (Fig 40) There was no difference in reaction intensity between duodenum, jejunum, ileum and rectum. In the colon a light activity

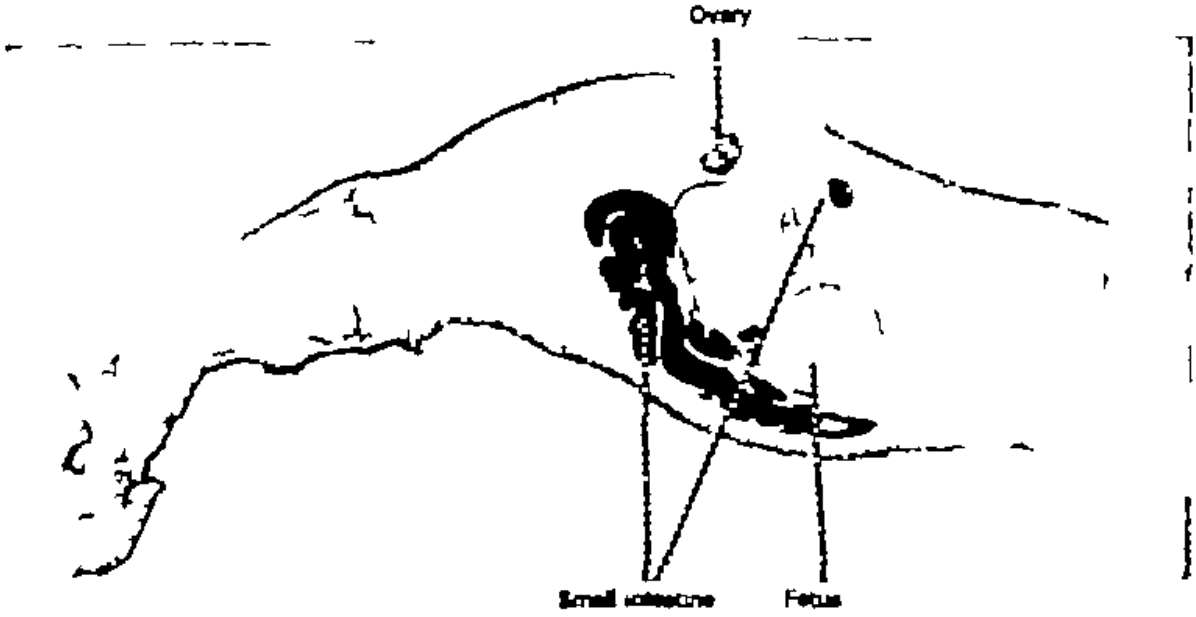


Fig. 38. Whole-body section of a pregnant mouse showing the MAO-activity There is very high enzyme activity (dark areas) in the intestinal mucosa.

was present at the surface epithelium. In the crypts the activity was still weaker In the stomach (Fig 41) a moderate enzyme activity could be seen in the squamous epithelium of the forestomach. In the glandular part of the stomach the strongest enzyme activity was present basally in the glands.

Formazan deposits could also be seen at the location of the myenteric plexus both in the stomach and in the intestine (Fig. 41)

In the adrenal cortex (Fig 4) the highest activity was present in the zona fasciculata. In the zona glomerulosa the activity was somewhat less intense and in the zona reticularis and the x zone even weaker In the adrenal medulla the activity was low

In the testicles the strongest enzyme activity was seen in the interstitial cells (Fig 43) The epithelial lining of the epididymal duct and of ductus deferens, showed a relatively high activity



Fig. 39 Detail of whole-body section of mouse showing the MAO-activity in sympathetic ganglion. A strong enzyme activity is present in all cells in the ganglion. (×20)



Fig. 40. Detail of whole-body section of mouse showing the MAO-activity in the mucosa of the small intestine. A high enzyme activity can be seen at the striated border of the villous epithelium. The epithelial cells of the crypts show a less intense activity (×60)

In the ovary the interstitial cells showed the strongest activity (Fig 44)

In the thyroid the highest enzyme activity was seen in cells scattered between the follicles (Fig. 45) The cells had a localization similar to the parafollicular cells.

In the skin the hair follicles showed a strong enzyme activity

In control sections, obtained by either of the three mentioned methods, the deposition of formazan was low and evenly distributed (Fig. 46)

Serotonin ^{14}C-incubations

A strong accumulation of radioactivity could be seen in the adrenal cortex. In the adrenal medulla the activity was low (Fig 47 a)

Sympathetic ganglia and the intestinal mucosa were other instances where a strong radioactivity could be seen.

A relatively high activity could be seen in the bone marrow the red pulp of the spleen and the epithelium of ductus deferens. A rather high radioactivity was also seen in the central nervous system and in brown fat.

In sections pre incubated with nialamide there was only a low radioactivity in the adrenal cortex. Other organs accumulated a similar amount of activity as without the MAO inhibition (Fig 47 b)



Fig. 41 Detail of whole-body section of mouse showing the MAO-activity in the wall of the stomach at the junction between the forestomach (to the left in the picture) and the fundic gland area (to the right in the picture). The highest enzyme activity can be seen in the squamous epithelium of the forestomach and at the base of the fundic glands. Formazan deposits can also be seen in the picture at the location of Auerbach plexus. (×40)



Fig. 42. Detail of whole-body section of a mouse showing the MAO-activity in the adrenal gland. There is high enzyme activity in the adrenal cortex, especially in the zona fasciculata. In the adrenal medulla the enzyme activity is low (×20)

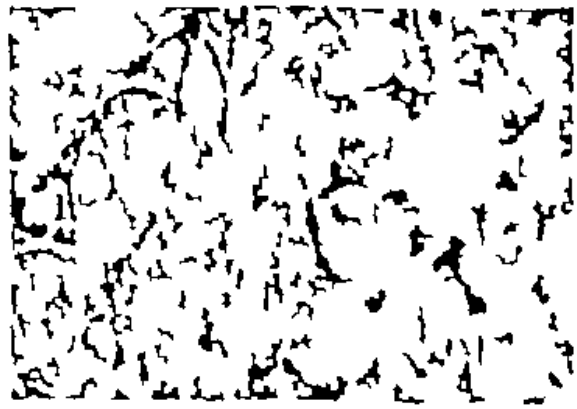


Fig. 43. Detail of whole-body section of mouse showing the MAO-activity in testicle. The highest enzyme activity is present in the interstitial cells. (×70)



Fig. 44 Detail of a whole-body section of mouse showing the MAO-activity in an ovary The highest enzyme activity is present in the interstitial cells. (×50)

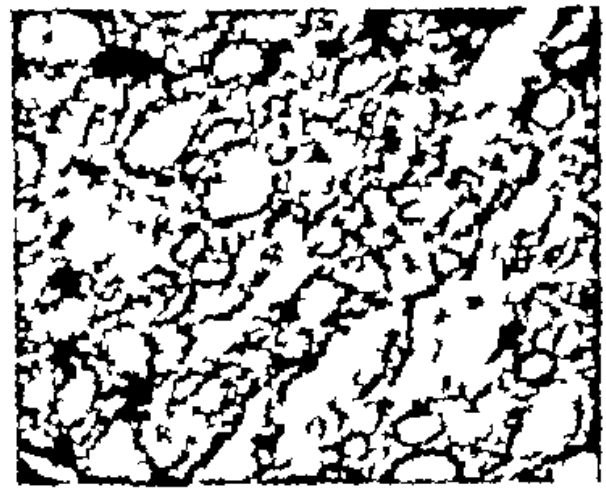


Fig. 45. Detail of a whole-body section of a mouse showing the MAO-activity in the thyroid. The highest enzyme activity can be seen in cells scattered between the follicles, probably the parafollicular cells. (×50)

Discussion

The use of whole-body sections for the demonstration of MAO-activity as in the present investigation allows a direct comparison of the enzyme activity in different organs treated in exactly the same way

In the cells the mitochondria contain the greatest part of the MAO-activity (see Gorkin 1966) The formazan precipitations have with a histochemical method for MAO localization in the electron microscope, also been shown to be localized in association to the outer mitochondrial membrane (Boadle and Bloom 1969)

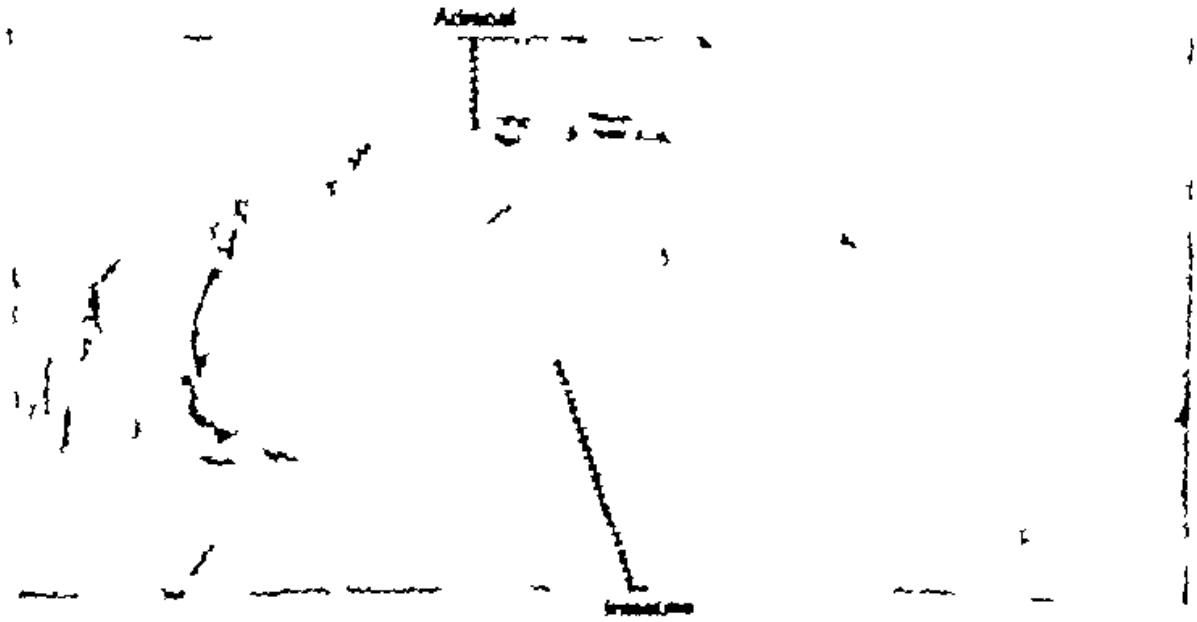


Fig. 4. Whole-body section of mouse which has been pre-treated with the MAO-inhibitor nialamide 500 mg/kg i.p. 4 hours before was killed. The section has been treated for MAO-histochemistry. The enzyme reaction is weak in all tissues

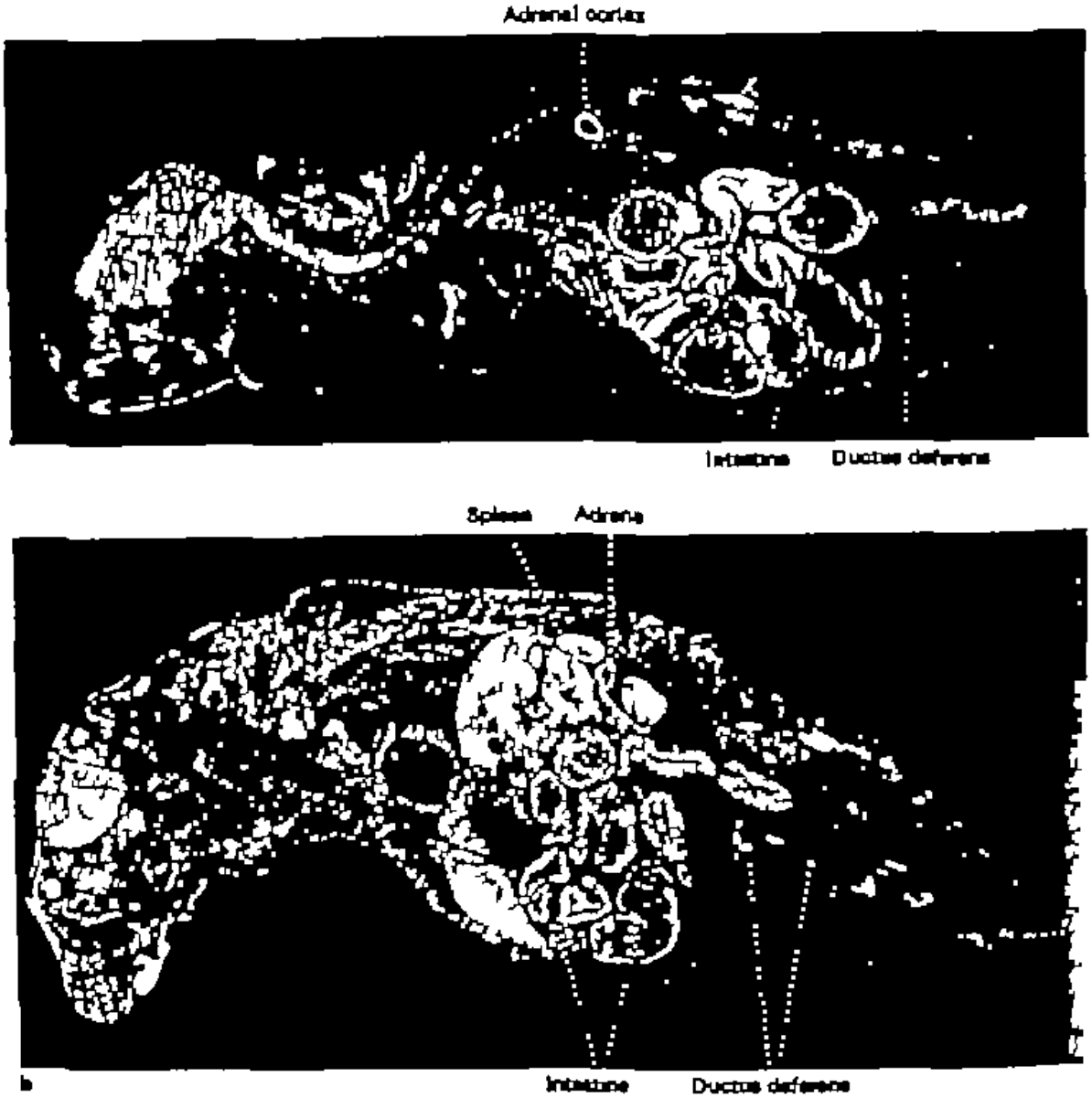


Fig. 47 a. Autoradiogram of whole-body section of mouse. The section has been incubated in buffer containing serotonin- ^{14}C.

b A toradiogram of a section, which has been pre incubated with the MAO-inhibitor nialamide before it was incubated with serotonin- C. A strong accumulation of radioactivity (light areas) can be seen in the adrenal cortex in the section, which has not been pre incubated with nialamide ( ) In the pre-incubated section the radioactivity in the adrenal cortex is low (b)

The highest MAO-activity could be observed in the sympathetic ganglia A high activity in sympathetic ganglia has also been shown in other species (Koelle and Valk 1954 Glenner *et al* 1957 Klingman and Klingman 1966, Consolo *et al.* 1968 Giacobini and Kerpel Fronius 1970) In spinal ganglia the MAO-activity was low Similar is the case in the cat (Giacobini and Kerpel-Fronius 1970)

The MAO-content in different ganglia has been correlated to the number of adrenergic neurons in the ganglia (Consolo *et al.* 1968)

A high MAO-activity could be observed in the myenteric plexa of the stomach and the intestine. This has also been observed in other species (see Glenner *et al.* 1957 Ruckebuch and Santini 1968)

In the mucosa of the small intestine and the rectum the enzyme activity was very high. The results are in agreement with an earlier investigation in the mouse (Iwai 1969) Also in other species a high activity has been shown in various parts of the intestine, although there are some species variations (Koelle and Valk 1954 Glenner *et al* 1957 Klingman *et al.* 1964 Penttilä 1968 Iwai 1969 Murali and Radhakrishnan 1970) The enterochromaffin cells in the intestine are mainly localized basally in the mucosa (see Penttilä 1966) where the MAO-activity is lowest. In the stomach the highest activity is present basally in the glands. The situation has been shown to be the same in the guinea pig and in the rat (Eder 1957 Håkanson *et al.* 1967 a) In this case the strongest MAO-activity is present in the area where most enterochromaffin and enterochromaffin-like cells are found (see Chapter 3) In the gastrointestinal tract most of the endogenous serotonin stores are present in the enterochromaffin cells (Penttilä 1966, Vialli 1966) The storage of serotonin thus seems to be in accordance with the occurrence of MAO in the stomach, but not in the intestine.

