HEMOPHILIA and OTHER HEMORRHAGIC STATES

ACKNOWLEDGMENTS

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HEMOPHILIA AND OTHER HEMORRHAGIC STATES

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PREFACE

THIS volume is based upon a Symposium on Hemophilia held in Rome in connection with the VIIth Congress of the International Society of Hematology on September 12 1958 The many recent advances which have occurred in this general field including progress in the purification and administration of plasma antihemophilic factor to hemophilic subjects the better delineation of vascular hemophilia and a deeper understanding of the physiologic and pathologic roles of many of the procoagulant clotting factors particularly Hageman and Stuart factors certainly justify the choice by Prof G Di Guglielmo President of the Congress of hemophilia as an appropriate and timely topic for discussion The program itself evolved from the contributions and suggestions of a panel who accepted the invitation of Prof Di Guglielmo to participate in this undertaking Included were W Achenbach (Cologne) B Alexander (Boston) E Deutsch (Vienna) R Feissly (Lausanne) J B Graham (Chapel Hill) R Gross (Marburg/Lahn) E Jorpes (Stockholm) F Koller (Zurich) R Marx (Munich) A Pavlovsky (Buenos Aires) A J Quick (Milwaukee) W H Seegers (Detroit) U M Serafini (Rome) J P Soulier (Paris) T H Spaet (New York) S van Creveld (Amsterdam) with Prof P de Nicola (Pavia) as secretary and your Editor as chairman Planning for separate publication of the various contributions to the Symposium was stimulated by the encouragement of Mr Robert Lee Henry of New York City whose support made the earlier Symposium volume on hemophilia and the hemophiloid diseases (1) possible Interest in a separate volume spread not only because of the desirability of having the contributions of many of the most active workers in the field readily accessible in one volume but also because the spoken word is a poor substitute for the recording of the material in print Publication has had other advantages as well One of these is that certain invited papers which could not be presented at the Symposium proper because of lack of time can now be made available Another is that an opportunity is provided for inclusion of material from some investigators who were unable to be in Rome at the time of the Symposium

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It is hoped that the present status of the hemophilia problem broadly conceived may be evident from this publication including as it does the work and the ideas emanating from many if not most of the laboratories actively pursuing investigations in this field

This volume was made possible by the help and support of many people and organizations Particularly helpful were Dr de Nicola assistant Editor of this volume as well as Miss Silvia Hinnom Mrs Elisabeth Sommer and Mrs Anne Payne who aided with both the translation of several papers into English and the bibliographies proof reading and indexing In addition this volume could be published only with the grants in aid provided by the New York Blood Transfusion Association and the National Heart Institute Public Health Service (HTS 5327)

Kenneth M Brinkhous M D

Chapel Hill N C
June 1959

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GLOSSARY, SYNONYMS, and SYMBOLS

Accelerator globulin Factor V Symbol AcG

AHF Antihemophilic factor Factor VIII

AHF B Factor IX Christmas factor

Antihemophilic factor plasma factor which corrects hemostatic defect in hemophilia Symbol AHF Factor VIII

Angiohemophilia vascular hemophilia also angiohemophilia A

Angiohemophilia B pseudohemophilia with Factor IX deficiency

Antihemophilic globulin plasma globulin possessing AHF activity Symbol AHG Factor VIII

Bleeder congenital hemorrhagic diathesis

Bridge anticoagulant a plasma inhibitor described in patients with either hemophilia or Christmas disease prototype patient named Bridge

BT bleeding time

Cephalin time PTT with cephalin as partial thromboplastin

Christmas factor plasma factor which corrects hemostatic defect in Christmas disease Factor IX

Christmas disease a hemophilioid disease prototype patient named Christmas Factor IX deficiency

Classical hemophilia hemophilia with severe AHF deficiency

Convertin active Factor V

Cothromboplastin Factor VII

CT clotting time

Factor V procoagulant plasma factor deficient in parahemophilia

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Factor X

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Platelet factor 4 antiheparin activity of platelets

Platelet metamorphosis agglutination and fusion of platelets

Proaccelerin Factor V

Proconvertin Factor VII

Prothrombin consumption time serum PT

Prower factor deficient factor in plasma and serum associated with a hemophilioid disease in a patient named Prower believed to be identical to Stuart factor

Pseudohemophilia general term for bleeders with normal number of platelets and long BT (coagulation defect not constant)

Pseudohemophilia A one type of pseudohemophilia with a capillary type of hemorrhagic diathesis

Pseudohemophilia B one type of pseudohemophilia synonymous with vascular hemophilia

PT prothrombin time

PTA plasma thromboplastin antecedent

PTC plasma thromboplastin component Factor IX

PTT partial thromboplastin time

Quick test prothrombin time

Russell s viper venom clotting time Stypven time

SPCA serum prothrombin conversion accelerator Factor VII

Stable factor Factor VII

Stuart factor deficient factor in plasma and serum in one of the hemophilioid diseases prototype a patient named Stuart

Stuart Prower factor Stuart factor

Stypven time one stage clotting test with recalcified plasma and Russell s viper venom

TAg thrombocyte agglutinating factor in plasma and serum

TAT thromboplastinogen activity time

TEG thrombelastogram

TGT thromboplastin generation test

Factor VII a procoagulant active in serum lacking in one of the hemophiloid diseases SPCA deficiency

Factor VIII AHF

Factor IX Christmas factor PTC

Factor X a serum clotting factor described as distinct and required for thromboplastin generation *See* Editor's note p 230

Fraction I fibrinogen-containing fraction of plasma proteins obtained by controlled low temperature ethanol plasma fractionation

Fraction II gamma globulin-containing plasma fraction obtained by controlled ethanol precipitation

Fraction III beta globulin containing plasma fraction

Fraction IV alpha globulin-containing plasma fraction

Glass factor a plasma factor activated by contact with glass which accelerates coagulation

Goethlin test a tourniquet test for eliciting petechiae

Hageman factor plasma factor deficient in a clotting anomaly (not a hemorrhagic diathesis) prototype subject named Hageman Symbol HF

Hageman trait clotting anomaly due to deficiency of HF

Hemmkorper hemophilia hemophilia with circulating anticoagulant

Hemophilia A inherited hemorrhagic diathesis with AHF deficiency and normal BT true hemophilia

Hemophilia B hemophiloid disease with PTC deficiency Christmas disease

Hemophiloid disease inherited hemorrhagic diathesis with impairment of an early phase of coagulation excludes true hemophilia

HF Hageman factor

Labile factor Factor V

Partial thromboplastin time plasma clotting time with a partial thromboplastin Symbol PTT

Plasma thromboplastin antecedent plasma factor deficient in one of the hemophiloid diseases Symbol PTA

Plasma thromboplastin component plasma factor deficient in Christmas disease Symbol PTC Factor IX

Platelet cofactor I plasma factor which with platelet factor 3 and plasma AcG functions in activation of prothrombin believed to be inhibited in hemophilia

Platelet cofactor II PTC Factor IX

Platelet factor 3 a procoagulant in phosphatide fraction of platelets acts with platelet cofactor I

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Stypven time one stage clotting test with recalcified plasma and Russell s viper venom

TAg thrombocyte agglutinating factor in plasma and serum

TAT thromboplastinogen activity time

TEG thrombelastogram

TGT thromboplastin generation test

Thrombopathy qualitative platelet defect constitutional thrombopathy von Willebrand s disease

Thromboplastin generation test test for thromboplastin formed with time in a mixture of prothrombin free plasma serum Ca^{++} and a partial thromboplastin Symbol TGT

Thromboplastinogen AHF

Tpl thromboplastin

Vascular hemophilia bleeders with reduced plasma AHF and long BT

von Willebrand s disease pseudohemophilic syndromes vascular hemophilia also v Willebrand Jurgens disease

Chairman's Comments

A. M. Brinkhous

IT IS my pleasure to welcome all of you to this symposium devoted to the recent advances in the field of hemophilia and the hemophiloid states. To my knowledge there have been two previous international symposia devoted to hemophilia, both held in close chronologic relation to but actually separate from the Congress of the International Society of Hematology. This Symposium on hemophilia then is the first held within the framework of the Congress, thanks to Prof. Dr. Guglielmo, President of the Congress. The fact that it appeared desirable to hold a second Symposium on this topic only two years after the New York gathering in 1956 (1) attests to the fact that there has been rapid progress in this field.