In connection with this the side effects observed in patients receiving MAO-inhibitors for treatment of depressive states can be pointed out (Sjöqvist 1967 Mayer 1968 Hammer *et al.* 1969) Hypertensive symptoms could be observed after the consumation of certain food stuffs, especially cheese. Tyramine is the principal pressor substance in cheese. When the MAO was inhibited, the tyramine was not destroyed and the hypertensive symptoms occurred. MAO thus has a detoxifying function in the gastrointestinal tract.

The observation that a high MAO-activity is present in cells of the thyroid which might correspond to the parafollicular cells is in agreement with an earlier investigation (Larsson *et al.* 1966)

A surprisingly high MAO-activity was found in the adrenal cortex, while the adrenal medulla showed a low activity Earlier reports of the MAO-activity in the adrenal gland show great variations.

Using the tetrazolium method Glenner *et al.* (1957) found a moderate activity in the cortex in guinea pig and rabbit. No activity could be observed in the medulla.

In a series of investigations Rosenkrantz and collaborators (Rosenkrantz 1959 Rosenkrantz and Laferte 1960 Connors and Rosenkrantz 1962) observed a substance in the cortex capable of nitro-blue tetrazolium reduction in the adrenal cortex of rabbit, guinea pig, rat, dog and cow after incubations with serotonin

They concluded that serotonin had a direct effect on the adrenal cortex, but they were unable to identify the reducing substance. It seems possible that the nitro-blue tetrazolium reduction was due to MAO-activity

Koelle and Valk (1954) observed an intense MAO-activity especially in the zona fasciculata of the adrenal cortex of the cat. The method used gave however an unspecific if considerably lower activity in control sections. Francis (1954) found an intense activity in singular medullar cells, possibly corresponding to ganglion cells in the guinea pig. The activity in the cortex was intense, but with the method used not specific. Langemann (1951) found MAO-activity in both cortex and medulla of the adrenal of the ox. Martinez and Toledano (1968) observed MAO-activity in the adrenal cortex of the cat, but no activity in the medulla.

In the testis the highest MAO-activity was found in the interstitial cells. Also the epithelium of the epididymal duct and ductus deferens showed a rather high activity. The results are in agreement with investigations in the rat (Penttilä and Kormano 1968, Spano *et al.* 1968). The uptake of DOPA in these structures has been discussed in Chapter 5.

In the ovary the highest MAO-activity was present in the interstitial cells. Adrenergic nerves are rather numerous in and restricted to the interstitial tissue of the ovary (see Sjöberg 1967). The uptake of injected DOPA is highest in the follicle walls and corpora lutea (see Chapter 5), where the MAO-activity is lowest.

Put together, there seems to be no correlation between the storage of monoamines or the uptake of DOPA and the occurrence of MAO in the steroid hormone producing organs. The same applies to the gastrointestinal tract. It is, however, obvious that monoaminergic mechanisms are present in the steroid hormone producing organs.

Effects of monoamines in the release of steroid hormones have also been observed. These have however in some cases been shown to be mediated via the release of the hormones in the hypophysis and the hypothalamus, but direct effects of monoamines on the steroid hormone producing organs have also been reported. Thus serotonin has been shown to stimulate the secretion of hydrocortisone by a direct effect on the adrenal gland (Verdesca *et al.* 1961). Direct effects of monoamines in the production of testosterone from the testis have also been reported (see Ellis 1969, Eik-Nes 1969). Reserpine inhibits ovulation (see Meyerson and Sawyer 1968). This effect, which is blocked by MAO-inhibitors, has earlier been proposed to be mediated via the hypophysis. Recently however it has been reported that the effect, at least to some extent must be produced via a direct action on the ovary (France 1970). Several MAO-inhibitors have been shown to depress the weight and function of the reproductive system. This action appears to be mediated both via the hypo-

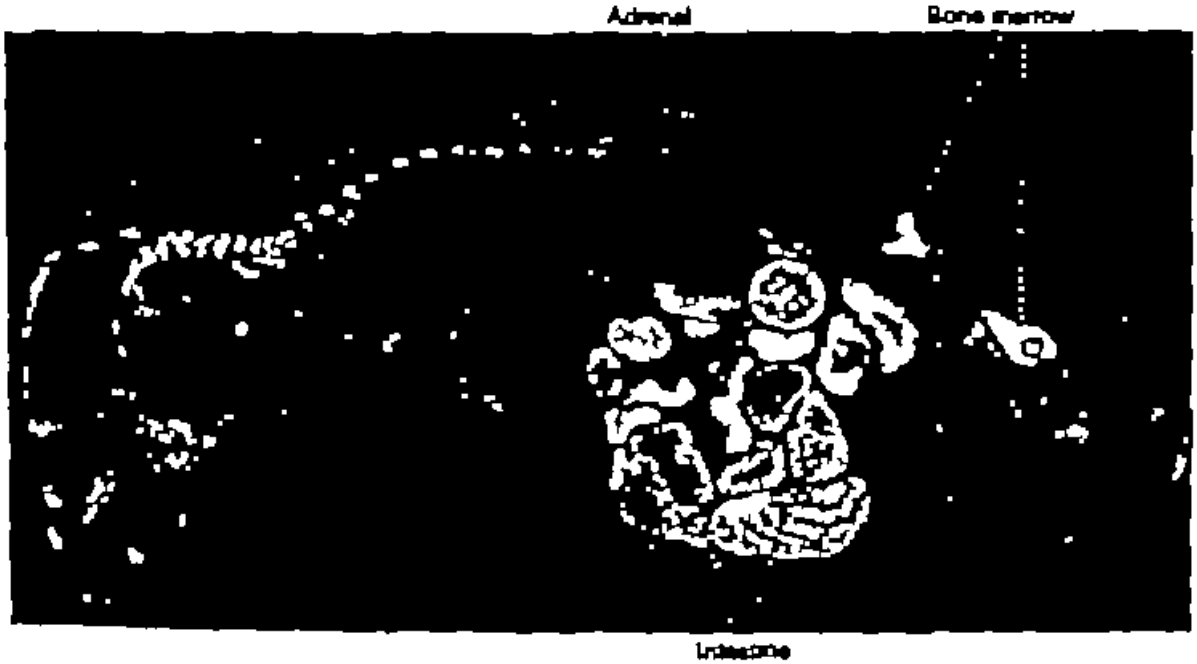


Fig. 48. Autoradiogram of a whole-body section of mouse. The section has been incubated in a buffer containing DOPA-2 ^{14}C. There is no accumulation of radioactivity in the adrenal.

physis and via a direct effect on the sex organs (see Zor *et al.* 1966, Forbes 1970)

In the incubations with serotonin- ^{14}C, there was a strong accumulation of radioactivity in the adrenal cortex. After pre-incubation with nialamide the uptake of serotonin- ^{14}C was considerably lower in the adrenal cortex. It might therefore be possible that in this case MAO is responsible for the binding of the serotonin to the tissue. The histochemical MAO-method in fact involves a binding of the substrate to the MAO-containing tissues, where the substrate is then oxidatively deaminated. The uptake of serotonin- ^{14}C may therefore partly be due to MAO in the tissues. In such tissues as the sympathetic ganglia the intestinal mucosa, the epithelium of ductus deferens the bone marrow and the spleen the activity was high, independent of MAO-inhibition. In these cases additional factors are obviously of importance for the *in vitro* binding of serotonin. It must be observed that incubations in the present investigation have been performed with sections, which have been frozen and dipped in acetone. It is impossible to make comparison between the uptake of substances in the tissues under these conditions and the uptake under *in vivo* conditions. *In vivo* injected serotonin is strongly accumulated in the adrenal medulla (see Ritzén *et al.* 1965) When whole-body sections from mice are incubated with DOPA- ^{14}C, there is no accumulation of radioactivity in the adrenal cortex (Fig 48) (Tjälve, unpublished) DOPA does not serve as a substrate for MAO (see Chapter 5) The tissues accumulating the highest radioactivity in this case are the bone marrow and the intestinal mucosa.



Fig. 49. Fluorescence photomicrograph of pancreatic islet of an untreated rabbit. A network of varicose adrenergic nerve terminals can be seen in the islet. (×130)

one hour. The fluorescence was however a little weaker and the islet cells with stronger fluorescence were fewer after two hours than after one hour (Fig. 51).

In the pieces incubated with DOPA *in vitro* there was a strong fluorescence in both the exocrine and endocrine pancreas (Fig. 52). In the islets all cells showed the same high fluorescence. In the exocrine pancreas a stronger fluorescence was seen in the zymogen granules than in the rest of the cells.

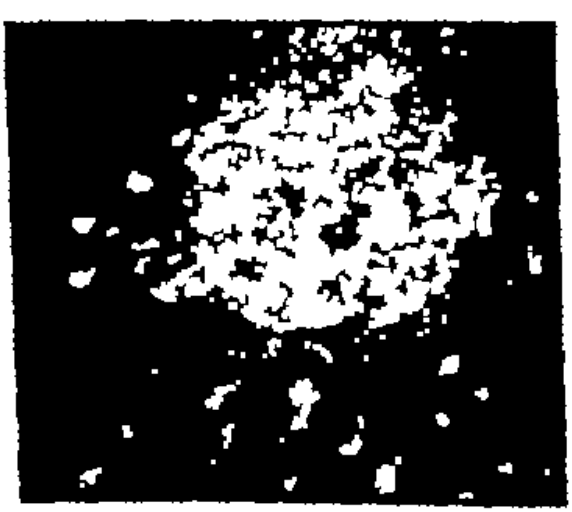


Fig. 50



Fig. 51

Fig. 50. Fluorescence photomicrograph of a pancreatic islet of a rabbit 1 hour after an intravenous injection of DOPA. Fluorescence can be seen in all cells of the islet, but is stronger in some cells. In the exocrine pancreas the fluorescence is limited to zymogen granules and excretory ducts. ×130)

Fig. 51. Fluorescence photomicrograph of a pancreatic islet of a rabbit 2 hours after an intravenous injection of DOPA. A strong fluorescence can be seen in a few cells of the islet. (×130)

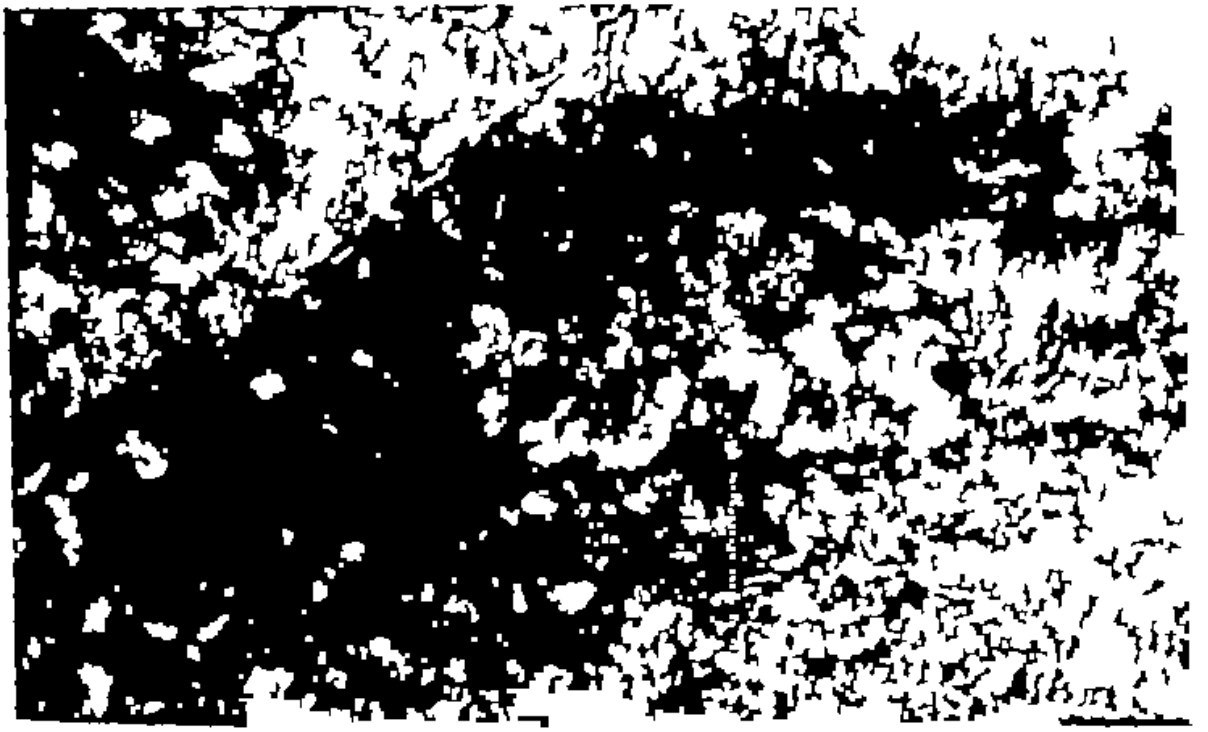


Fig. 52. Fluorescence photomicrograph of a section of a piece of pancreas from a rabbit. The pancreatic piece was incubated in buffer containing DOPA. Fluorescence can be seen in all cells of the pancreatic islet. In the exocrine pancreas the strongest fluorescence is present over the zymogen granules. (×150)

Part 2 Chromatographic investigation of the in vitro metabolism of DOPA 2 ^{14}C in the rabbit pancreas

Material and methods

Pieces of pancreas, weighing 20 mg each from two untreated Havanna rabbits were incubated for 30 minutes in 2 ml of the same buffer solution and under the same conditions as has been described in Part 1. Instead of non-labelled DOPA, DOPA ^{14}C was added to the incubation solution. The isotope (DL-3(3,4-dihydroxyphenyl)alanine 2 ^{14}C, specific activity 261 μCi/mg (52 mCi/mM) (The Radiochemical Centre Amersham, England) was added in a concentration of 2.5 10^{-3} M corresponding to 2.6 μCi in 2 ml buffer solution.

After the incubation the pieces were rinsed for some seconds in a buffer solution not containing DOPA. They were then immediately homogenized in a Potter Elvehjem homogenizer extracted twice with acid ethanol (1 ml conc. HCl/1000 ml ethanol) and filtered. The acid ethanol extracts were evaporated in N_2. Small amounts of the extracts were spotted on paper (What

They concluded that serotonin had a direct effect on the adrenal cortex, but they were unable to identify the reducing substance. It seems possible that the nitro-blue tetrazolium reduction was due to MAO-activity

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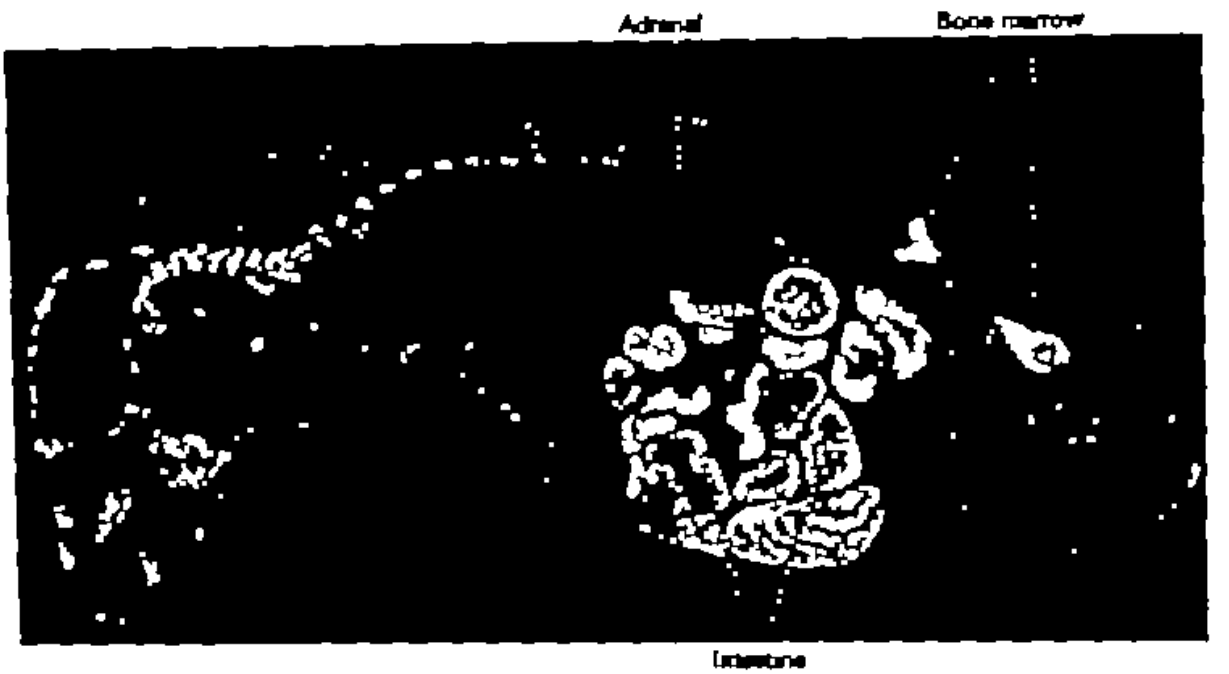


Fig. 48. Autoradiogram of a whole-body section of mouse. The section has been incubated in buffer containing DOPA- C. There is no accumulation of radioactivity in the adrenal.

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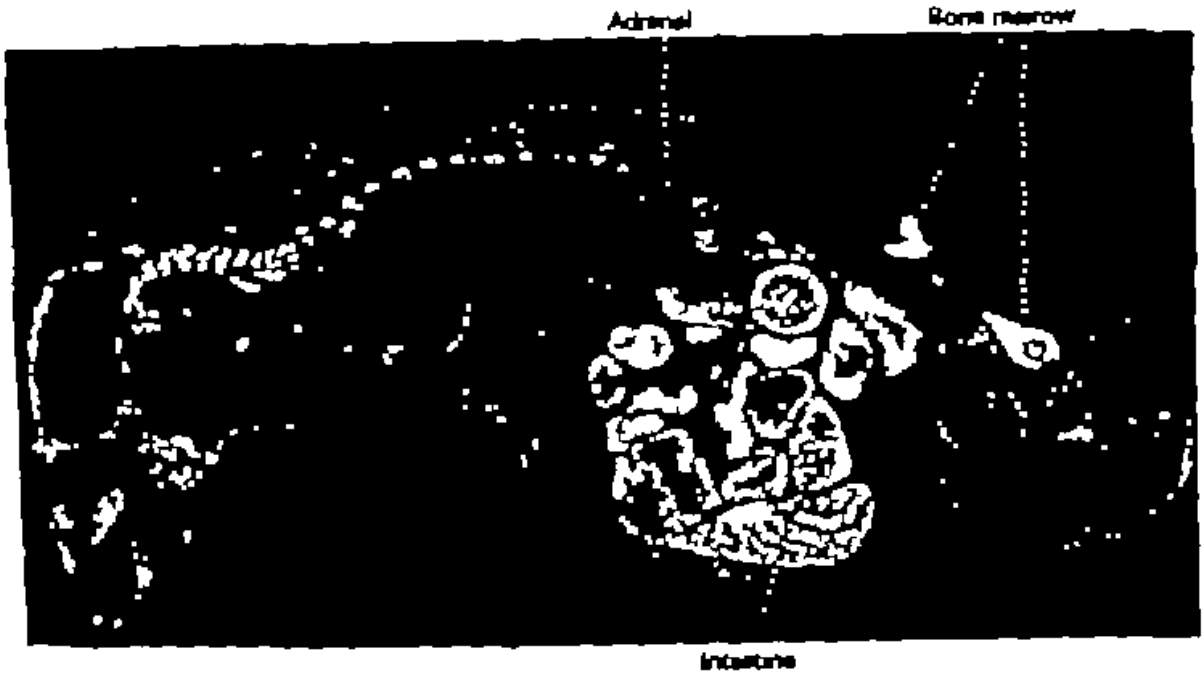


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Studies on the uptake and metabolism of DOPA in the rabbit pancreas

As has been shown and discussed in Chapter 1 the injection of DOPA and 5-HTP into mice leads to a monoamine formation in the pancreatic islets. In the exocrine pancreas monoamines are formed in the zymogen granules (Alm *et al.* 1969 Alm 1969)

The present study was performed in order to investigate whether these circumstances are the same in the rabbit as in the mouse. The *in vitro* uptake and metabolism of DOPA in the rabbit pancreas was also studied. The reason for this investigation was the fact that rabbits were used as experimental animals in Chapter 8 where the *in vitro* effect of monoamines and monoamine precursors on the insulin release was studied

The investigation is divided into two parts. In the first part fluorescence microscopy is employed to study an eventual endogenous occurrence of monoamines, and the *in vivo* and *in vitro* uptake of DOPA in the rabbit pancreas. In the second part DOPA 2 ^{1}C is used for a chromatographic investigation of the *in vitro* metabolism of DOPA in the rabbit pancreas. Separate descriptions of the experiments in the two parts will be given followed by a common discussion for the two parts.

Part 1 Fluorescence microscopic investigation of the in vivo and in vitro uptake of DOPA in the rabbit pancreas.

Material and methods

Male pigmented rabbits of the Havanna strain, weighing about 2 kg were used. The rabbits were kept on a diet consisting of carrots, Swedish turnips and cabbage *ad lib* and were given free access to water

Six rabbits were used for the *in vivo* DOPA investigation and two were used for the *in vitro* DOPA investigation. In addition two rabbits were used for control of eventual endogenous fluorescence.

For the *in vivo* experiments L-DOPA (Sigma) was dissolved in physiological saline (100 mg/6 ml physiological saline) and immediately injected intravenously in an ear vein. The dose injected corresponded to 100 mg L-DOPA/kg body weight. Two rabbits were killed respectively 10 minutes, 1 hour and 2

hours after the injection by a sharp blow on the back of the head. They were then bled by decapitation and small pieces of the tail and the body of the pancreas were quickly removed and frozen in isopentane cooled with liquid nitrogen. The pieces were then freeze-dried, treated according to the Falck-Hillarp fluorescence method and subjected to fluorescence microscopy as has been described in Chapter 1

In the *in vitro* experiments pieces of the pancreas, weighing about 20 mg each, were taken from untreated rabbits, killed as described above. Each piece was put in 2 ml of a buffer solution, pH 7.4 of a composition which is described in Chapter 8 (p. 88) L-DOPA was added to the buffer to give a concentration of 10^{-4} M. The buffer was also supplemented with 3 mg/ml of glucose.

The pieces were then incubated for 30 minutes at 37.5 C in a Dubnoff shaker The incubations were gassed with O_2+CO_2 (95.5) After the incubation the pieces were rinsed for some seconds in a buffer solution not containing DOPA. They were then blotted on a filter paper freeze dried and treated for fluorescence microscopy as described (Chapter 1)

For control of endogenous fluorescence pancreatic pieces of two untreated animals were taken and handled for fluorescence microscopy

For identification of β-cells in the pancreatic islets the sections were stained with aldehyde fuchsin, as described by Jennings (1965) after they had been submitted for fluorescence microscopy

Results

In untreated animals a weak fluorescing network of varicose adrenergic nerve terminals could be observed in the islets (Fig 49) Fluorescing islet cells could not be observed. Adrenergic nerves could also be observed in connection to vessels.

10 minutes after the injection of DOPA there was a distinct green fluorescence in both, the endocrine and exocrine pancreas. In the endocrine pancreas all cells emitted a fluorescence of similar intensity In the exocrine pancreas there was a slightly stronger fluorescence over zymogen granules in the cells than over the rest of the cells.

One hour after the injection most cells in the islets showed a moderate cytoplasmic fluorescence (Fig 50) There were, however some cells which showed a stronger fluorescence. On the aldehyde fuchsin staining showed that these cells were B cells. In the exocrine pancreas the fluorescence was confined to zymogen granules and pancreatic ducts.

Two hours after the injection was essentially the same as after

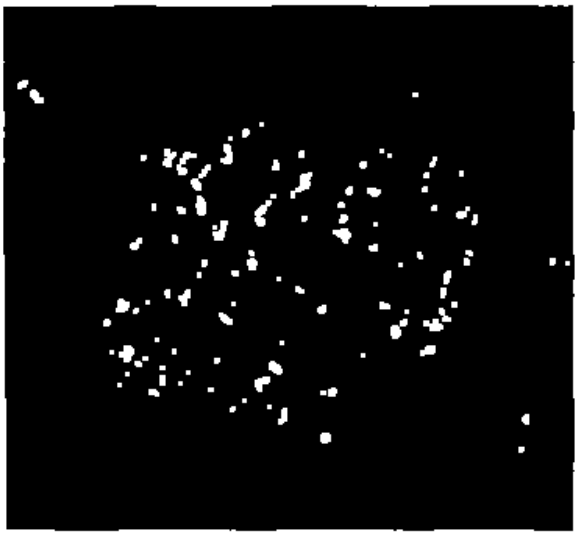


Fig. 19 Fluorescence photomicrograph of pancreatic islet of an untreated rabbit. A twork of varicose adrenergic nerv terminals can be seen in the islet. (×130)

one hour The fluorescence was, however a little weaker and the islet cells with stronger fluorescence were fewer after two hours than after one hour (Fig 51)

In the pieces incubated with DOPA *in vitro* there was a strong fluorescence in both the exocrine and endocrine pancreas (Fig 52) In the islets all cells showed the same high fluorescence. In the exocrine pancreas a stronger fluorescence was seen in the zymogen granules than in the rest of the cells.

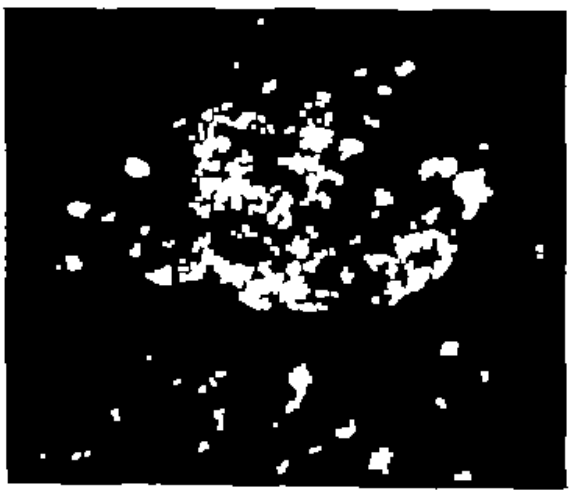


Fig 50



Fig 51

Fig. 50 Fluorescence photomicrograph of pancreatic islet of rabbit 1 hour after an intravenous injection of DOPA. Fluorescence can be seen in all cells of the islet, but is stronger in some cells. In the exocrine pancreas the fluorescence is limited to zymogen granules and excretory ducts. (×130)

Fig. 51 Fluorescence photomicrograph of pancreatic islet of a rabbit 2 hours after an intravenous injection of DOPA. A strong fluorescence can be seen in few cells of the islet (×130)

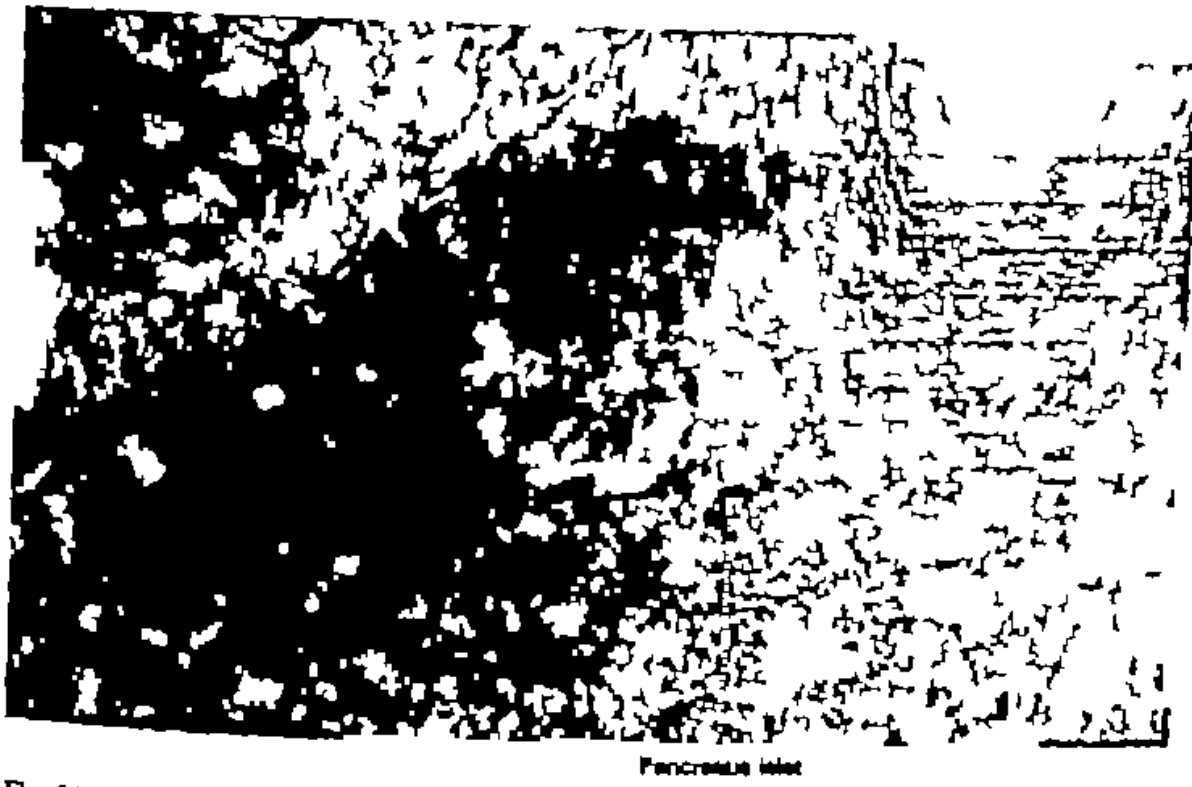


Fig. 32. Fluorescence photomicrograph of a section of piece of pancreas from rabbit. The pancreatic piece was incubated in buffer containing DOPA. Fluorescence can be seen in all cells of the pancreatic islet. In the exocrine pancreas the strongest fluorescence is present over the zymogen granules. (×150)

Part 2. Chromatographic investigation of the in vitro metabolism of DOPA-2-^{14}C in the rabbit pancreas

Material and methods

Pieces of pancreas, weighing 20 mg each from two untreated Dutch rabbits were incubated for 30 minutes in 2 ml of the same buffer solution and under the same conditions as has been described in Part 1. In addition from labelled DOPA, DOPA-2-^{14}C was added to the incubation solution. The substance (DL-3(3,4-dihydroxyphenyl)alanine-2-^{14}C, specific activity 50–60 μC mg/5–6 mCi/mM) (The Radiochemical Centre Amersham, England) was added in a concentration of $2.5 \cdot 10^{-4}$ M corresponding to 6 μCi in the buffer solution.

After the incubation the pieces were rinsed for some seconds in a buffer solution not containing DOPA. They were then immediately homogenized in a Potter Elvehjem homogenizer extracted twice with acid ethanol (1 ml conc. HCl/1000 ml ethanol) and filtered. The acid ethanol extracts were evaporated in N_2. Small amounts of the extract were spotted on paper (What

man no 1) Descent chromatograms were run for 20 hours in phenol-0 1 N HCl (85 15 w/v) The front was at this time about 40 cm from the origin. Reference substances were chromatographed parallelly with the tissue extracts.

The radioactivity of the chromatograms was detected with the aid of a radiochromatogram scanner (Packard) and by autoradiography The x ray film used for the autoradiography was Structurix (Gevaert) The non radioactive reference substances used were visualized by spraying the chromatograms with a diluted solution of Folin-Ciocalteau phenol reagent, which produced blue spots on the exposure of the paper with ammonia fumes.