The 1956 Symposium was opened on a brief historical note, and it seems appropriate to do this again—this time in Rome. The word hemophilia has become deeply embedded in our scientific as well as in our general literature. This is of interest because the word itself, literally meaning the love of blood, does not seem to be an especially appropriate term for this disease. Professor Johann Lucas Schonlein (see Frontispiece) is generally credited with the origination of the word in the late 1820's while he was Professor of Special Pathology and Therapy and Director of the Julius Spital in Wurzburg. He apparently coined the term hemophilia in connection with his lectures to medical students on nosology of disease. The exact origin of the word is clouded in obscurity however since Schonlein did not publish his lectures. There are two early publications however suggesting the origination of the term by Schonlein is correct. The first is a dissertation by one of Schonlein's pupils, Dr. Friedrich Hopff (2) who stated in 1828 that Schonlein had recently introduced the term hemophilia. The other one is anonymous (3) being the unauthorized publication in 1832 of his lectures. In this publication the term appears as haemorrhaphilia rather than hemophilia. It is interesting to speculate that haemorrhaphilia— the love of hemorrhages —was the original term of which hemophilia is but a contraction. Pagel however comments on this latter publication in which haemorrhaphilia is used as follows: Schonlein

never published anything himself on the nosological system Partly with his consent partly against his wishes some of his students had his lectures published some complete some fragmentary Some [i e (3)] were pub lished anonymously in the most inaccurate way from class notebooks (4) Regardless of Pagel s comments on possible inaccuracy Virchow some 25 years later (5) seems to use hemophilia and haemorrhaphilia synony mously

We are all aware that today the word hemophilia is used in a more specific sense than ever before Also with the increased understanding of the hemorrhagic diseases many hemophiloid states have been recognized A relative newcomer to this group of diseases is vascular hemophilia a major topic for discussion in this Symposium A consideration of this con dition along with many others will reflect I feel certain the great amount of thought and attention that is being given in many parts of the world today to the various bleeding diathesis

References

1 *Hemophilia and Hemophiloid Diseases* K M Brinkhous Ed Chapel Hill University of North Carolina Press 1957

2 Hopff F *Ueber die Haemophilie oder die erbliche Anlage zu todtlichen Blutungen* Wurzburg 1828 p 17

3 *Allgemeine und specielle Pathologie und Therapie Vorlesungen nieder geschrieben und herausgegeben von einem seiner Zuhorer* 2 Bd Wurz burg 1832

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5 Virchow R Die Bluterkrankheit (Blutsucht Haemophilia Haemorrha philia) *Handbuch der spe ellen Pathologie und Therapie* v 1 1854 pp 263 270

Part 1

HEMOPHILIA

A The Antihemophilic Factor Purification and Properties

B The Antihemophilic Factor Management of Hemophilic Hemorrhages

C Inhibitor Reactions and Hemophilia

D Inheritance

E Diagnosis

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5 Virchow R Die Bluterkrankheit (Blutsucht Haemophilia Haemorrhaphilia) *Handbuch der speziellen Pathologie und Therapie* v 1 1854 pp 263 270

A The Antihemophilic Factor Purification and Properties

1

Antihemophilic Factor Some Problems in Its Study*

Robert H Wagner Kenneth M Brinkhous and George D Penick

Departments of Pathology and Biochemistry University of North Carolina Chapel Hill

THE present paper is an attempt to present the current status of some problems concerned with the study of AHF Problems concerning assay purification stability properties and metabolism will be considered briefly

Assay There are still many problems concerned with assay of AHF and numerous new assays have been devised The five desiderata—specificity sensitivity accuracy simplicity and ease of obtaining materials —are not to be found equally in any of the present assays

A number of coagulant factors are able to shorten the prolonged clotting time of hemophilic blood without altering the fundamental defect the block in prothrombin utilization Serum eluates from $BaSO_4$ have this effect (1) and also certain serum fractions (2) Thrombin behaves in a similar manner thrombin in trace amounts greatly shortens the partial thromboplastin time of hemophilic plasma (3) For a long time it was believed that asbestos treatment of hemophilic plasma seemingly caused unmasking of AHF activity since the clotting time became normal however it was found that such treated plasmas did not result in normalization of prothrombin consumption either *in vitro* or *in vivo* (4) Finally it was

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In addition to the need for a specific and reproducible assay procedure another requirement for successful purification of AHF is a satisfactory source Plasma contains only traces of AHF and is therefore a poor source for purification work Examination of various tissues might be fruitful if AHF could be assayed in the presence of tissue thromboplastic activity Attempts to isolate AHF from tissues with various techniques have yielded products active in the thromboplastin generation test (8 9) but the identity of the activity must be questioned

Instability Another problem is the instability of AHF that has been observed repeatedly in fractions and variably in blood and plasma We

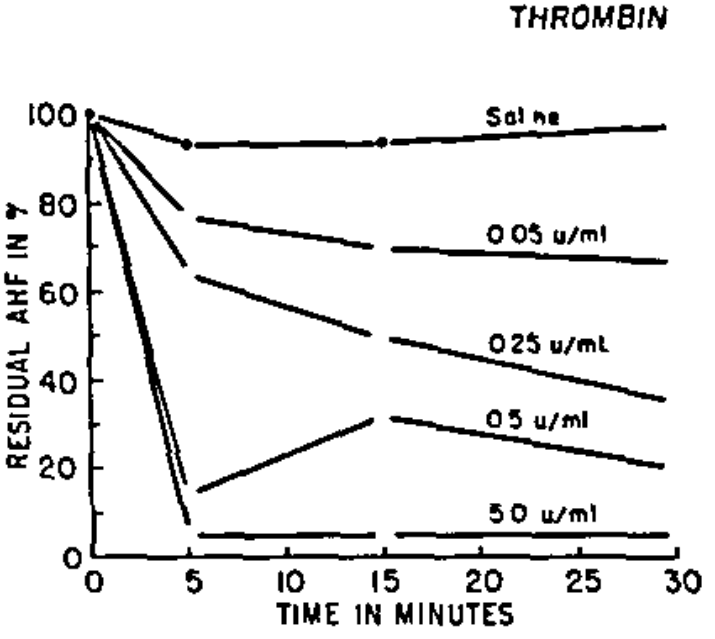


FIG 2 Effect of Purified Thrombin on AHF in $BaSO_4$ treated Plasma Effect of various concentrations of thrombin on disappearance of AHF activity in the presence of calcium ions Temp 28 C siliconе treated tubes

have studied two enzyme preparations fibrinolysin (10) and thrombin that when present in only trace amounts will inactivate AHF In Fig 1 is shown an experiment in which fibrinolysin at a relatively low concentration 0 8 Loomis units per ml was found to inactivate 70% of the AHF in one minute An experiment by one of us is shown in Fig 2 (11) As little as 0 5 Iowa units of highly purified thrombin per ml of plasma caused nearly complete inactivation of AHF in 10 minutes The presence of fibrinogen another substrate for thrombin did not alter this inactivation phenomenon These and other experiments (6 12) have led to the suggestion that incipient thrombin formation and traces of fibrinolysin are two causes of AHF instability (13) Perhaps related to this is the marked lability of AHF in sodium oxalate (14 15) The recent report of Mustard

found in 1958 that Hageman factor preparations have a shortening effect on the partial thromboplastin times of hemophilic plasma (5)

AHF is not adsorbed onto $BaSO_4$ (6) and it is only poorly adsorbed by aluminum hydroxide (7) Therefore a preliminary adsorption of plasma with a suitable adsorbant can remove many interfering coagulant factors before assay Unfortunately Hageman factor is not removed by $BaSO_4$ and its possible effect on AHF assays has not yet been resolved

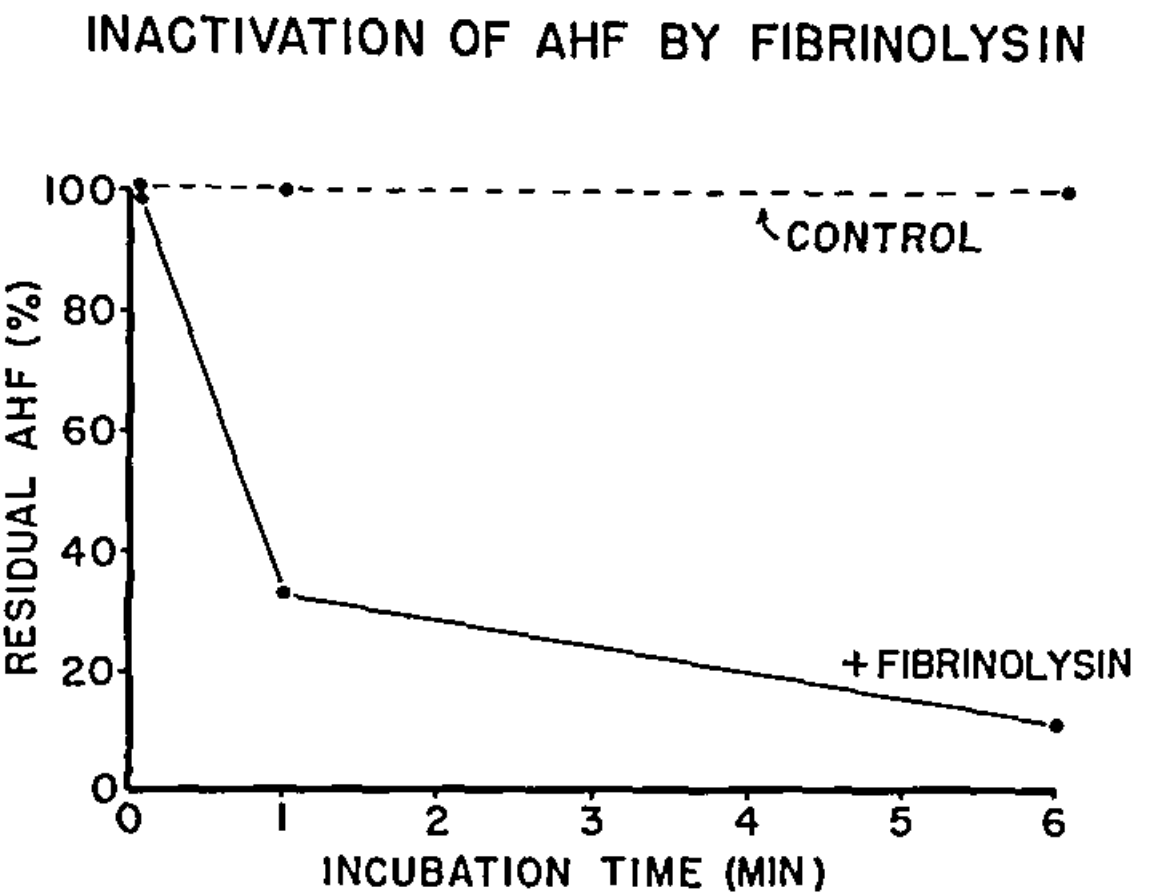


FIG 1 Effect of Fibrinolysin on Activity of AHF Purified Canine AHF preparation 3 6 ml was mixed with 0 4 ml bovine fibrinolysin Final fibrinolysin conc 0 8 Loomis units per ml Control 3 6 ml AHF solution mixed with 0 4 ml saline solution 0 02 M with sodium oxalate Mixtures let stand at 37°C At intervals samples were withdrawn and frozen at −25 C Diluent added to samples before thawing samples were then thawed diluted further and assayed for AHF activity

Correction of the hemostatic defect in hemophilic subjects when given *in vivo* as well as correction of the basic clotting defect of hemophilic blood or plasma *in vitro* appears necessary for positive identification of AHF It is obviously difficult to carry out both *in vitro* and *in vivo* tests regularly As a minimum we believe that hemophilic plasma should be used as a substrate with appropriate *in vivo* checks to establish the validity of the assay

and Steele (21) using the method of Walton and Pennell (22) selectively precipitate fibrinogen with dextran sulfate but this is admittedly a difficultly reproducible procedure The Blombacks have selectively precipitated an AHF fraction low in fibrinogen giving a good human plasma fraction (23) Bidwell has done outstanding work in purifying animal AHF (24) We have been working with a simple selective precipitation of AHF using amino acids with considerable success Our greatest purification of AHF has been obtained by selective removal of fibrinogen and by use of salting out procedures The highest degree of purification obtained with porcine plasma has been 800 times in terms of protein nitrogen and 2000 times for canine plasma (25) On the basis of these findings it is estimated that AHF contributes less than 3 to 5 mg protein per 100 ml of plasma

Properties of AHF Next we should like to consider some properties of AHF in plasma and in partially purified preparations Electrophoretic studies have been reported that suggest mobility with either the beta or alpha globulins Our own studies on electrophoresis have been hampered by three obstacles—low solubility of AHF so that with standard procedures AHF was below the level of detection irreversible adsorption of isolated AHF onto the paper when this technique was used and considerable protein protein interaction (25) Work on continuous curtain electrophoresis of plasma has shown AHF activity in pre albumins and alpha 1 and alpha 2 globulins (26 27) This procedure surmounts largely the irreversible adsorption troubles which one encounters with ordinary paper electrophoresis Bernfeld and Stefanini have reported that hemophilic plasma is different electrophoretically from normal plasma (28) We have been unable to confirm this with a number of studies on hemophilic dogs and other workers have been unable to confirm this with hemophilic human patients We reported in centrifugation experiments with plasma that AHF sediments much more rapidly than does fibrinogen in plasma (25) Work with Mr Thelin has not shown this rapid sedimentation with purified preparations (29)

Problems of Function The exact function of AHF in clotting is still uncertain This procoagulant appears essential for a fully effective hemostatic mechanism Also AHF is essential in the test tube for rapid and complete conversion of prothrombin to thrombin This has been explained as a need for AHF in the elaboration of a prothrombinase which leads to conversion of prothrombin to thrombin (30) Other theories abound as to the biochemical reactions in which AHF participates to bring about prothrombin conversion but definitive data are still needed The role of AHF in hemostasis is likewise uncertain It has been suggested that the hemostatic action of AHF is mediated through the platelet agglutinating mechanism This is a particularly attractive and simple hypothesis A thrombocyte agglutinating factor or TAg in normal canine plasma has been described recently In the presence of magnesium ion TAg agglu

concerning lability of AHF in plasma collected in EDTA is also noteworthy (16)

Methods of Stabilizing AHF Blood collected carefully in sodium citrate maintains most of its AHF for many days at 4°C (13) Adsorption of plasma with $BaSO_4$ or a similar adsorbant stabilizes the AHF This is shown in Fig 3 (11) In this experiment Ca^{++} and crude cephalin were added to $BaSO_4$ adsorbed plasma and the titer of AHF remained unaltered When an eluate from the $BaSO_4$ was added back consumption of AHF

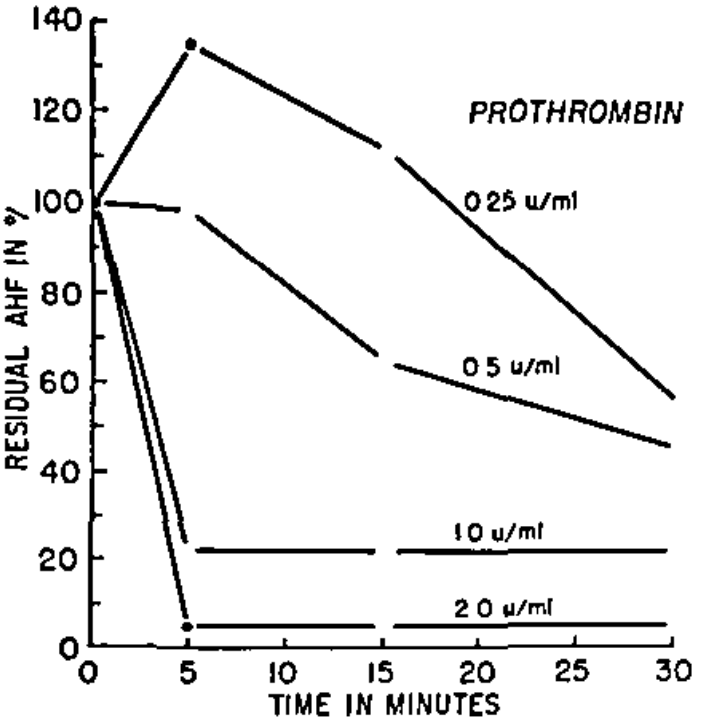


FIG 3 Effect of Purified Prothrombin on AHF Activity of $BaSO_4$ treated plasma Effect of various concentrations of purified prothrombin on disappearance of AHF activity in the presence of cephalin preparations and calcium ions Temp 28°C silicone treated tubes

occurred In a similar manner and for probably similar reasons AHF disappears from native platelet poor plasma more slowly than from native plasma with a full complement of platelets (17) It has been stressed that the stability of the AHF in fraction I is improved if the AHF is separated promptly from the bulk of the fraction (18) Fantl has reported that AHF is stable if the pH is maintained at 6 4 (19 20) These procedures with the possible exception of the last one appear to be effective by preventing enzymatic destruction of AHF rather than by prevention of physical denaturation

Purification Another problem is that of removing fibrinogen This removal is necessary in order to obtain a material that can be dissolved in a very small volume to give a highly concentrated AHF fraction Surgenor

rate of fall off of AHF was then determined The half life now appears to be in the order of 24 hours Fig 5 shows another type of experiment Purified AHF was injected into the subcutaneous or intramuscular tissues of hemophilic subjects Assayable levels of AHF promptly appeared in the plasma These findings again suggest that a rapid exchange of AHF occurred