The extraction procedure was controled by measurement of the radioactivity of the residues on the filters in a liquid scintillation counter (Packard) For this purpose the residues were dissolved in 1 ml Soluene 100 (Packard) and transferred to liquid scintillation vials containing 15 ml of 0,5 % PPO and 0,05 % POPOP in toluene The activity in the residues was found to be very low

Results

Four radioactive spots were seen in the chromatograms with R_f-values corresponding to DOPA (0 09) noradrenaline (0 14) dopamine (0.30) and dihydroxyphenylacetic acid (0 49) (Fig. 53) Quantitation of the spots, other than estimation of the peaks obtained in the radiochromatogram scanner and by estimation of the blackening of the films, was not tried. From these estima-

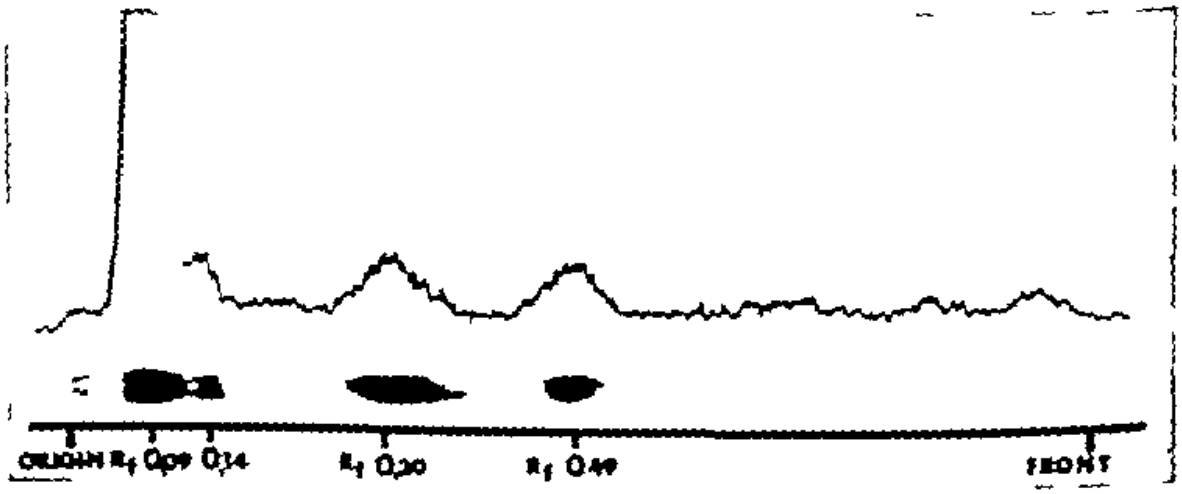


Fig 53 Diagram of radioactive paper chromatogram scanned in a radiochromatogram scanner (Packard) and autoradiogram of the same chromatogram. Tissue extract from pieces of rabbit pancreas incubated in buffer containing DOPA-2 ^{14}C was chromatographed in phenol-0.1 N HCl (85 15 w/) Four radioactiv spots with Rf-values corresponding to DOPA (0 09) noradrenaline (0 14) dopamine (0.30) and dihydroxyphenylacetic acid (0 49) ere egistered

tions, it as, however obvious that the DOPA-spot dominated, while the amounts of noradrenaline, dopamine and dihydroxyphenylacetic acid were of a smaller magnitude

Discussion

The results of the fluorescence microscopic investigation show that the *in vitro* uptake of DOPA in the rabbit pancreas is essentially the same as has been described in the mouse (see Chapter 1) In the islets an initial catecholamine formation takes place in all islet cells. The catecholamines then disappear first from the β-cells, leaving the α-cells with the highest catecholamine concentration.

In untreated rabbits a network adrenergic nerves could be observed within the islets. Cegrell (1968) showed similar networks in the golden hamster dog and cat, but could not demonstrate them in the rabbit. The reason for this discrepancy might be that the fluorescence as it was observed in the present investigation, is weak and is only visible under optimal fluorescence conditions. The islets of the rabbits have also been shown to be richly innervated with hollinergic nerves (Coupland 1958, Lihman and Sutherland 1965)

The results from the *in vitro* fluorescence microscopic investigation indicate that the monoaminergic mechanisms observed *in vivo* also function *in vitro* Fluorescence was observed in all islet cells. In the exocrine pancreas a stronger fluorescence was seen over the zymogen granules than in the rest of the cells. The parafollicular cells of the thyroid and the enterochromaffin-like cells of the stomach have similarly been shown *in vitro* to possess functioning monoaminergic mechanisms when the tissues are incubated with the monoamine precursors (Owman and Sundler 1967 Håkanson *et al.* 1969 b)

The results from the *in vitro* chromatographic investigation show that both dopamine and noradrenaline are formed. Apart from the fluorescence methods, the catecholamine formation *in vitro* has also been studied with chromatographic methods. *In vitro* the injection of DOPA into mice has thus been shown to give a dopamine formation in the pancreas (Rosell *et al* 1963) and the injection of dopamine into rats has been shown to give a formation of noradrenaline (Symchowicz *et al* 1968 The formation of noradrenaline from dopamine probably takes place in the adrenergic nerves as has been shown to be the case in rat adrenal gland Jonason 1969)

The dihydroxyphenylacetic acid is formed as a result of the action of MAO on dopamine. As has been shown (Chapter 1) MAO is present in the parenchymal cells of both the exocrine pancreas. Dopamine is a better substrate for MAO than (Weiner 1960) The *in vivo*

catabolism of dopamine has also been shown to proceed at a higher rate than the catabolism of noradrenaline in rat salivary glands and in rabbit brain *in vitro* (Jonason and Rutledge 1968 Jonason 1969) In the rat salivary glands the extraneuronal MAO seems to be of greatest importance in the catabolism (Jonason 1969) The same should be the case in the pancreas.

The catabolism through the action of COMT seems in the present investigation to be of lesser importance than the catabolism through the action of MAO This was also the case in the rat salivary glands and the rabbit brain *in vitro* (Jonason and Rutledge 1968 Jonason 1969)

In the present investigation radioactive DL-DOPA was used. Dopamine is not obtained from the D-form and therefore the radioactive DOPA-spot on the chromatograms should contain more D- than L-DOPA.

CHAPTER 8

Radioimmunological studies on the effect of catechol and indolamines and of their precursors on the insulin secretion from rabbit pancreas *in vitro*

The capability of the pancreatic islets, the parafollicular cells of the thyroid and the enterochromaffin cell system of the gastrointestinal tract, to form and store biogenic amines, has led to the mentioned assumptions that the biogenic amines might be of importance in the formation storage and/or release of the hormones formed in these cell systems. Effects of monoamines on the release of insulin have also been shown (see Chapter 1 and Discussion). This has been possible with the use of radioimmunological methods for hormone estimation.

The introduction of methods for the estimation of insulin levels by means of radioimmunoassay was made by Yalow and Berson (1960). The method allows a precise and reliable estimation of insulin secretion *in vivo* and *in vitro*. Several investigations of the effect of a variety of substances on the insulin secretion have been performed. Radioimmunological methods for the estimation of hormones other than insulin have also been developed, but these methods are still difficult to use and the number of such investigations few as yet.

In the present work a radioimmunological method (Hales and Randle 1963) has been used to study some parameters of the monoamine and the monoamine precursor influence on the insulin release.

The investigation has been performed on rabbit pancreas *in vitro*. The *in vitro* method used is the one described by Coore and Randle (1964). The rabbit pancreas has been shown to be especially suitable for *in vitro* studies of this type, mainly due to the high concentration of insulin in the pancreas relative to that of other species (Marks and Young 1960). An *in vitro* preparation has been chosen to avoid influences on the insulin secretion of other organs which might occur *in vivo* upon injections of monoamines or monoamine precursors.

Material and methods

Procedure

Male pigmented rabbits of the Havanna strain were used. The weight of the animals was about kg and they were kept under the same conditions as described in Chapter 7. Approximately 30 animals were used.

The rabbits were killed by a sharp blow on the back of the head and then bled by decapitation. The tail and the body of the pancreas were rapidly removed and dropped into a Petri dish containing an ice-chilled medium of the same composition as in the incubations (see below). After trimming the pancreas of blood vessels and mesentery it was divided into 10—16 pieces, weighing about 20 mg each. Each piece was placed in an Erlenmeyer flask in 2 ml of the buffer (see below). They were then incubated in a Dubnoff shaker at 37.5° C for 30 minutes before the incubation used for the radioimmunoassay commenced. This pre-incubation was found to be necessary to establish a steady rate of insulin secretion.

After the pre-incubation each piece was transferred with forceps to new flasks and was taken individually through a series of three incubations of 30 minutes duration each. The first of these flasks contained 2 ml of the same medium as in the pre-incubation-flasks. The flasks used for the second and the third incubations were supplemented with substances, the effects of which on the insulin release were to be investigated. These flasks contained an, between themselves, identical medium. Between each individual incubation period the pieces were rinsed for 15 seconds in a small quantity of the medium in which they were to be incubated. At least four pieces of pancreas were treated identically for each substance during a series of incubations.

Insulin which had been secreted into the medium was measured after removal of the pancreas from the flask. The insulin content in the first medium was used as a control. The insulin content in the second and the third incubation solution was then compared with that in the first solution. The amount of insulin in these solutions was expressed as percentage of the content in the first solution. This was done in order to avoid errors caused by the variation in insulin-output between different pieces, depending on their varying number of islets. This variation in turn is caused by an irregular distribution of the islets dispersed among the cells of the exocrine pancreas. In spite of the fact that the islets only form a small proportion of the total pancreatic mass, the preparation used has been found to be very useful for *in vitro* studies of insulin release (see Discussion and Results).

Incubation medium

The medium used for all incubations was a bicarbonate-buffered salt solution pH 7.4 (Krebs 1950) of the following composition (m-equiv/l): Na^+ 141.0 K^+ 5.9 Ca^{2+} 5.1 Mg^{2+} 2.4 PO_4^{3-} 2.2 SO_4^{2-} 2.4 HCO_3^- 24.9 Cl^- 109.6 pyruvate 4.9 glutamate 4.9 and fumarate 5.4. This composition was obtained by mixing the following chemicals with distilled water to a volume of one liter:

$MgSO_4$ 144 4 mg CaCl 283 3 mg KCl 440.3 mg Na pyruvate 538.9 mg Na-glutamate 468.1 mg Na fumarate 437.3 mg Na_2PO_4 12 H_2O 279.5 mg NaHCO 2090.8 mg and NaCl 5763 0 mg The buffer was supplemented with 1 mg/ml of bovine albumin, fraction V (Sigma) The albumin serves the double purpose of preventing adhesion of insulin to the walls of the vessels used and of diminishing the breakdown of insulin caused by the enzymes of the exocrine pancreas (see Candela and Coore 1967) The media were also supplemented with glucose in the concentration of 3 mg/ml for most incubation series and 0 6 mg/ml for some other incubation series. The incubations were gassed with $O_2 + CO_2$ (95 5)

Substances investigated

The effects on the insulin secretion of the following substances (in the concentrations given in the figures in the results) were studied* L-DOPA, D-DOPA, dopamine noradrenaline, L-5-HTP serotonin (serotonin creatinine sulphate) L-tyrosine, L tryptophan, dihydroxyphenylacetic acid (DOPAc) L-DOPA + the DOPA-decarboxylase inhibitor MK 485 (DL-α-hydrazino-α-methyl-3 4-dihydroxyphenylpropionic acid) L DOPA + the dopamine β-hydroxylase inhibitor DDTC (Na-diethyldithiocarbamate, Fluka AG Buchs, Switzerland) L-DOPA + the α-receptor blocking substance phenoxybenzamine (phenoxybenzamine hydrochloride, (Dibenyline) Smith Kline and French Labs, Ltd Herts, England) L-DOPA + the β-receptor blocking substance H56/28 (1 (o-allylphenoxy) 3-isopropylamino-2 propanol hydrochloride (Aptin) AB Hässle, Mölndal Sweden) L-DOPA + the β-receptor blocking substance propanolol (propanolol hydrochloride, ICI Macclesfield England) 5-HTP + MK 485 noradrenaline + phenoxybenzamine adrenaline, adrenaline + phenoxybenzamine and adrenaline + H56/28. The effects of MK 485 DDTC phenoxybenzamine, H56/28 and propanolol were also investigated separately in the concentrations which were used in the combinations with the monoamines and the monoamine precursors.

The effects of L-DOPA and noradrenaline on the insulin secretion were also studied as described, using pancreatic pieces from two rabbits treated with 6-hydroxydopamine (6OH-dopamine) (100 mg/kg intravenously) 1 hours before they were killed. 6OH-dopamine has been shown to cause a selective destruction of adrenergic nerve terminals (Thoenen and Tranzer 1968) with rapidly occurring and long lasting inability of storage and uptake of noradrenaline (Jonsson and Sachs 1970)

* The substances were obtained from Sigma Chemical Co, USA, unless otherwise stated

Measurement of insulin

The concentration of insulin in the incubation medium was measured by radioimmunoassay according to the double antibody technique described by Hales and Randle (1963) The radioimmunoassay was initiated immediately after the incubations. The method is based on the competition of insulin in the sample to be assayed and of radioactive insulin for reaction with an antibody which is specific to insulin. The amount of radioactive insulin bound to antibody therefore varies inversely with the concentration of insulin in the assay sample. In the present method the complex of insulin and anti insulin is rendered insoluble by a second antibody The precipitate obtained is separated from free insulin by filtration. The concentrations of insulin in the samples were found by referring to standard curves prepared from measurements on pure rabbit insulin. Twice crystallized rabbit insulin was used (supplied by Novo Research Institute, Copenhagen, Denmark) The antibodies (insulin binding reagent) and the ^{125}I insulin, which were used, were supplied by the Radiochemical Centre Amersham, England.

The precipitates in the solutions were collected by filtration through Milli pore cellulose acetate membrane filter discs (Oxoid Ltd, England) The radioactivity in the filter discs was measured by liquid scintillation counting After the filtration the filters were transferred to empty counting vials and dried 20 ml of a scintillant of the following composition was then added to each vial ethanol 150 ml, dioxan 300 ml, naphtalene 50 g, POP 5 g toluene to 1 litre. The vials were then transferred to a liquid scintillation counter (Packard) for determination of radioactivity For each sample the filter discs in the immunoassay clean filters for background count rate determination and unfiltered samples of iodinated insulin for total count-rate determination were counted.

The filter discs in the immunoassay were obtained from the unknown insulin solutions, the standard insulin solutions and from samples to which no unlabelled insulin was added (Zero for control of the total insulin binding capacity of the antibodies) The filtration procedure was controlled by the counting of filters from samples to which no insulin binding reagent had been added. The assays were always performed in duplicate samples.