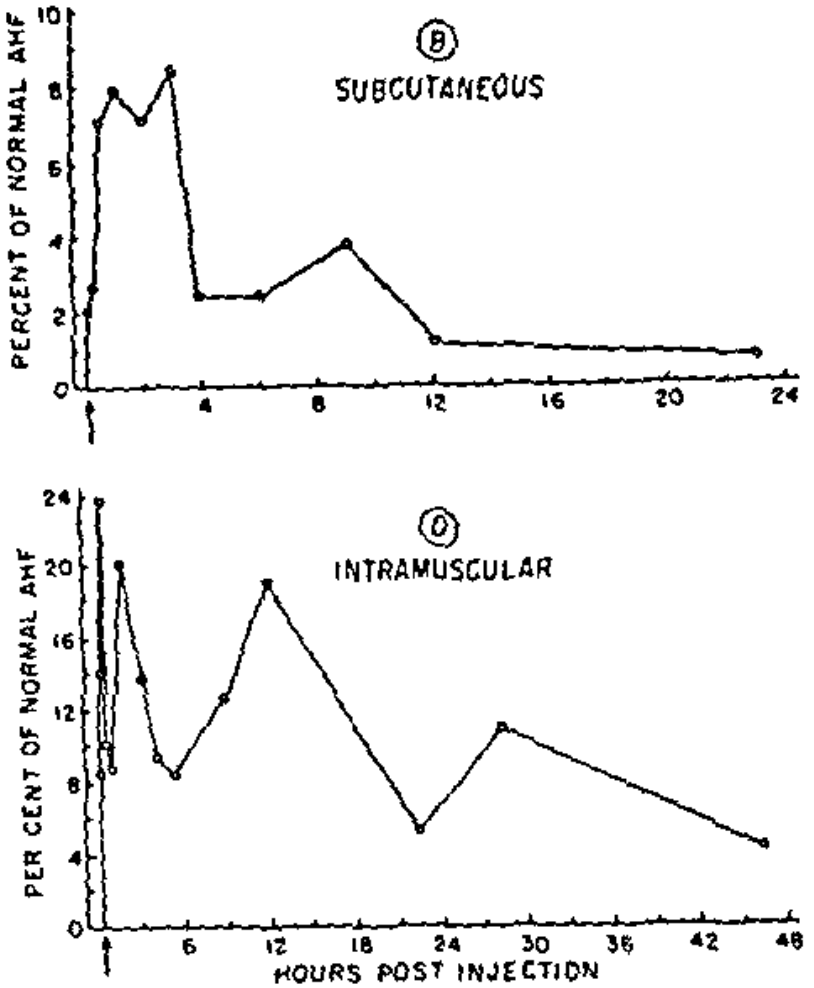


FIG 5 Plasma AHF Levels Following Injections of AHF Preparations (B) Subcutaneous 11 9 kg female hemophilic dog was given 25 ml AHF preparation 19 times the activity of dog plasma in 4 equal doses each thigh Inj time 4 min (D) Intramuscular 13 2 kg female hemophilic dog given 62 ml AHF preparation 15 times the activity of dog plasma intramusc Inj time 11 min

between the vascular and extra vascular compartments These findings also suggest that a significant tissue source of AHF may exist for purification work

Conclusions

It is obvious from the account presented here that there are certain discrepancies AHF sediments more rapidly than fibrinogen in plasma but not in relatively purified form In comparison to fibrinogen and gamma

tinates platelets in a matter of seconds In the tests used hemophilic plasma had as much TAg activity as normal plasma either with normal platelets or with hemophilic platelets (31) In this regard it is of interest that hemophilic dogs were relatively refractory to the thrombocytopenic effect and other coagulant effects of thromboplastin (32) Astrup and Sjölin (33) have suggested that the low thromboplastic content of synovial and joint capsular tissues combined with a low plasma thromboplastic potency is responsible for the characteristic localization of hemophilic hemorrhages

The site of formation of AHF is still undetermined A recent survey of autopsy tissues of human hemophiliacs revealed a normal complement of plasma cells in the lamina propria of the small intestine (34) This is in

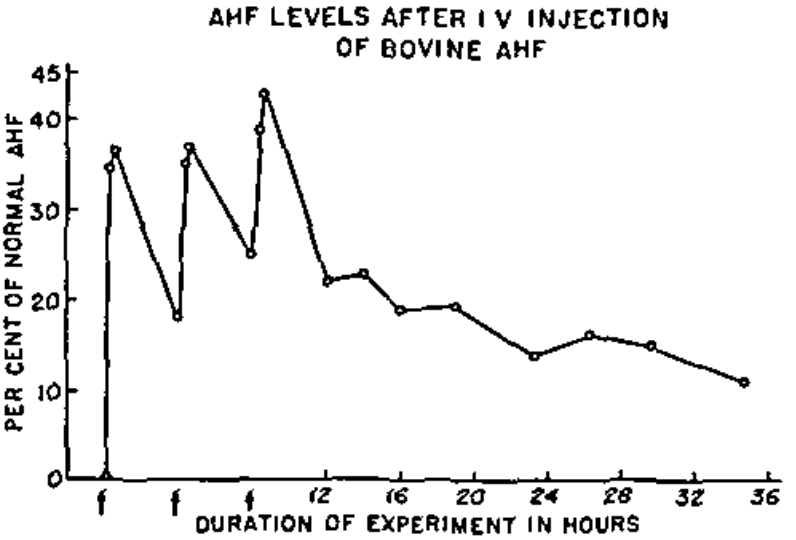


FIG 4 AHF Levels Following Repeated Closely Spaced Intravenous injections of AHF fractions At zero time 12 6 kg female hemophilic dog was given a priming dose of 58 ml AHF in solution 4 times as active as normal dog plasma Twenty nine ml doses injected at 4 and 8 hr respectively

contrast to the findings in patients with agammaglobulinemia Contrary to earlier studies from our laboratory Penick and associates have shown that AHF is moderately depressed with severe damage to the liver by hepato toxins (32) Whether this depression is due to increased utilization of AHF in the presence of necrotic tissue or is indicative of impaired manu facture of AHF by the damaged liver is still uncertain

It has been found that there is a rapid loss of AHF from the circulation of both normal and hemophilic subjects following injection of AHF into the circulation (35 36) To what extent this rapid loss of AHF is due to metabolic utilization and how much represents loss to extravascular spaces has been investigated further by two types of experiments First as shown in Fig 4 repeated injections of AHF were given to a hemophilic dog in order to maintain its plasma AHF above the 20% level for 12 hours The

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globulin the rate of equilibration of AHF between plasma and tissue compartments appears to be especially rapid The apparent half life is very short Is AHF merely more rapidly utilized than other proteins or is it a small molecule occurring in the plasma as a complex with a large protein entity?

We must conclude that many of the important questions regarding AHF remain to be answered Where is it synthesized in the body what is its precise mode of action and what is the nature of the genetic defect causing the disease?

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2

Further Studies on Antihemophilic Globulin and Related Substances

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AS IS known fraction 1 of Cohn has been used in the treatment of hemophilia A patients Some authors for instance Professor Marotta of Italy have reported good results Many difficulties however have been met with by several authors The yield of antihemophilic activity in fraction I has often been found to be extremely low Furthermore due to contamination with prothrombin and plasmin fraction I is rather instable

By extracting human fraction I with an aqueous mixture of glycine citrate and ethanol at pH 6 we could remove prothrombin and other contaminating proteins (Fig 1) We thus obtained a fibrinogen residue denoted fraction I O which was stable in solution (1) In addition to fibrinogen it contained almost quantitatively the antihemophilic activity (AHG) of plasma It was therefore administered to hemophilia A patients and to patients with von Willebrand s disease (2 4)

It was found that the fraction did not only increase the AHG level in both types of patients but also corrected the prolonged bleeding time in the latter Further results in these patients will be reported by Dr Nilsson This and the following observations made us suggest that fraction I O in addition to fibrinogen and AHG contained a plasma factor correcting the prolonged bleeding time in von Willebrand s disease

As to the yield of AHG in fraction I O it was sometimes found to be low The variations have been found to depend mainly on the following