Calculation and expression of result

The count rate for each determination was expressed in counts per minute. Computation of the mean count rates for replicates was then made. The amount of bound iodinated insulin (as given by the observed count rate) at each concentration of insulin in the standards and for the Zero was plotted

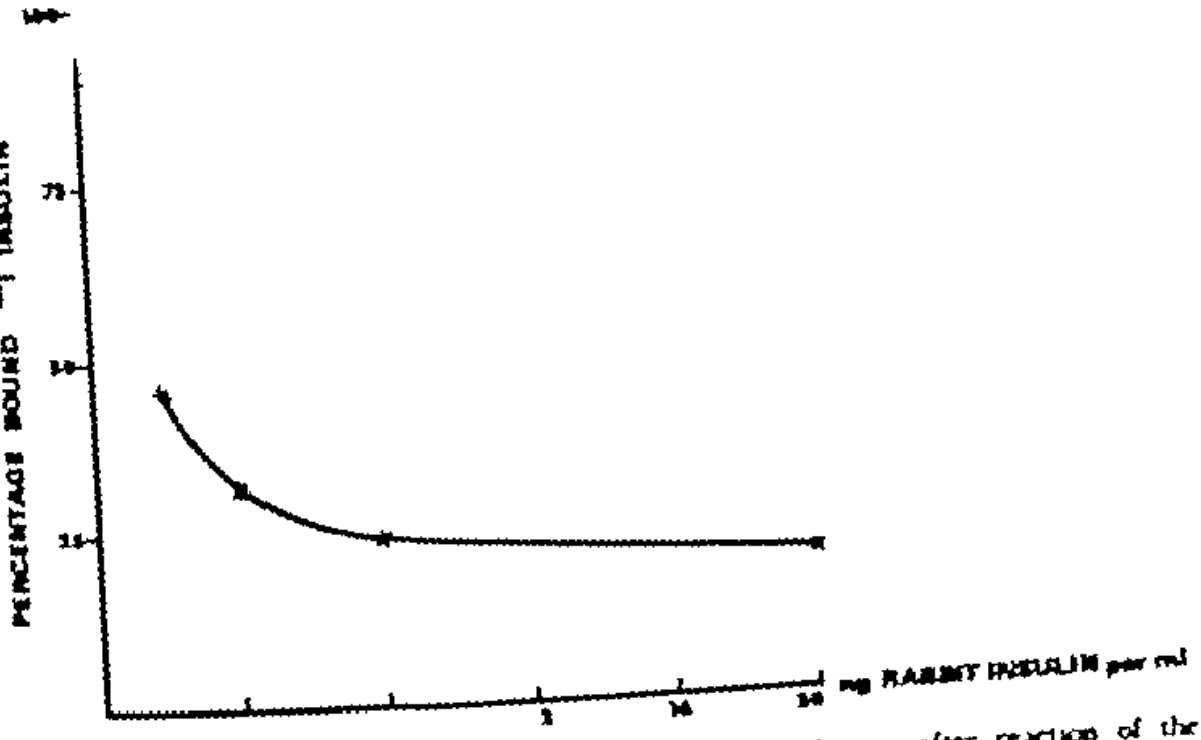


Fig. 54 Binding of ^{125}I-insulin in insulin-antibod precipitate after reaction of the insulin-antibodies with solutions containing differen amounts of rabbit insulin (the antibodies for this curv were from batch N 9996, /The Radiochemical Centr /)

on a graph. The concentration of insulin in each of the unknowns could then be read directly from th graph (see Fig 54)

The effect of the substances on the insulin secretion was calculated as follows

The mean concentration of insulin in the second and the third incubation solution compared with the insulin concentration of the first incubation solution The differences were expressed as percentage of insulin in the second and th ird solution in relation to the first solution. Student t test was used for the tistical evaluat on of the differences

Ve erification of the reliabil ty of th method incubations of pancreatic piece er performed in suc cessiv 30 minut periods in media containing in- er a e amount of glucose which is known to be the major stimulus of in- sulin sec etion. An increase of glucose f rm 0.6 mg/ml to 3.0 mg ml caused an rease in the radioimmunologically measurabl ins lin of a magnitude of abo t seven times. When panc e tic pieces were incubated for consecutive 30 m tr periods w th th same glucose content in the incubation solutions, the

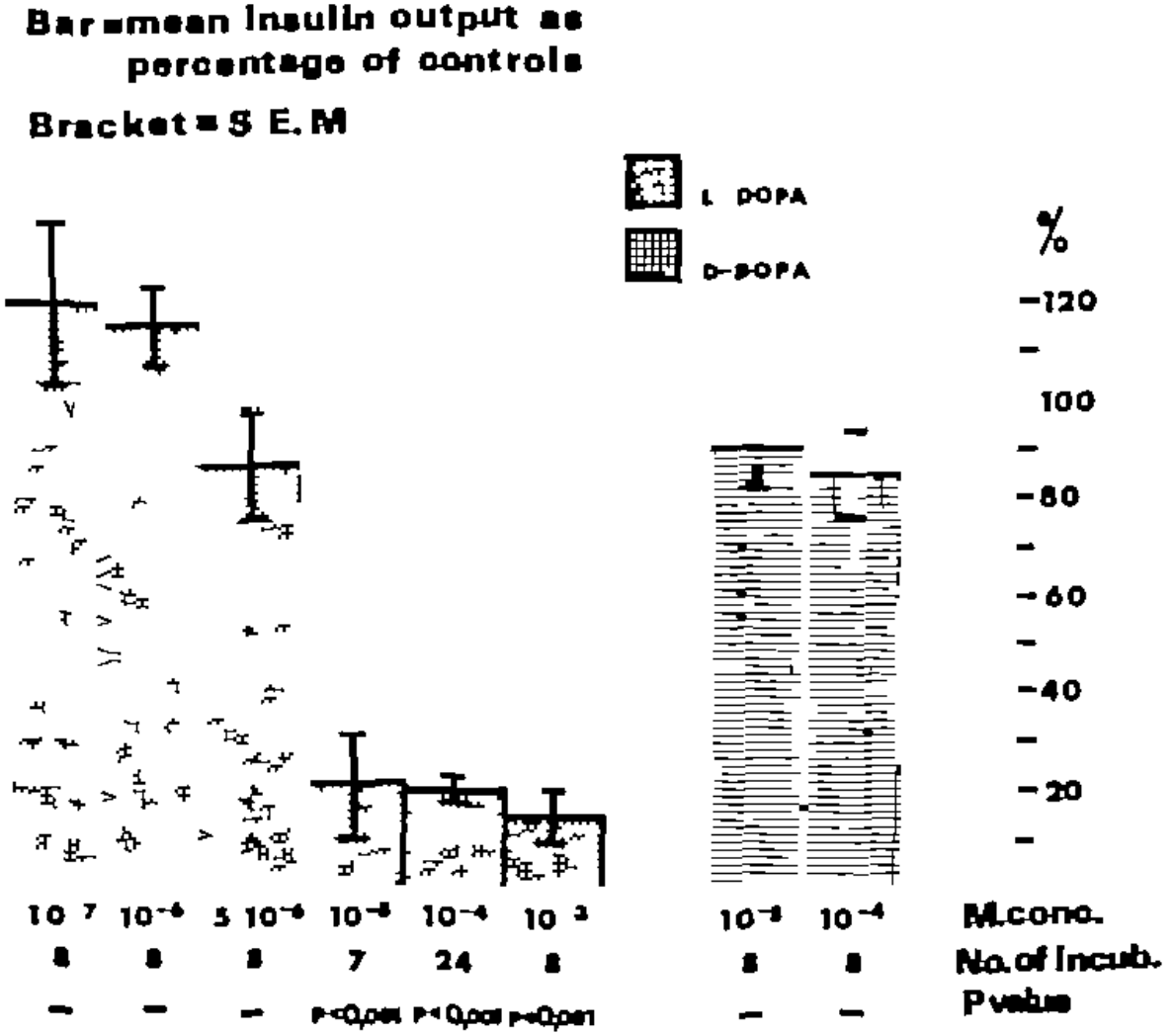


Fig. 55 Effect of different concentrations of L DOPA and D-DOPA in the incubation solutions on the release of insulin from pieces of rabbit pancreas *in vit* Glucose-concentration in the incubation solutions 3 mg/ml.

insulin output was found to be very constant up to three hours of incubation. which was the longest time period investigated.

By preparing standards containing the highest concentrations of the substances used in the incubations, it was ascertained that these substances had no effect on the radioimmunological measurement of the insulin

Results

The results from the incubations with glucose concentrations of 3 mg/ml and 0 6 mg/ml respecti vely will be presented separately

Glucose concentration 3 mg/ml

Fig. 55 shows the effect of L-DOP \ and D-DOPA on the insulin secretion. As can be seen L-DOPA in a concentration of 10^{-5} M or more in the incubation

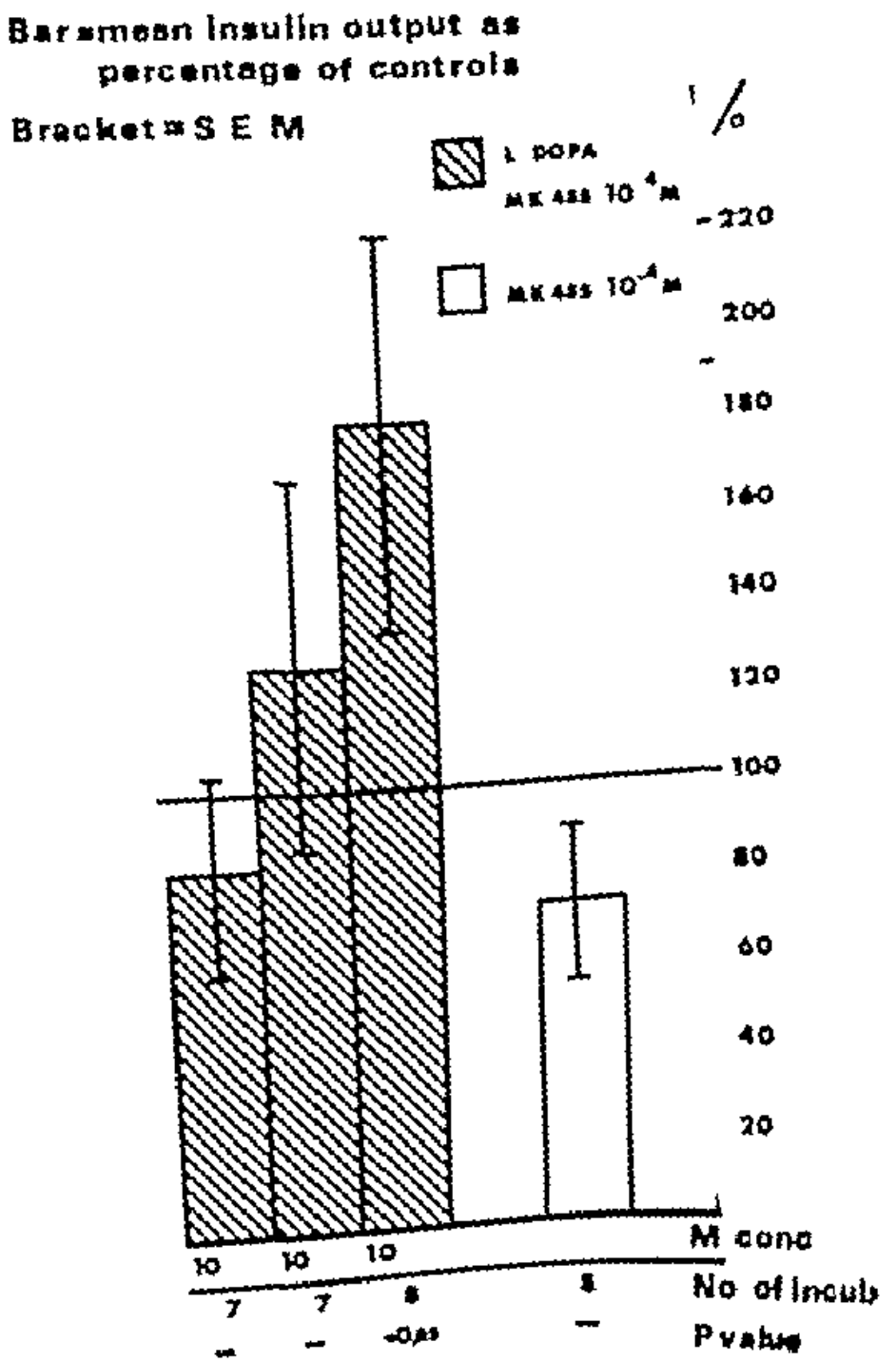


Fig. 56 ffect of differen concentrations of 1 DOPA in I ubation with th e tanning I M MK 485 DOPA-decarboxylase inhibitor) on the lease of i sulin I rn perfus ubbit pancreas ure The effect of 10 M MK 485 on the insul le is also s en. Glucose-concentr tion in the inc bation solution: 3 mg/ml.

medi m strongly inhibits the insulin s retion. D-DOPA s ure significant inhibition in concentration (10 3 M and 10 4 M) whe l DOP has trong inhibitory effect.

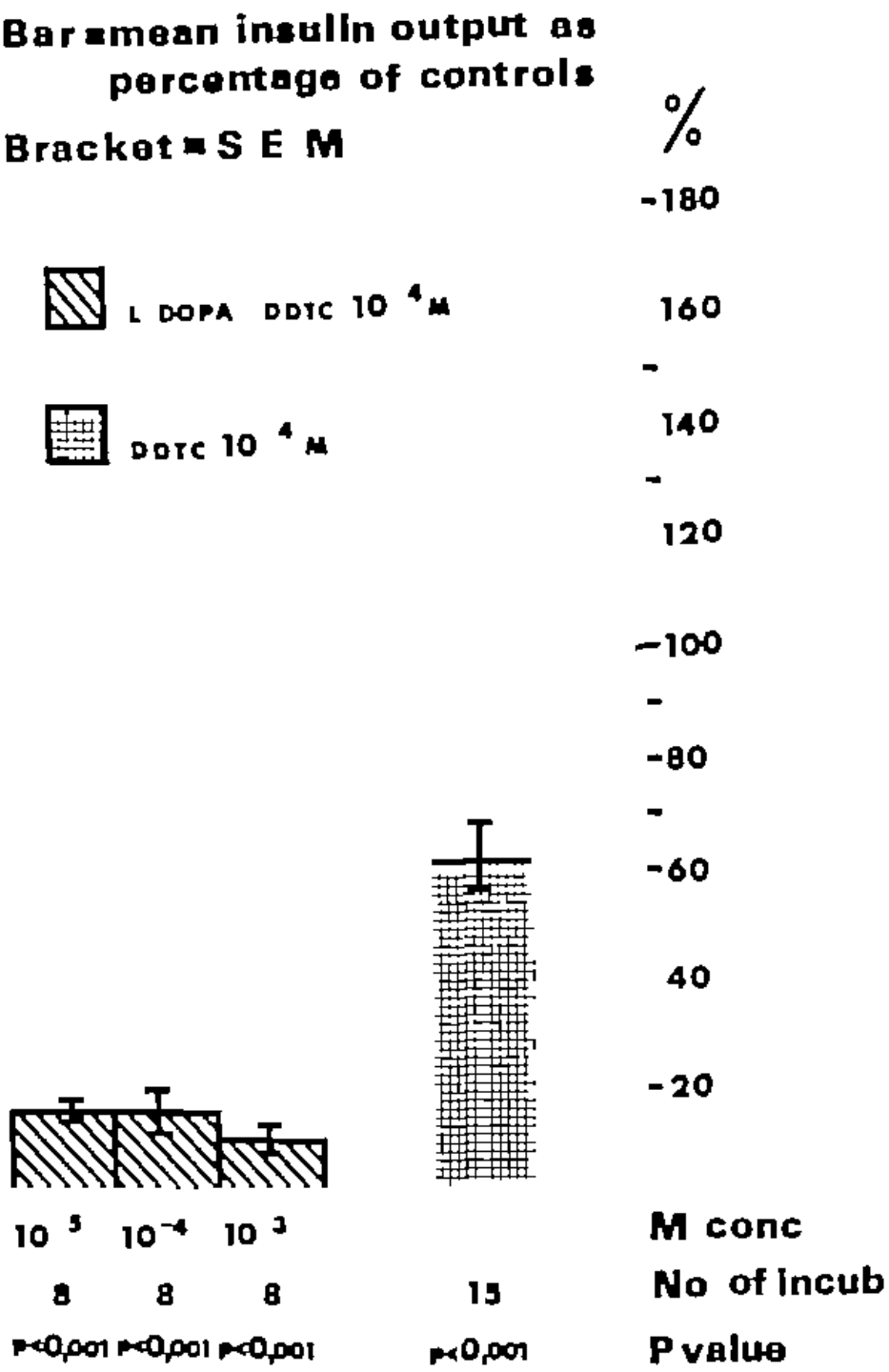


Fig. 57. Effect of different concentrations of L-DOPA in incubation solutions containing 10^{-4} M DDTC (dopamine-β-hydroxylase inhibitor) on the release of insulin from pieces of rabbit pancreas in vitro. The effect of 10^{-4} M DDTC on the insulin release is also given. Glucose-concentration in the incubation solutions 3 mg/100 ml.

If the DOPA-decarboxylase inhibitor MK 485 is present in the medium the inhibiting effect of L-DOPA is abolished and at the concentration of 10^{-5} M L-DOPA even stimulates the insulin secretion (Fig. 56).

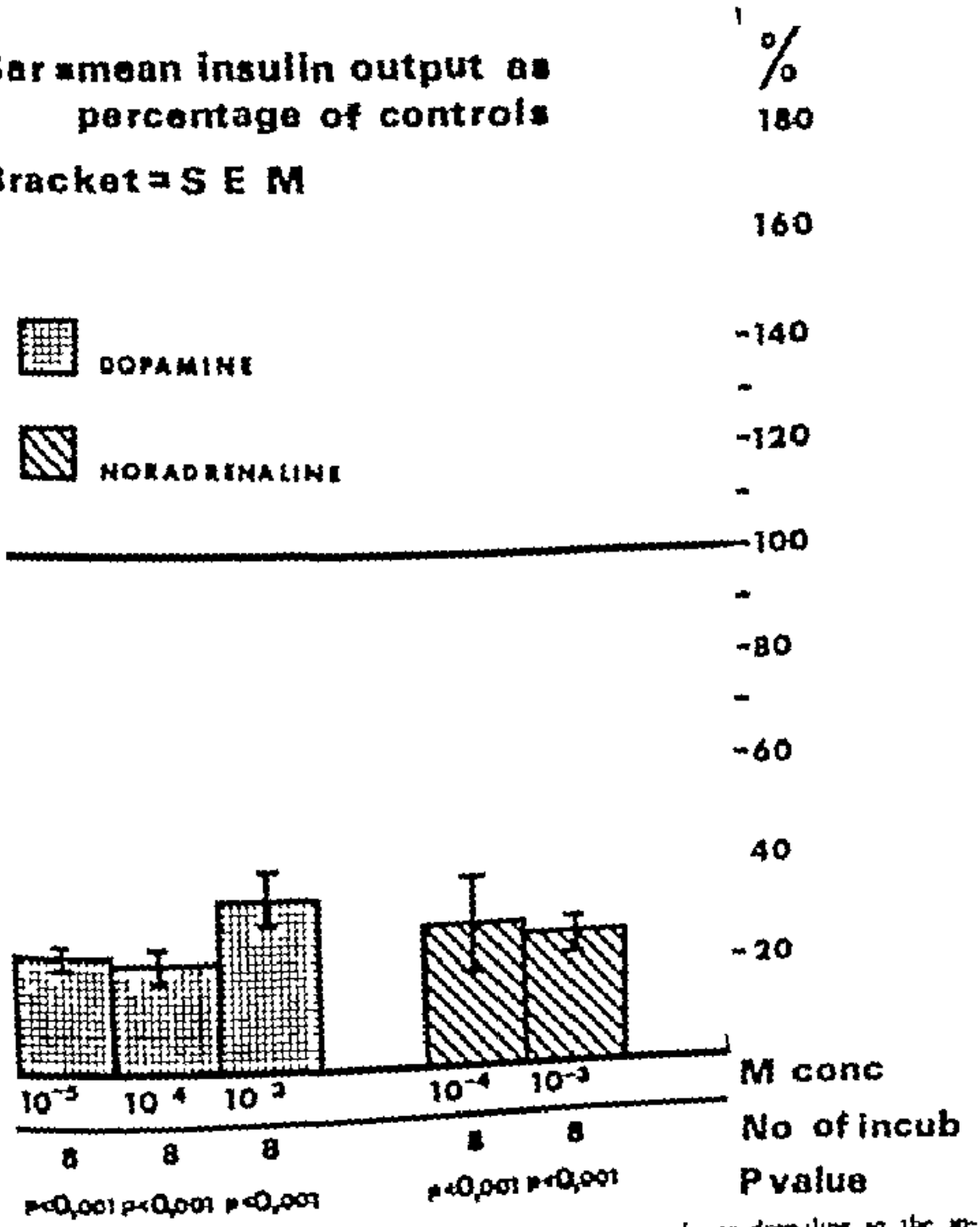


Fig. 38 Effect of different concentrations of dopamine and noradrenaline on the insulin secretion from isolated rat pancreatic islets incubated in a medium containing 3 mg/ml Glucose. Concentrations in the incubation solutions 3 mg/ml

In the presence of the dopamine β-hydroxylase inhibitor DDTC the inhibiting effect of L-DOPA persists (Fig. 37). DDTC itself inhibits the insulin secretion although this effect is not so strong as the L-DOPA-effect.

As can be seen from Fig. 38 dopamine (concentrations 10^{-5} M 10^{-3} M

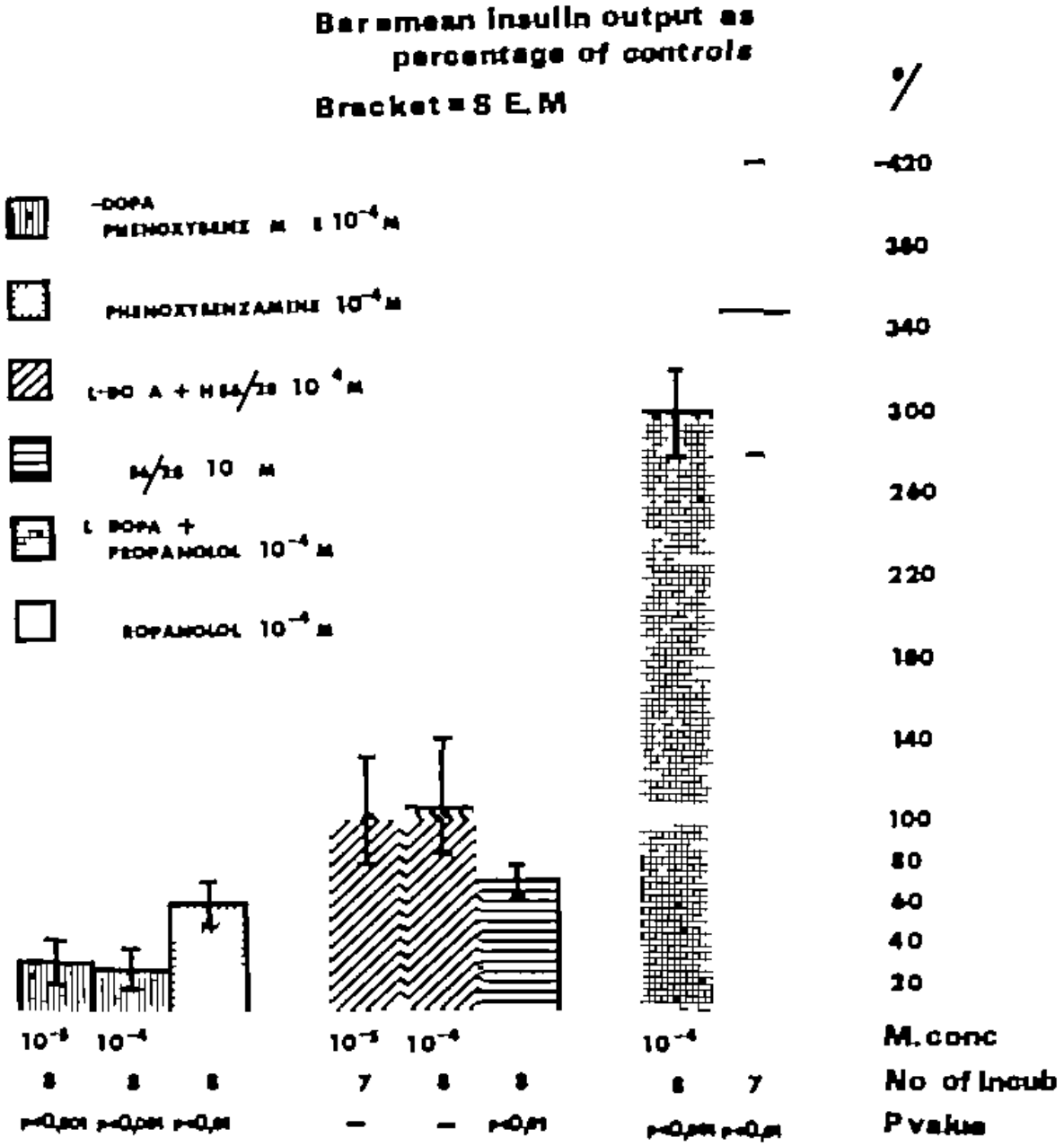


Fig. 59 Effect of different concentrations of L-DOPA in incubation solutions containing 10 M phenoxybenzamine (α-receptor blocking substance) 10 M H56/28 (β-receptor blocking substance) or 10 M propanolol (β-receptor blocking substance) on the release of insulin from pieces of rabbit pancreas *in vitro*. The effect of 10^{-4} M phenoxybenzamine 10 M H56/28 and 10 M propanolol on the insulin release is also given. Glucose-concentration in the incubation solutions 3 mg/ml

10^{-3} M) and noradrenaline (concentration 10^{-4} M, 10^{-3} M) strongly inhibit the insulin secretion.

When L-DOPA is incubated in a medium containing the α receptor blocking substance phenoxybenzamine the inhibiting effect of L-DOPA is still present (Fig. 59). The β-receptor blocking substance H56/28 abolishes the

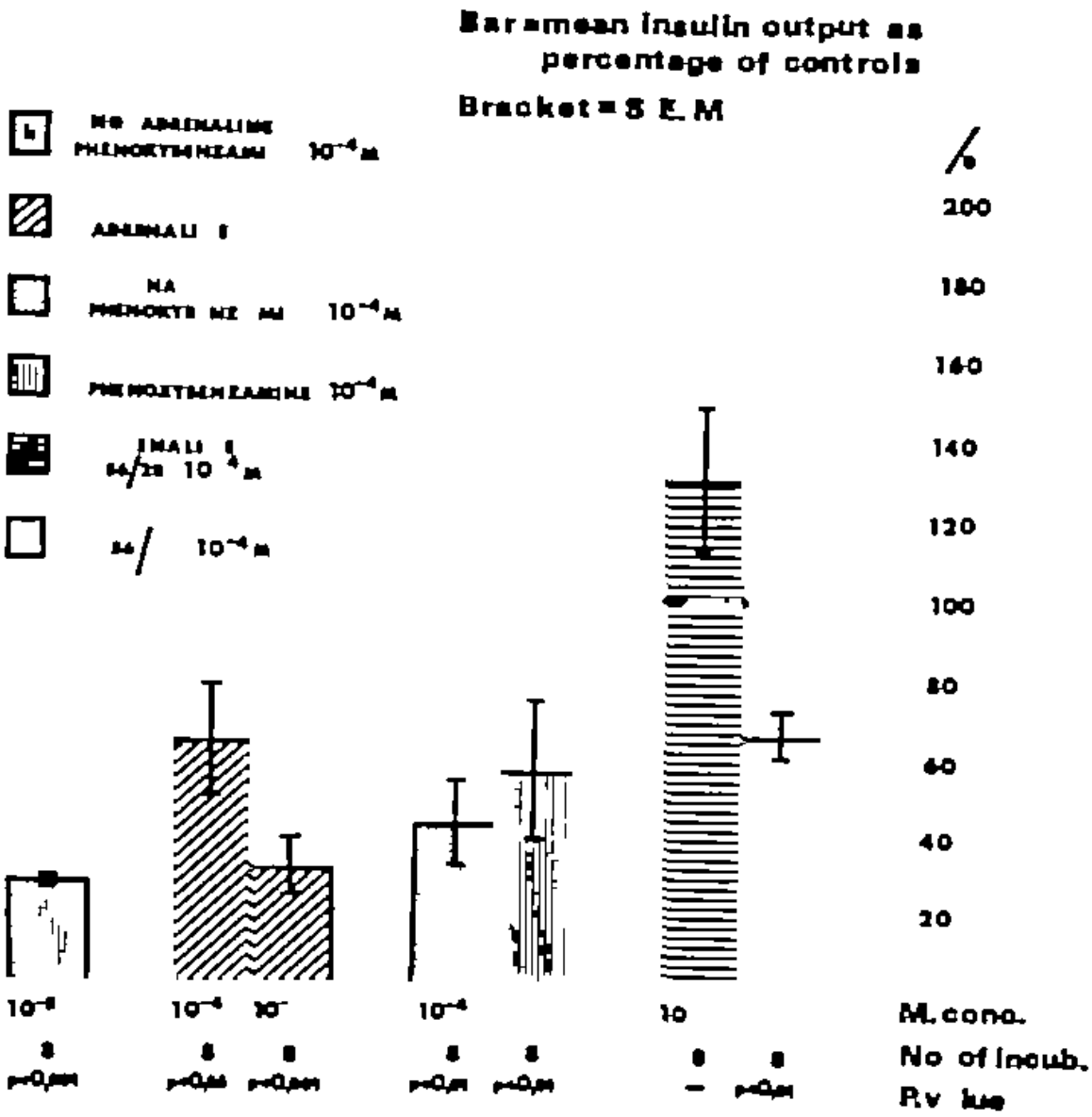


Fig. 60. Effect of adrenaline in the incubation solutions, and of noradrenaline and adrenaline in combination with 10^{-4} M concentrations of α- and β-receptor blocking substances in the incubation solutions, on the release of insulin from pieces of rabbit pancreas in vitro. The effect of 10^{-4} M concentrations of the α- and β-receptor blocking substances (phenoxybenzamine and H56/28) on the insulin release is also given. Glucose-concentration in the incubation solutions 3 mg/ml.

L-DOPA-effect. Both phenoxybenzamine and H56/28 inhibit the secretion of insulin to some extent (Fig. 59). The β-receptor blocking substance propanolol strongly stimulates the insulin secretion and this effect is still observable in the presence of L-DOPA (Fig. 59).

As was the case for L-DOPA, noradrenaline inhibits the insulin secretion also in the presence of phenoxybenzamine (Fig. 60).

Adrenaline was found to inhibit the insulin secretion (Fig. 60). This effect

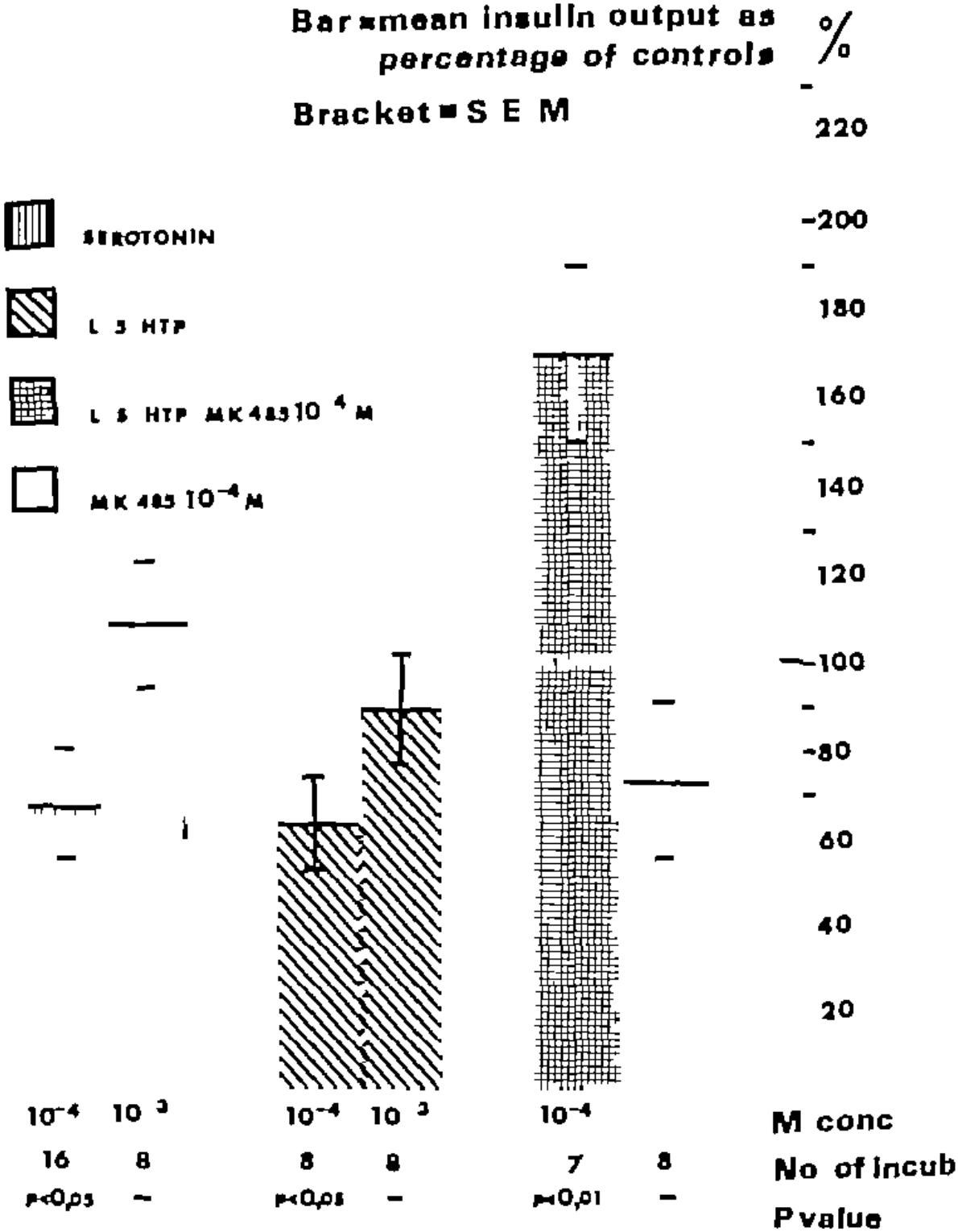


Fig. 61 *Effect of* different concentrations of serotonin and L-5-HTP in the incubation solutions and of L 5-HTP in combination with 10 M Mk 485 in the incubation solutions on the insulin release from pieces of rabbit pancreas *in vitro* The effect of 10^{-4} M Mk 485 (DOPA-decarboxylase inhibitor) on the insulin release is also given. Glucose-concentration in the incubation solutions 3 mg/ml