(cf 2 5) (1) the blood collection procedure (2) the rate of the addition of ethanol in precipitating fraction I (3) the pH of the glycine solution used for the extractions and (4) the adsorption of AHG to the sterile filters used As to the blood collection procedure silicone technique as well as magnetic stirring were introduced in order to avoid incipient clot ting As to the precipitation of fraction I the addition of ethanol had to be rapid otherwise the AHG did not quantitatively precipitate together with the fibrinogen in fraction I The explanation of this phenomenon we do not know The optimum pH of the extraction mixture for obtaining a good yield of AHG in fraction I O was found to be pH 6 8 and pH was accordingly changed from pH 6 0 which was originally used to pH 6 8 (5)

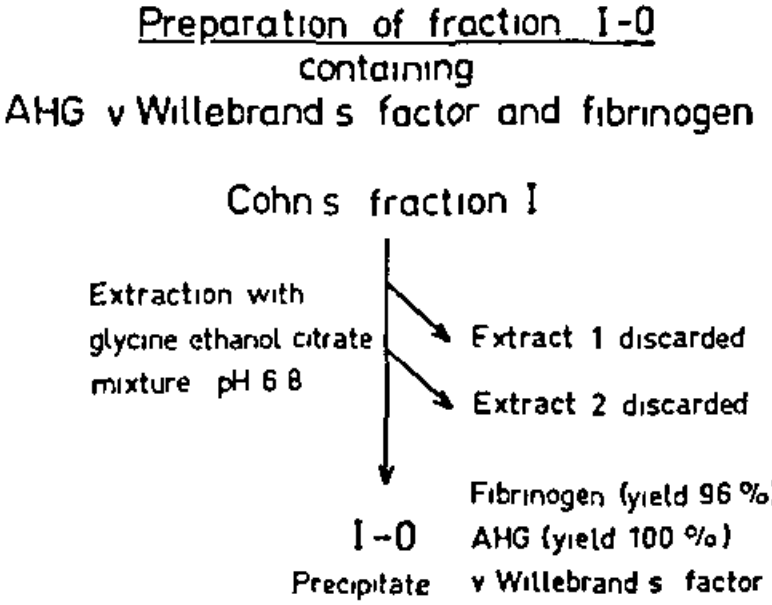


FIG 1

Already at an early stage the losses of AHG at sterile filtrations were avoided by introducing a sterile or aseptic technique similar to that of Dr Kekwick In addition to these findings some other conditions were also found to be of importance for instance the rate at which the AHG samples were frozen before freeze drying Slow freezing at low temperatures (between −5 to −15°C) gave a considerable loss of AHG activity (5)

As mentioned fraction I O has been administered to hemophilia A patients among others A recalcification method using hemophilia A plasma has been used to measure the activity *in vitro* and *in vivo* A fact emphasiz ing the advantage of this method is that it has been possible with its use to demonstrate a low AHG content (20 60% of normal) in the plasma of 30 definite carriers of hemophilia A before the menopause (This work has been performed in co operation with A Thilén and I von Francken Stockholm)

One dose of fraction I O has been prepared from eight donors i e from about 1400 ml of fresh normal human plasma and it contains the AHG activity of between 1100 and 1600 ml of normal human plasma and about 3 gm of protein It has generally been administered in a total volume of 200 ml

Fraction I O has been given in all 79 times to 14 hemophilia A patients The patients were treated in connection with bleeding episodes such as gastrointestinal bleedings hematuria and joint bleedings as well as in connection with surgical procedures such as joint bleeding punctures and tooth extractions An appendectomy (2) and an operation for nephro-

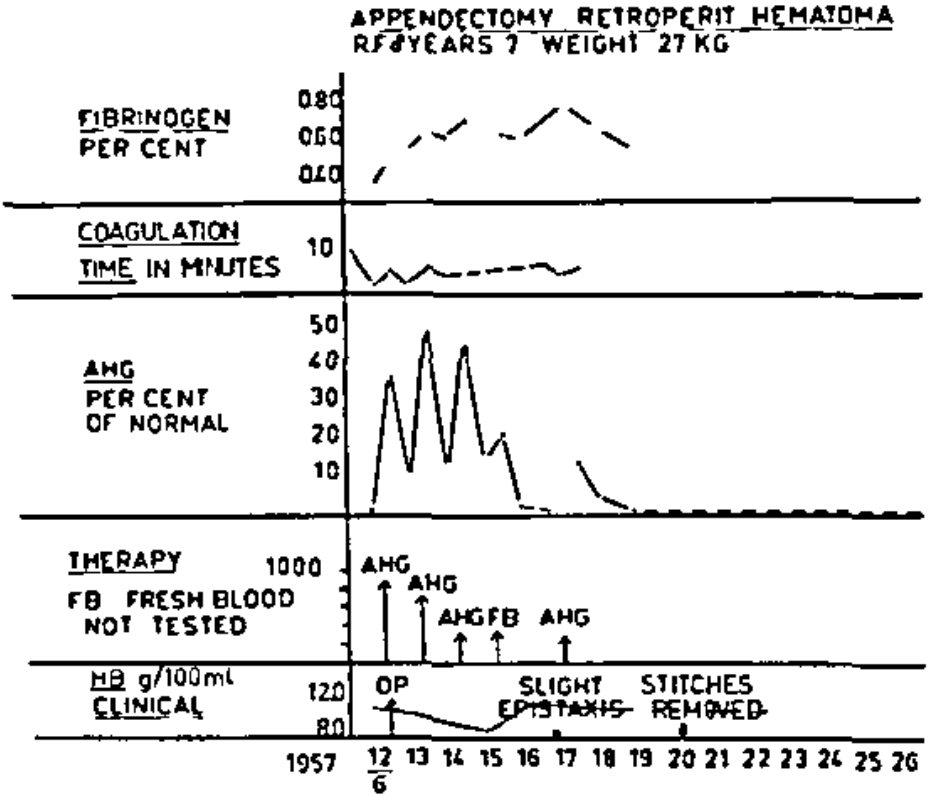


FIG 2 Use of Fraction I O

lithiasis have been performed under cover of fraction I O The bleedings were arrested and the surgical procedures could be performed without any increased bleeding tendency One boy has by now received the fraction 38 times No side reactions have been seen and no resistance has de veloped

As has been pointed out by several authors it was found suitable to increase the plasma AHG of the patient to 40-80% of normal before operation and then maintain the AHG level at about 20 30% during the time of healing This was possible to achieve in an adult by giving one dose of fraction I O just before operation one dose on the next day and 3-4 doses during the subsequent postoperative course until the stitches had been removed

The appendectomy was performed under cover of fraction I O essentially according to the above mentioned scheme (Fig 2) This was also the case in the operation for nephrolithiasis It is recommended that the last dose be given immediately before the removal of the stitches

Finally we would like to mention something about the further purification of AHG in fraction I O (Fig 3) From fraction I O in solution a fraction denoted I 1 A containing most of the AHG activity of fraction I O can be precipitated at low ionic strength in the presence of glycine

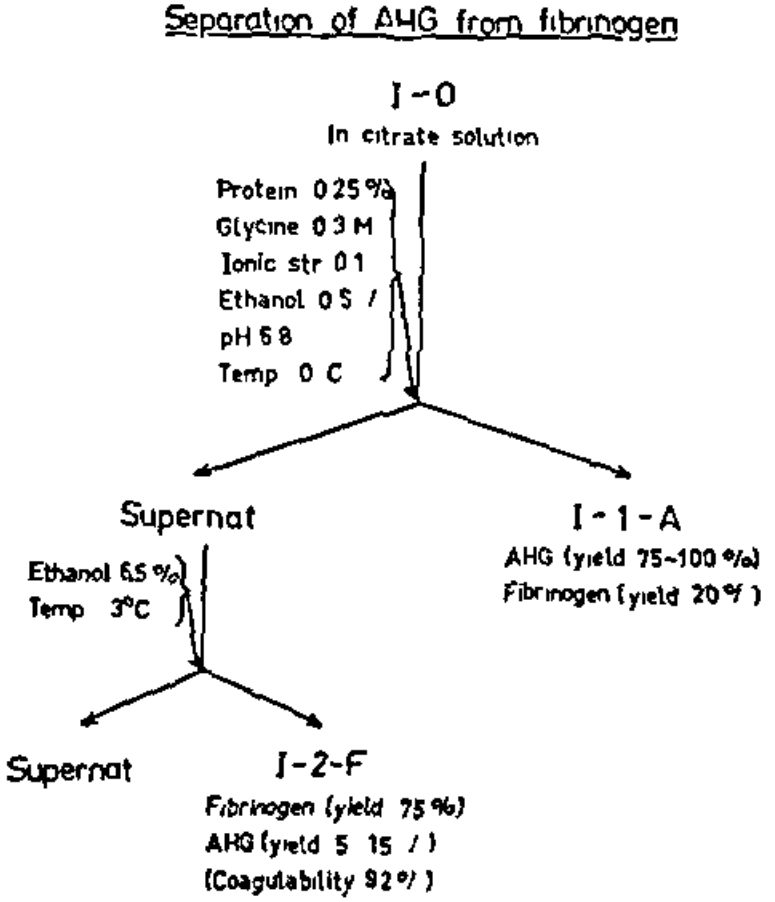


FIG 3

and small amounts of ethanol (5) The fibrinogen can then be recovered from the supernate of the AHG fraction by increasing the ethanol concentration The AHG activity in fraction I 1 A is 200 times that of plasma per mg protein i e about 4 5 times that in fraction I O The yield of AHG is about 80% of that in fraction I O

In order to verify the results obtained *in vitro* this separated AHG fraction has been given once to a hemophilia A patient (*Fig* 4) This figure shows the result in the hemophilic boy mentioned above who has by now been treated 38 times with fraction I O The protein (0 7 gm) in the fraction increased his AHG to about 90% of normal A dose of the unseparated fraction I O with good activity containing 2 gm of protein

increased his AHG to 50 60% of normal The injection of an earlier batch of fraction I O containing about 4 gm of protein in which the AHG activity was low *in vitro* did not increase his AHG to more than half of this content A response equally as good as in the hemophilic boy was obtained by injecting fraction I 1 A into a von Willebrand patient but no correction of BT was seen

It can also be mentioned that it has been possible to purify by a simple method the AHG of fraction I 1 A another ten times

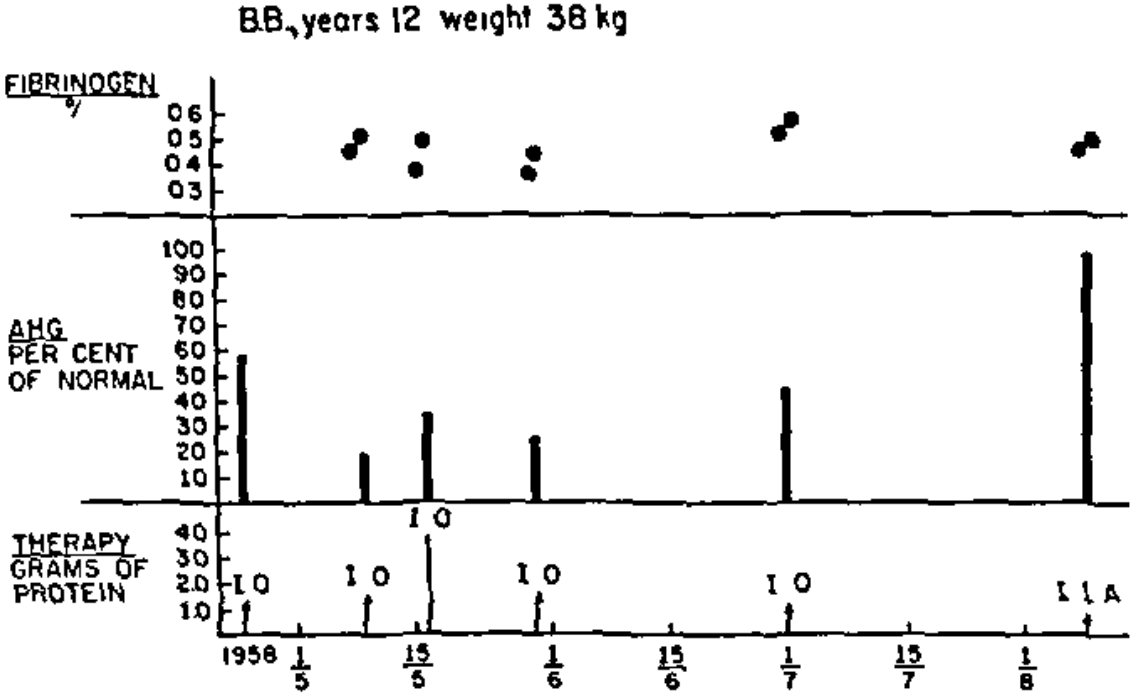


FIG 4 Use of Fractions I O and I 1 A

In summary it has been possible to separate the AHG from the bulk of fibrinogen with good yield of AHG in fraction I 1 A and a good yield of fibrinogen in the fibrinogen fraction I 2 F The bleeding time correcting factor lacking in von Willebrand's disease is found in fraction I O but cannot be demonstrated in the separated AHG or fibrinogen fractions

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B The Antihemophilic Factor Management of Hemophilic Hemorrhages

3

The Treatment of Haemophilic Patients with Animal Antihaemophilic Globulin

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THERE are conflicting theories on the basic defect in haemophilia but one fact seems to be established A fraction of normal human beef or pig plasma will correct the clotting defect of haemophilic blood *in vitro* and will establish normal haemostasis for a time after its injection *in vivo* This fact is usually taken as evidence that normal plasma contains an essential clotting factor which is deficient in haemophilic blood this has been called antihaemophilic factor (AHF) Factor VIII or less specifically antihaemophilic globulin (AHG) It may be that there is a deficient production of such a factor in haemophilia or over production of an inhibitor or that AHG works for some quite unexpected reason the important fact is that it works as a therapeutic agent

Recognition of the therapeutic importance of AHG has been slow mainly becau e it was not realized that while very small amounts were sufficient to correct the clotting defect of haemophilic blood *in vitro* very large amounts were needed to correct the haemostatic defect in the patient About 1% of the normal blood level of AHG is often enough to make the clotting time normal but over 30% may be needed for efficient haemo stasis and this is difficult to achieve in practice because of the rapid loss of AHG from the circulation In different patients at different times it may be necessary to give AHG equivalent to between 2 10 l of plasma to obtain this level It is not surprising therefore that many attempts to control bleeding by blood or plasma transfusions or by the use of AHG preparations of doubtful potency and in inadequate amounts should have been unsuccessful

In order to achieve therapeutic levels of AHG in a severely affected haemophilic patient concentrated AHG is essential Without overloading the patient s circulation it is impossible to transfuse several litres of plasma at the rate needed to reach the necessary AHG level against con tinuous and rapid loss But the general use of concentrated human AHG raises the problem of supply Its preparation involves loss of activity and even if it were used for emergencies only the already over taxed transfusion services would find it difficult to provide the fresh blood required There is no prospect of there ever being enough human blood to provide con tinuous prophylactic treatment with AHG for the haemophilic population On the other hand the supply of ox and pig blood is almost unlimited and both happen to have 10 15 times the antihaemophilic activity of human blood Bidwell (1 2) has produced ox and pig AHG which is up to 8000 times as active as the same weight of fresh human plasma protein The disadvantage of animal AHG in its present form is its antigenicity This is no more of a deterrent in emergency use than is the antigenicity of animal antitoxins used in the treatment and prophylaxis of diphtheria tetanus and gas gangrene It does mean however that a particular animal AHG can be used safely for only one course of treatment lasting about 10 days and for this reason we now reserve it for serious situations or essential operations There is reason to hope that the antigenicity of these prepara tions is due to removable impurities and that at some future date a rela tively non antigenic factor may be produced

Meanwhile we consider that the use of animal AHG in a series of emergencies has in most cases proved its therapeutic effectiveness and has saved patients who almost certainly would have died had it not been available

Bleeding and the Level of AHG In untreated haemophilic patients the amount of AHG in the blood varies All severely affected patients whose symptoms include spontaneous internal bleeding massive intramuscular haematomata without sufficient cause and repeated joint haemorrhages with residual deformity have no AHG to be detected in their blood Patients who have between 1 5% of AHG are variably affected They may *have occasional haemarthroses but there is usually no permanent* joint damage they have severe bleeding after dental extraction and operations often requiring multiple transfusions but true spontaneous bleeding is rare Those with more than 5% are all mild bleeders In this category the patients have no undue haemorrhage unless quite severely injured They bleed after dental extractions and operations and may have unduly large *traumatic haematomata for example following football injuries* A study of these patients suggests that the AHG level is the primary factor deter mining the haemorrhagic state though other as yet unidentified factors also influence the expression of the disease Thus different affected members of a family may require hospital treatment with variable frequency Some can

undertake quite heavy work and act as family wage earners whereas others remain permanently unemployable from repeated disabling haemorrhages Little is known about the cause of these differences