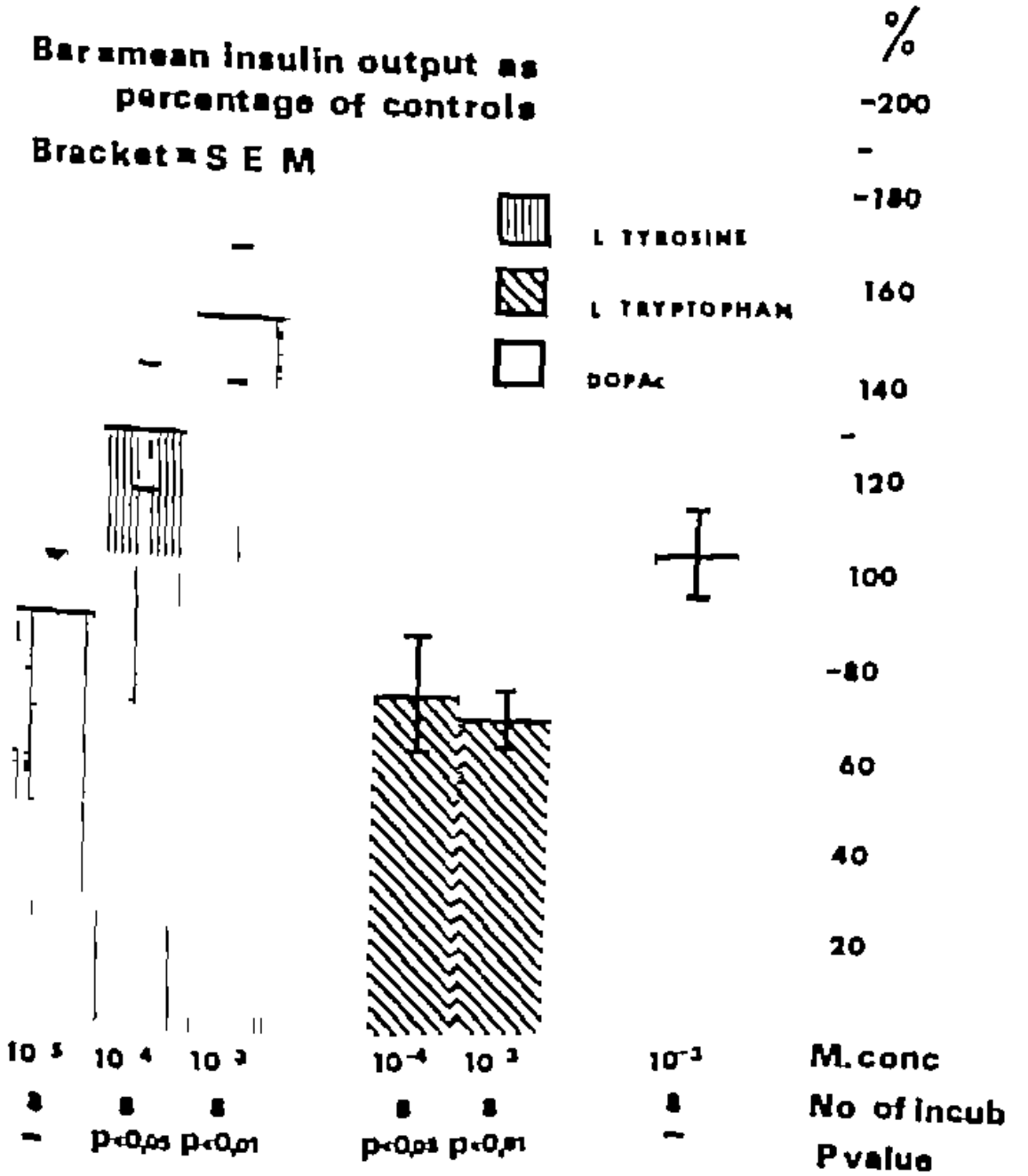


Fig. 62. Effect of different concentrations of L-tyrosine L-tryptophan and dihydroxy phenylacetic acid (DOPAc) in the incubation solutions on the release of insulin from pieces of adult pancreas in vitro Glucose-concentration in the incubation solutions 3 mg/ml.

persisted in the presence of phenoxybenzamine but was abolished when H156/28 was present in the medium (Fig 60)

The effects of L-5-HTP and serotonin on the insulin secretion are shown in Fig 61 A can be seen that was a small inhibiting effect of L-5-HTP and serotonin in a concentration of 10 M while a concentration of 10 M of the

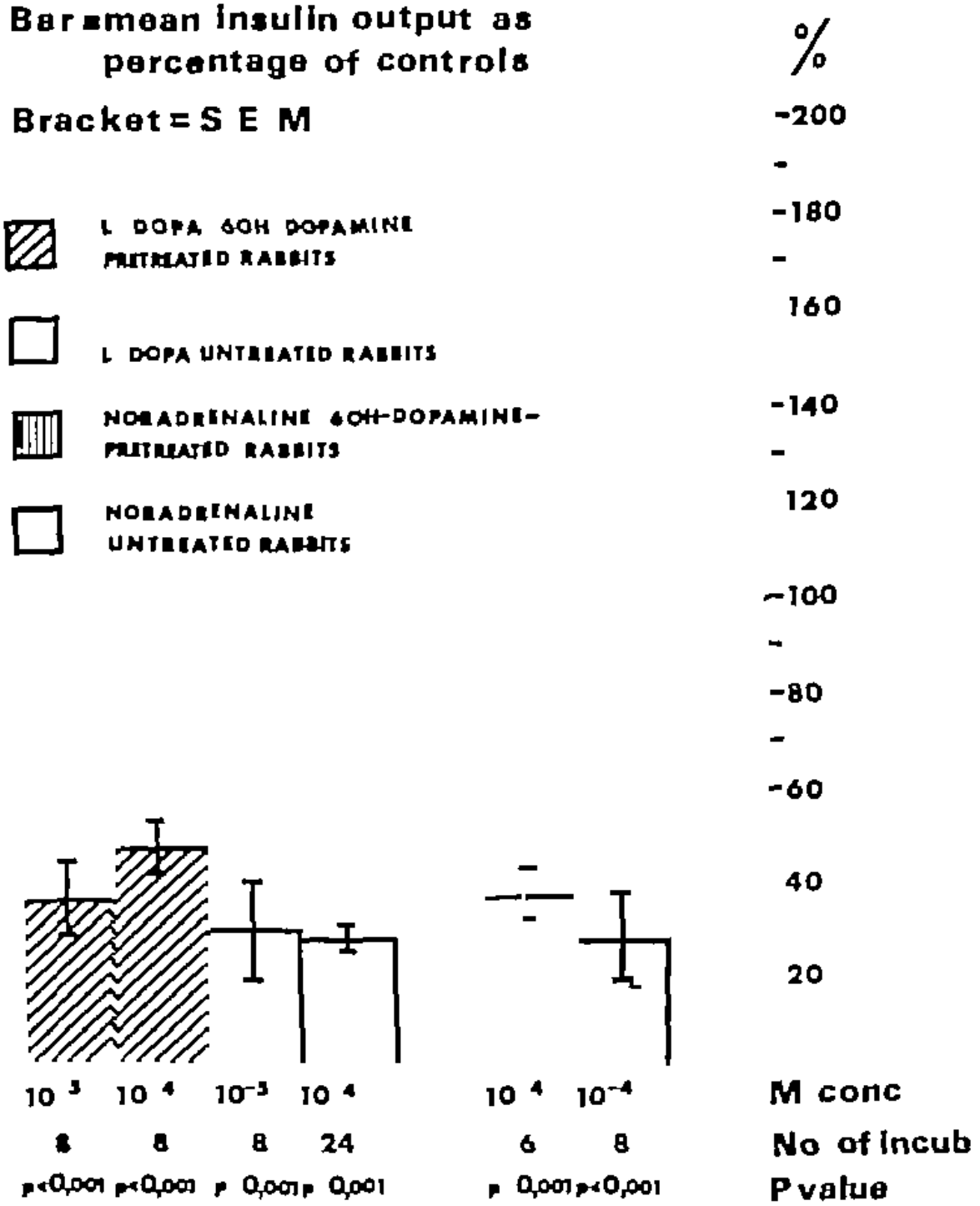


Fig. 63 Effect of L-DOPA and noradrenaline in the incubation solutions on the *in vitro* release of insulin from pieces of pancreas of untreated rabbits and of rabbits pre-treated with 6OH-dopamine. Glucose-concentration in the incubation solutions 3 mg/ml.

substances had no effect on the insulin secretion. When L-5-HTP in a concentration of 10 M was incubated together with MK 485 the inhibiting effect observed on the insulin secretion without MK 485 had changed to a stimulating effect (Fig. 61).

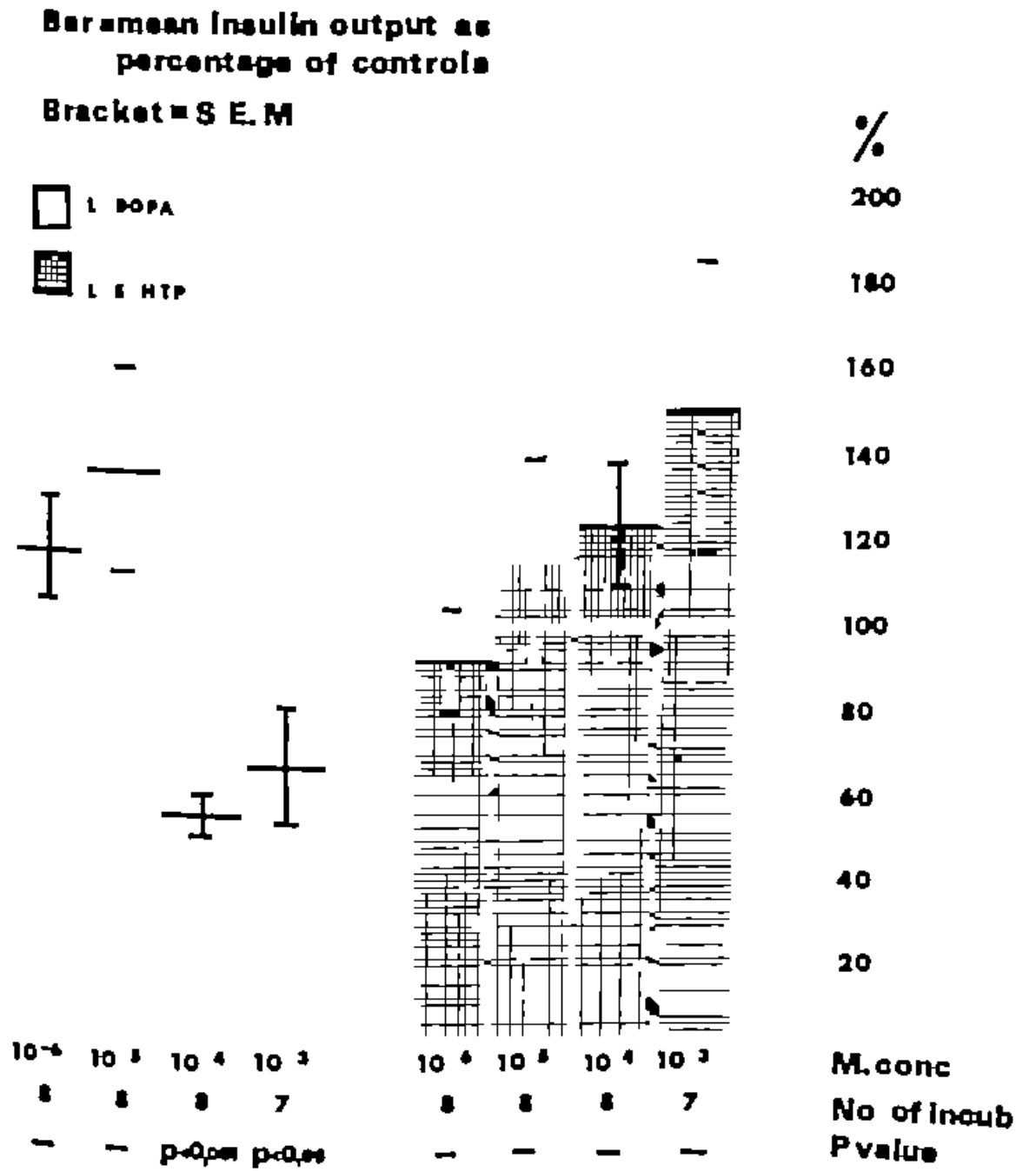


Fig. 64. Effect of different concentrations of L-DOPA and L-5-HTP in the incubation solutions on the release of insulin from pieces of rabbit pancreas *in vitro*. Glucose-concentration in the incubation solutions 0.6 mg/ml.

L-tyrosine in a concentration of 10^{-4} M had no effect on the insulin secretion. In a concentration of 10^{-3} M and 10^{-2} M, it stimulated the insulin secretion (Fig. 62). L-tryptophan in a concentration of 10^{-4} M and 10^{-3} M inhibited the insulin secretion to some extent (Fig. 62).

Dihydroxyphenylacetic acid (DOPAc) was found to have no effect on the insulin secretion in a concentration of 10^{-4} M (Fig. 62).

The results of the incubations performed on the 60H-dopamine pre treated rabbits are shown in Fig 63 As can be seen both L-DOPA and noradrenaline in the concentrations tried, inhibit the insulin secretion also in this case.

Glucose concentration 0.6 mg/ml

In the concentrations of 10^{-4} M and 10^{-3} M L-DOPA also had an inhibiting effect when the glucose concentration was 0 6 mg/ml (Fig 64) The incubations with L-5-HTP on the other hand indicated a small stimulating effect on the insulin secretion, although this effect was not statistically significant (Fig. 64)

Discussion

The preparation used in the present investigation has been shown to be very useful for the study of the *in vitro* release of insulin. This preparation, which was originally introduced by Coore and Randle (1964) has been used in several investigations. Telib and Pfeiffer (1970) especially studied the dynamics of insulin secretion using this *in vitro* preparation. They found a constant insulin secretion for five hours when pancreatic pieces were incubated in consecutive periods in solutions containing the same substances. When the effect of certain substance on the insulin secretion has been studied, it has been found to be most reliable to use the first incubation as a control and compare the obtained insulin secretion with the secretion in the following incubations, where the substance is present (see Coore and Randle 1964 Hales and Milner 1968 Telib and Pfeiffer 1970) This is the most appropriate way to express the results, because variation in the number of islet per piece causes variation in the absolute amount of insulin secreted by the different pieces. When, as in the present investigation, insulin is measured in an incubation medium, the immunological reaction between insulin and antibody has been found to be remarkably robust in tolerating without alteration in reaction considerable changes in the composition of the incubation medium (see Hunter 1968)

From the results it is obvious that the incubation of pancreas in the presence of L-DOPA causes a strong inhibition in the secretion of insulin. As was shown in Chapter 7 both dopamine and noradrenaline are formed in the pancreas under these circumstances. D-DOPA, which is not decarboxylated, does not inhibit the insulin secretion.

When the DOPA-decarboxylase inhibitor MK 485 is present in the incubation solution, the inhibitory effect of L-DOPA is blocked. This indicates that dopamine or noradrenaline are responsible for the effect. Both dopamine

and noradrenaline were also found in the pre
inhibiting effect on the insulin secretion.