The Objectives of Therapy The objectives of therapy are to arrest spontaneous bleeding and to restrict the blood lost after injury to that expected in normal people At the outset it must be admitted that it is not always easy to know when these objectives have been achieved Haematuria for example may cease during therapy but this does not necessarily mean that the therapy has caused the cessation Again clinical experience with untreated haemophilic patients has led physicians to expect the worst following dental extraction and operation Thus a patient who is free of bleeding in a week or two following the extraction of 4 or 5 teeth and who in this time has not required more than one or two transfusions may be regarded as a satisfactorily treated case a case which has given rise to no trouble But of course such a patient s bleeding has been far in excess of the normal an extraction of 4 5 teeth from an entirely normal person often gives rise to bleeding only for a matter of minutes Similarly clean surgical incisions should in normal people heal by primary union in 7 10 days with no haematoma formation It has always been our policy to view the success or failure of treatment in haemophilic patients in terms of the bleeding to be expected in normal people rather than from the point of view of the improvement over the expected haemophilic bleeding For testing a therapeutic material we have also never relied on the apparent effect on spontaneous bleeding but only on the control of traumatic bleeding Mildly affected haemophilic patients (those with more than 5% of AHG in their blood) do not have spontaneous bleeding and it is our experience that these spontaneous haemorrhages in severely affected patients are often controlled much more easily than traumatic bleeding It appears that AHG levels of 5 10% may suffice to prevent or arrest such bleeding whereas patients with 25% of AHG may have persistent traumatic bleeding and levels above this are required to prevent or stop it Thus the effectiveness against traumatic bleeding is the best test of a new material

We have now treated many patients for dental extraction with transfusions of fresh frozen plasma The plasma is separated from the whole blood by centrifuging within an hour or two of collection It is frozen immediately at −20° C until required Such plasma does not always maintain its AHG activity but if used within a week of collection it is usually satisfactory We customarily give 1000 ml of this plasma to an adult patient during the 45 min immediately preceding the planned extraction This treatment raises the AHG blood level to between 10 and 25% of normal and is repeated as often as is thought necessary In our experience such treatment very seldom reduces bleeding to the normal level Even with local protection by acrylic splints it is rare for a severely

affected haemophilic patient to have more than 3 teeth removed at one time without quite troublesome bleeding often necessitating transfusion The bleeding is much less than in the untreated patient but it is nevertheless excessive Plasma infusions are however quite often satisfactory for controlling spontaneous bleeding such as haematuria or effusions into the tongue or throat causing respiratory obstruction

The Use of Animal AHG It is our opinion that unconcentrated AHG is practically never satisfactory for the prevention or treatment of traumatic haemorrhage Concentrated AHG may be derived from human (3) or animal blood and in this context we shall discuss only the material derived from beef and pig blood by the process of Bidwell (1, 2)

In early studies the material was used for 3 volunteers requiring dental extractions and in two the bleeding was reduced to that of normal people (4) The third patient developed transient thrombocytopenic bleeding and it was found that the beef AHG used had reduced his platelets and in this instance it took 3 weeks for the platelet count to be restored to normal In treating a further 14 cases we have always found a transient thrombocytopenia using beef AHG but we have not again seen thrombocytopenic bleeding The pig AHG does not cause thrombocytopenia

In treating fourteen major operations in ten haemophilic patients many new problems and difficulties have naturally been encountered and the degree of success (viewed in terms of normal haemostasis) has been variable

Two patients were given animal AHG for the treatment of head injuries one injury which was caused by a motorcycle accident (5 case 12) had an extensive scalp laceration and haematoma but no intracerebral damage The second patient was injured in a car accident he had a scalp haematoma and also a diffuse intracerebral swelling presumably due to numerous small haemorrhages causing hemiplegia The first patient was treated by simple evacuation of the scalp haematoma and suture of the laceration The second patient had a craniotomy in an attempt to find a localized blood clot but none was found Both patients showed steady uninterrupted healing with no bleeding or bruising In the first of these patients blood AHG levels above 25% were achieved daily for 6 days by which time the wound appeared nearly healed In the second patient levels above 25% were achieved daily for 7 days and thereafter levels from 14 30% were achieved for a further 7 days The success in these two cases was probably due to the adequate daily rise in AHG level and to the site of injury We have found that complete immobilization of the injured site greatly facilitates healing and this is easily achieved in the case of head injuries

Two patients had abdominal operations one a colostomy for the relief of intestinal obstruction and the second patient had two polypi removed from his colon Neither had any excessive bleeding and the abdominal

wounds healed by first intention Levels of AHG above 25% were achieved daily in each case until healing was advanced

Three patients had amputation of the leg Two of these were operated on for massive cystic haematomata of the thigh muscles and one for osteogenic sarcoma of the tibia All three patients died but not from bleeding The patient with the osteogenic sarcoma died of secondary deposits One patient with a cyst of the thigh died from anuria following profound circulatory collapse during operation The third patient (5 case 11) was 59 at the time of his first operation he acquired a serious postoperative infection and had in all 4 major operations including disarticulation through the hip joint This last patient bled when a ligature was dislodged from his femoral artery In neither of the other two cases was abnormal haemorrhage encountered

One patient (5 case 9) had a laminectomy involving 12 vertebrae this patient had some episodes of bleeding and a wound haematoma developed Apart from the massive extent of the wound there were two obvious causes of bleeding One was that the AHG level was allowed to fall to inadequate haemostatic levels between the sixth and eighth days and during the first 3 weeks of treatment no attempt was made to immobilize the wound

One patient had a radical haemorrhoidectomy having suffered incapacitatingly frequent episodes of rectal bleeding This patient had no excessive post-operative bleeding for the first five days when the AHG level was raised to well above 25% every day using beef AHG From the sixth to the eighth day he bled not seriously but enough to require transfusion and during this time the blood AHG level remained at less than 5% Thereafter the dose of AHG was greatly increased and higher levels were obtained for 4 days bleeding ceased and healing was complete

The tenth case has been reported in great detail previously (5 6) This patient s history records recurrent bleeding from an extensive gunshot wound when high daily AHG levels had not been achieved and satisfactory healing with no bleeding after the excision of an arteriovenous aneurism when satisfactory AHG levels (above 25%) were achieved daily

The Causes of Haemostatic Failure Experience with these patients has led us to believe that if the AHG level is raised to above 25% of normal once daily for the duration of the healing process and if the wound can be immobilized reasonably efficiently healing in haemophilic patients can be normal and uncomplicated by abnormal bleeding Immobilization is necessary because with intermittent daily treatment the level is not maintained and the patient s haemostatic mechanism is abnormal for much of the day It may seem surprising that given a reasonably concentrated AHG preparation this rather modest objective has not been achieved with certainty in every case The reason for this is that haemophilic patients

differ very much one from another in their reaction to AHG treatment or the same patient reacts differently at different stages of treatment More over the amount of AHG required may be very large and unless this is anticipated adequate stocks of material may not be available These difficulties may be illustrated from one or two cases

For example the case of the patient (*5* case *11*) who had 4 operations for a cystic haematoma of the thigh the last of which was a disarticulation through the hip was very unusual Amounts of AHG equivalent to 1000 ml of fresh human plasma were sufficient to raise his AHG level to 70% of normal Apart from massive arterial bleeding haemorrhage was never a problem in this patient Had this patient been encountered early in our study of haemostasis in haemophilic *patients* a *very unrepresentative* picture of future difficulties would have been visualized For all we know there may be parts of the world where this type of haemophilic patient is the rule rather than the exception In such areas the treatment of haemophilia might be very easy a pint or two of blood sufficing to arrest haemorrhage following major surgery

The majority of our cases have required much more AHG than this patient Macfarlane and co workers (5) suggested that single doses equivalent to 3500 4700 ml of fresh plasma might be required and this opinion has been supported by more recent studies Moreover although such doses may be adequate initially the patient may suddenly become resistant to treatment and the dose requirement may suddenly increase enormously For example a recent patient was well controlled on doses equivalent to between 4 6000 ml of plasma when suddenly his requirements increased fourfold It took two days to acquire a sufficient supply of AHG to adjust the dose and during this time the patient bled In all this patient received animal AHG equivalent to about 150 l of fresh human plasma

Add to these experiences the patient who had thrombocytopenic bleeding following treatment and another who had a long bleeding time and positive tourniquet test before treatment was started and it will be seen that the patients at present classified as having haemophilia (who all have marked AHG deficiency) do not form a uniform group Failure to realize this fact together with failure to appreciate the importance of immobilization have been the two causes of such bleeding as has occurred in our cases

The Selection of Cases for Treatment with Animal AHG With the exception of the three initial volunteers who had animal AHG as an untried treatment for dental extraction we have used this material *only* for seriously injured patients or those requiring a life saving operation We think that at present it should be reserved for such cases