Injections of L-DOPA into mice and rabb t
hyperglycaemia, which has been proposed to be
son *et al.* 1967 b Hayashi *et al.* 1968) When the
inhibited, the hyperglycaemia was abolished The
findings in the present investigation.

In the presence of MK 485 L-DOPA actually
tion to some extent. This might be explained by
L-DOPA. Most amino acids are known to have a
insulin secretion (Floyd *et al.* 1966 Edgar *et al.* 1969 M
1970)

When L-DOPA was incubated in the presence of the dopamine β-hydroxylase inhibitor DDTC, the inhibitory effect of L-DOPA persisted. This indicates that the formation of noradrenaline is not necessary for the inhibition of the insulin secretion. A dopamine formation occurs in the pancreas both *in vivo* and *in vitro* after the administration of L-DOPA and this probably takes place both in the islets and in the exocrine pancreas, while a formation of noradrenaline only seems to occur in the adrenergic nerves (see Chapter 1 5 and 7) In line with the assumption that a formation of noradrenaline is not necessary for the inhibitory effect of L-DOPA, is the observation that this effect was not influenced by the prior administration of 6OH-dopamine to the rabbits. 6OH-dopamine is known to specifically abolish the capability of the adrenergic nerves to form and store catecholamines (Thoenen and Tranzer 1968, Jonsson and Sachs 1970) The inhibitory effect of noradrenaline was also present after 6OH-dopamine-pre treatment. 6OH-dopamine, however does not affect the adrenergic receptor sites (Thoenen *et al.* 1970) and the effect of exogenous noradrenaline therefore persists also after 6OH-dopamine pre-treatment.

Adrenaline was in the present investigation also shown to cause an inhibition of the insulin secretion.

The inhibitory effect of catecholamines on the insulin secretion has been observed earlier Coore and Randle (1964) observed an inhibitory effect of adrenaline in the rabbit. Porte and co-workers ha studied the inhibitory effect of adrenaline and noradrenaline in man (see Port *et al.* 1965 Porte and Williams 1966 Port 1969) The inhibitory effect of adrenaline and noradrenaline has also been shown in rat and mouse (see Malaisse *et al.* 1967 Loubatières *et al.* 1967 Lernmark and Hellman 1970) Wong *et al.* (1967) showed an inhibitory effect of dopamine, noradrenaline and adrenaline in the rabbit.

In the present investigation L-5-HTP and serotonin were found to inhibit

the insulin secretion in a glucose concentration of 3 mg/ml in the medium. This effect could be observed in a concentration of 10^{-4} M of L-5-HTP and serotonin, but not in a concentration of 10^{-4} M. When MK 485 was present in the medium L-5-HTP stimulated the insulin secretion. It thus seems probable that the effect of L-5-HTP is due to an inhibitory effect of serotonin formed in the pancreas from L-5-HTP. The stimulatory effect of L-5-HTP in the presence of MK 485 might, as with L-DOPA, be an amino acid-effect of L-5-HTP. In a glucose concentration of 0.6 mg/ml L-5-HTP in a concentration of 10^{-3} M had a small stimulating effect on the insulin secretion.

The effect of serotonin on the insulin secretion has earlier been studied in the rabbit and the golden hamster (Telib *et al.* 1968, Feldman and Lebovitz 1970 a, b, c). Telib *et al.* (1968) who studied the insulin secretion from rabbit pancreas *in vitro* found that when the glucose concentration was 3 mg/ml, serotonin, in one concentration, corresponding to 10^{-4} M in the present investigation, inhibited the insulin secretion, while it had a stimulating effect in a higher concentration. In the absence of glucose in the medium serotonin had a stimulating effect on the insulin secretion. In *in vitro* experiments in golden hamster serotonin, in a concentration of 10 M and with a glucose concentration of 3 mg/ml in the medium, had an inhibitory effect on the insulin secretion (Feldman and Lebovitz 1970 a). The results of these investigations thus largely support the findings in the present investigation.

It is interesting to notice that dopamine probably in itself inhibits the insulin secretion.

The observation that dopamine not only is a precursor of noradrenaline and adrenalin but also might have a function of its own, was first clearly expressed by Blaschko (1957). There is now considerable evidence that dopamine has a specific function, both in the central nervous system and in the periphery (see Klawans 1968). Serotonin has also been demonstrated to have many functions separate from the functions of catecholamines (see Garong and Lorenzen 1967).

The capability of the cells of the pancreatic islets to store and form dopamine and serotonin together with the observation that dopamine and serotonin affect the insulin secretion might indicate that these two amines play a role in the intrinsic regulation of the hormone production by these cells.

As has been demonstrated in the present investigation and in several earlier investigations, noradrenaline and adrenaline, however also affect the insulin secretion. As has been shown in Chapter 7 the islets of the rabbit in addition are richly innervated with adrenergic nerves. Also in other species, such as golden hamster cat and dog the islets are richly innervated with adrenergic nerves (Cegrell 1968).

Besides the adrenergic innervation, the islets of cat, rat, rabbit, golden hamster and guinea pig have been shown to be richly innervated with cholinergic nerves (Coupland 1958, Libman and Sutherland 1965). Stimulation of the vagus nerve and administration of parasympathomimetic agents have been shown to cause a stimulation of the insulin secretion (Kaneto *et al.* 1967, 1968; Malaisse *et al.* 1967; Mayhew *et al.* 1968).

It thus seems probable that the sympathetic and parasympathetic nervous systems also may influence the insulin secretion.

Another interesting fact is that the islet cells of the rabbit have been demonstrated to have a strong positive reaction for both true and pseudocholinesterase (Coupland 1958). True or pseudocholinesterase have also been demonstrated in the parafollicular cells and the enterochromaffin cells in many species (see Pearse 1968). Pearse correlates these findings with high levels of phospholipid synthesis in the cells. Another possibility is that acetylcholine plays a role in the intrinsic regulation of the polypeptide hormone production in these cells.

In addition injected atropine has been shown to be accumulated in the pancreatic islets, the parafollicular cells and the enterochromaffin cell system in mice (Albanus *et al.* 1968; Slanina and Tjälve, in preparation).

Since the actions of monoamines and acetylcholine in connection with neuronal functions are assumed to be based on the changes they induce in the permeability of membranes, a similar function might be present also in extraneuronal tissues. Dopamine, serotonin and acetylcholine might be functionally integrating with each other within the cells. The possibility of a synergistic effect of several biogenic amines in these cells has been discussed by Ullberg and Hammarström (1969). The possible role of acetylcholine in regulation of membrane permeability and active transport has been discussed by Koelle (1963).

It is interesting to notice that dopamine and serotonin have been shown to affect the release of the hypothalamic releasing factors such as FSH-releasing factor, LH releasing factor and luteinizing hormone releasing factor (see Kamberi *et al.* 1970, Schneider and McCann 1969 a, b, 1970). Dopamine and serotonin have also been reported to affect the release of ACTH from the hypophysis (Gromova *et al.* 1967; King and Thomas 1968, King 1969).

The inhibitory effect of adrenaline and noradrenaline on the insulin secretion has been shown to be affected by α- and β-receptor blocking substances. In most cases the α-receptor blocking agents have been reported to abolish the inhibitory effects, while in the presence of β-receptor blocking agents, the inhibitory effects persist (see Malaisse *et al.* 1967; Porte 1969).

As adrenaline and noradrenaline are known to affect the generation of 3'5' cyclic AMP (adenosine-3'5' cyclic phosphate), it has also been proposed that

their effects on the insulin secretion might be mediated via this mononucleotide (see Robison *et al.* 1968) Exogenous 3 5 cyclic AMP and substances, such as theophylline, which increase the endogenous content of 3 5 cyclic AMP have been shown to stimulate the insulin secretion in the rat (Sussman and Vaughn 1967 Turtle *et al.* 1967) A lowering effect of adrenaline on the endogenous 3'5 cyclic AMP level in pancreatic islets of the rat, has been shown to be blocked by phenoxybenzamine (α-blocker) (Turtle and Kipnis 1967) Phenoxybenzamine was also shown to prevent the inhibitory effect of adrenaline on the insulin secretion. In conclusion, these findings might indicate that the effect of adrenaline and noradrenaline on the insulin secretion are mediated via generation of 3 5 cyclic AMP which in turn takes place via an action of adrenaline and noradrenaline on α and β-receptors.

Recently data have been presented on the inhibitory mechanism of action of adrenaline and serotonin on the insulin secretion, which indicate that these substances act rather via a specific receptor site on the islet β-cells, than via a generating action on 3 5 cyclic AMP (Feldman and Lebovitz 1970 c)

In the present investigation it was found that the inhibitory effect of L-DOPA and catecholamines on the insulin secretion was blocked by H56/28 (β-blocker) but was not affected by phenoxybenzamine (α-blocker) Propanolol was found to be a potent stimulator of the insulin secretion in itself

The effects reported of β-receptor blocking agents are very divergent. In some investigations in the rat, propanolol has been reported as in the present investigation, to cause a stimulation of the insulin release (Sussman *et al* 1967 Mariani *et al.* 1969 a) In studies in intact mouse, dog and man an impairment in the insulin secretion has been reported after the administration of β-receptor blocking agents (Bressler *et al.* 1969 Sirek *et al.* 1969 Cerasi *et al.* 1969) The hypoglycaemic effect of tolbutamide has been reported to persist after block of the β-receptors in rabbit, but not in man and mouse (Erill 1969 de Divitis *et al.* 1968 Bressler *et al.* 1969) In man there are several reports of hypoglycaemia as a result of the administration of propanolol (Kotler *et al.* 1966, Abramson *et al.* 1966, Simpson 1967 Machintosh 1967) In the rabbit propanolol has been shown to cause hypoglycaemia and to increase the hypoglycaemic response to insulin (Calvey and Summerill 1968)

In the present investigation it was observed that phenoxybenzamine blocked the inhibitory effect of L-DOPA and catecholamines. Phenoxybenzamine was also in itself observed to have a small inhibitory effect on the insulin secretion. An inhibitory effect of phenoxybenzamine has also been reported on an isolated perfused rat pancreas preparation (Mariani *et al.* 1969 b) In intact man and mouse α-receptor blocking agents have been reported to cause a stimulation of the insulin secretion (Cerasi *et al* 1969 Bressler *et al.* 1969) In the rabbit

the α-receptor blocking substance phentolamine has been shown to cause hyperglycaemia, while in rat, dog and man it has been shown to cause hypoglycaemia (Trapold *et al.* 1950 Sherif *et al.* 1957 Salvador *et al.* 1968, Calvey and Summerill 1968) In the rabbit phentolamine also reduces the hypoglycaemic response to insulin (Calvey and Summerill 1968)

A possible explanation for the varying results reported by different authors on the effects of adrenergic blocking agents on the insulin secretion may be, that if their effects are truly manifestations of adrenergic blocking actions, the types of adrenergic receptors, which mediate the insulin release, vary from species to species. It is also possible that the results reported from intact organisms are due to metabolic effects, which secondarily may affect the insulin secretion.

L-tyrosine was in the present investigation found to have a stimulatory effect on the insulin secretion, while L-tryptophan inhibited the insulin secretion to some extent. As has been mentioned most amino acids have been reported to stimulate the insulin secretion (Floyd *et al.* 1966 Edgar *et al.* 1968 Milner 1970 Feldman and Lebovitz 1970 b) In *in vitro* investigations a condition precedent for the effects of amino acids on the insulin secretion has been shown to be the presence of glucose in the incubation medium (Edgar *et al.* 1969 Milner 1970) There seems to be some species variation in the insulin response to amino acids. Tryptophan has been shown to cause a stimulation of the insulin secretion in golden samster (Feldman and Lebovitz 1970 b) an effect which could not be demonstrated in the present investigation, nor in another investigation in the rabbit (Milner 1970) In the rabbit phenylalanine has been shown to have an inhibitory effect on the insulin secretion (Milner 1970)

Summary

In recent years the interest has been focused on extraneuronal localizations and functions of catechol- and indolamines. Special attention has been paid to the ability of some endocrine cell systems, such as the pancreatic islets, the para follicular cells of the thyroid and the enterochromaffin cell system of the gastrointestinal tract, to form and store dopamine and serotonin. It has been assumed that catechol and indolamines might play a role in the regulation of the polypeptide hormones, which are known to be or assumed to be produced by these cell systems.

The present work has been performed to elucidate some of the extraneuronal localizations and functions of catechol and indolamines and then especially the localization and function in the mentioned endocrine cell systems.

The formation of dopamine and serotonin in the pancreatic islets, the parafollicular cells of the thyroid and the mucosa of the stomach after administration of the monoamine precursors dihydroxyphenylalanine (DOPA) and 5-hydroxytryptophan (5-HTP) into mice was studied with microautoradiography fluorescence microscopy and combined microautoradiography fluorescense microscopy (Chapter 1—3) The combination of microautoradiography and fluorescence microscopy allowed a concomitant demonstration of catechol- and indolamines in the cells.

It was found that there was a good correspondence between the localization of dopamine and serotonin in these cell systems.

The monoamines were rapidly formed in the cells after the injection of the precursors.

In the islets all cells contained monoamines shortly after the injection. Later the monoamines disappeared first from the β-cells. Some α-cells contained more monoamines than others. The enzyme monoamine oxidase (MAO) which was demonstrated by a histochemical method appeared to have the strongest activity in the cells from which the monoamines first disappeared. In the stomach endogenous fluorescence was present in mast cells in the fundic gland area and in enterochromaffin cells in the pyloric gland area. After injection of the precursors, monoamines seemed to be formed in these two cell types as well as in the so called enterochromaffin-like cells of the fundic gland area.

The combination of microautoradiography and fluorescence microscopy showed that the same cells in the pancreatic islets, in the thyroid and in the stomach formed catechol and indolamines after the injection of the precursors.

With electron microscope autoradiography it was shown that the incorporation in the fundic gland area took place in enterochromaffin cells of two types (Chapter 4). The silver grains were found predominantly over the specific granules in the cytoplasm of the cells.

Whole-body autoradiography was used to compare the distribution patterns obtained after the injection of DOPA 2-^{14}C and DOPA 1-^{14}C before and after the inhibition of DOPA-decarboxylase (Chapter 5). When DOPA 1-^{14}C is decarboxylated, $^{14}CO_2$ is formed and exhaled. Comparison of the distribution patterns allows a conception of the places, where catecholamines are formed. In the adrenal medulla catecholamines were very rapidly formed. DOPA in itself was not accumulated in the medulla. The condition was the same in the pancreatic islets and in the parafollicular cells. DOPA in itself was moderately accumulated in the exocrine pancreas. A relatively strong accumulation could also be seen in parts of the caput epididymidis and in structures of the ovary such as follicle walls and corpora lutea. In ganglia DOPA in itself was accumulated to some extent, although catecholamines were formed much less in sympathetic than in spinal ganglia.

A histochemical method was used to study MAO in whole-body sections of mice (Chapter 6). The highest enzyme activity was found in sympathetic ganglia, the intestinal mucosa and the adrenal cortex. Serotonin-^{14}C incubated with whole-body sections of mice, was shown to be accumulated in the adrenal cortex. This binding could not be observed when MAO was inhibited, thus MAO in this case might be responsible for the binding.

Fluorescence microscopy was used to show that the formation of dopamine in the pancreatic islets of the rabbit after the administration of DOPA occurred in a similar way as in the mouse (Chapter 7). The uptake-storage mechanisms appeared to function also *in vitro*. By means of chromatography dopamine, noradrenaline and dihydroxyphenylacetic acid were demonstrated to be formed from DOPA *in vitro* in the rabbit pancreas (Chapter 7).

The effects of monoamines and monoamine precursors on the insulin secretion from rabbit pancreas were studied *in vitro* with a perifusion technical method (Chapter 8). The results indicated that dopamine, noradrenaline and DOPA *in vitro* inhibited the insulin secretion. Noradrenaline and adrenaline also inhibited the insulin secretion. The effects of catecholamines were like those of a β-receptor blocking substance, but not by an α-receptor blocking substance. The results also indicated that serotonin formed from 5-HTP in vitro inhibited the insulin secretion in a high glucose concentration. In low glucose concentration it seemed to have a small stimulating effect on the insulin secretion. Tyrosine had a stimulating, and tryptophan a depressing effect on the insulin secretion.

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