Some haemophilic patients develop circulating anticoagulants which destroy AHG These anticoagulants probably differ aetiologically from the states of resistance to AHG which not infrequently follow transfusion or *AHG therapy* These circulating anticoagulants have been studied by

many workers including Biggs and Bidwell (7) who suggested a quantitative method for their measurement based on the speed with which the anticoagulant neutralizes AHG An inhibitor of potency such that 75% of added AHG is destroyed in 1 hour *in vitro* can sometimes be neutralized and overcome by an excess of AHG given *in vivo* Inhibitors of greater strength may be impossible to neutralize and surgery in such patients may be impracticable

Regimen for Treatment When it is proposed to treat previously diagnosed and severely affected adult haemophilic patients for an acute surgical emergency the first necessity is an adequate supply of potent animal AHG The maximum requirements are about 20 000 units of beef AHG and 20 000 units of pig AHG each unit being roughly equivalent to 4 ml of fresh human plasma This material is at present supplied commercially in ampoules each containing 200 units or the equivalent of about 800 ml plasma An initial trial dose of 4 ampoules of this material (or proportionately more if the potency is less) is given to the patient two hours before the operation is planned The infusion is given by intravenous drip and the contents of the ampoules are diluted to about 200 ml with 0 85% NaCl After treatment a sample of blood is tested by a reliable method for measuring AHG If the level achieved is over 50 % no further treatment will be required on that day If a level of between 25 50% is achieved a further dose of 2 ampoules is given during the operation If the level is 20 25% a further 4 ampoules are given pre-operatively If the level is much below 20 it is probable that the patient has an anticoagulant and major surgery may have to be avoided The dose to be given daily depends on the levels achieved and it must be remembered that at any stage resistance may develop and necessitate a three or four fold increase in the dose Treatment with one antigen (beef or pig AHG) can be continued for 7 10 days Thereafter if more treatment is required the preparation from the second animal source must be used

Reactions In only one case has a serious reaction to animal AHG occurred This was an anaphylactic type reaction with broncho-spasm and hypotension which took place immediately after the first injection of ox AHG in a patient who unknown to us had previously received injection of a proprietary haemostatic derived from ox platelets (6) In a few cases there were mild pyrexial reactions Towards the end of a course of treatment some allergic signs such as urticaria also occurred which could be controlled by antihistamine drugs or steroids but their onset usually coincided with increasing difficulty in raising the blood level of AHG No cases of serum sickness occurred There has been no indication of any worsening of the haemophilic condition following animal AHG treatment

Conclusion

We have now treated 17 patients with animal AHG ten of whom required major surgery involving 14 operations In 7 of these operations there was by *normal* standards no excessive bleeding and we do not believe that such results have been achieved without the use of concentrated AHG In the remainder there were for reasons which have been discussed some episodes of abnormal bleeding It appears however that such bleeding does not occur if the AHG level is above 30% provided that there is no other haemostatic defect

The resistance to treatment which may develop in some cases is at present unexplained It does not seem to be due to a circulating anticoagulant in the usual sense of the word

It might be thought unwise to use AHG from an animal source because this is antigenic It is true that animal AHG is antigenic but so are many other animal preparations which are or have been used in medicine such as antitoxins and gland extracts In due course it may prove possible to render AHG non antigenic In the meantime the available preparations have undoubtedly saved lives

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4

Management of Hemophilia *

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RECENTLY our insight into the deviations of the clotting mechanism in classic hemophilia or hemophilia A has greatly increased This has induced a more intensive study of the problems which arise in the effective fight against bleeding occurring in this form of hemorrhagic diathesis

At first the aim was to normalize the clotting time (CT) However this appeared not to be efficient in all cases the more so as in classic hemophilia the CT is not always prolonged Many hemophiliacs A in whom the CT is not or only slightly prolonged show clinically a different morbid picture (1 2) In the history of these mild cases of hemophilia bleeding in joints is often lacking The tendency to bleed usually does not become manifest in such cases without real trauma (extraction of teeth surgical intervention)

The present conception is that the determination of the prothrombin consumption during clotting which is decreased in hemophilia gives a more profound insight into the effect of transfusions of blood and plasma Thus this determination of prothrombin consumption appears to be a more sensitive method for judging the degree of the deviation in clotting and thus also for judging the effectiveness of treatment Nevertheless it appeared that in many cases the correction of the disturbed prothrombin consumption to normal values did not correspond to corrected hemostasis

The thromboplastin generation test (TGT) (3) enables us to make a closer examination of the clotting defect in hemophilia than formerly was

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possible Results of the TGT indicate that in hemophilic hemorrhages one should try to increase the concentration of the antihemophilic factor (AHF) to a level which will prevent the appearance of hemorrhages and will check hemorrhages already present Recently methods for the quantitative determination of AHF in the blood have been elaborated (4 7) making it possible to answer more exactly some questions concerning the treatment of patients with classic hemophilia by means of blood and plasma therapy These questions are the following

1 What is the lowest level of AHF necessary to prevent hemorrhages in hemophilia (e g in cases of operation)?

2 How can this requisite level in the blood be obtained and for how long should it be maintained in cases of hemorrhages or injuries?

3 Which preparation or preparations aside from blood and plasma have to be considered in order to maintain the AHF level?

Experience has shown that to combat hemorrhages in patients with classic hemophilia transfusions of a large quantity of fresh blood or plasma applied within a short time or repeated small transfusions are not always efficient Hemostasis can often be maintained much better by administration of the blood during a longer time and as fresh as possible in the form of a drip transfusion eventually alternated with administration of fresh plasma or AHF preparations (8) However in applying this form of therapy only rarely has the fact been considered that in hemophilic hemorrhages the AHF consumption varies greatly This can explain why in severe hemorrhages blood transfusions are often inadequate and also why recently non toxic concentrated AHF preparations have been tried In this way one should be able to combat the AHF deficiency more effectively and the treatment of the very severe hemorrhages in hemophiliacs should be more successful

It makes considerable difference whether one is dealing with a hemophiliac to be operated upon or a hemophiliac to be treated for an extensive spontaneous hemorrhage The maintenance of the AHF level during an operation is not a simple problem as becomes clear from the case described by one of us with Hoorweg and Stijn (9) But the difficulties were less in a hemophilic baby operated for pyloric hypertrophy and described by one of us with De Bruijne and Steendijk (10) In the latter case hemostasis could be maintained more easily by controlling the AHF level of the blood

Rather frequently we have to deal with hemorrhages which require rapid elevation of the AHF level of the blood and maintenance of this level for a long period This is needed to prevent serious complications such as extensive subcutaneous and intramuscular hemorrhages which by pressure on nerves or arteries may give rise to paralysis or gangrene further hemorrhages in the neck with the danger of asphyxia and cerebral hemorrhages

Recent researches have demonstrated that the disturbed TGT and also the so-called partial thromboplastin time (PTT) (11) in hemophiliacs can usually be turned to normal when the AHF level of the plasma—determined with one of the methods given by Brinkhous *et al* (4 7) *—is raised to 4 6% of the normal value The AHF content of fresh blood and of fresh plasma decreases when kept at room temperature in our experience this diminution amounts to 15 20% when the blood or plasma is kept at room temperature for 3 hours the time usually necessary for the transfusion of blood or plasma Lyophilized dried plasma has a varying AHF concentration which however is always much smaller than that in fresh plasma unless the plasma after the withdrawal from the donor has been frozen immediately This fact should be kept in mind and taken into account

If the AHF level of normal fresh blood is supposed to be 100%—in reality Brinkhous and others have demonstrated that the level may greatly vary—and the quantity of plasma circulating in the body amounts to 50 ml per kg of body weight then theoretically and neglecting the varying AHF consumption about 5 ml of fresh blood or plasma per kg of body weight should be required in order to realize the necessary minimal AHF level When such a quantity of plasma is transfused in a hemophiliac at a rate of about 100 ml per hour and the AHF level of the blood is determined at certain intervals we should schematically obtain the accompanying curve (Fig 1)

From this curve and from our further experience it follows that after such a transfusion about half of the injected AHF will have disappeared in about 3 5 hours This time nearly corresponds with the experiences of Brinkhous *et al* Here we have to make some remarks

Laboratory researches have shown that when the AHF level is 4 6% (determined after the method of Brinkhous *et al*) the TGT becomes normal so we have to maintain at least this level But we must also take into account the following factors (a) when the hemorrhage is persistent or when there is a great loss of blood within a short time the symptoms of shock will also determine the quantity and the rate of the required transfusion Under such circumstances one must start by giving blood (not plasma or an AHF preparation) and the rate of infusion should be quicker than usual When no symptoms of shock are present a slow transfusion of fresh blood or plasma is sufficient or still better an AHF preparation should be given initially It is more important that the AHF concentration should be increased and maintained for a long time at a level somewhat higher than the required minimum so that efficient hemostasis will become

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*This method of Brinkhous *et al* can be clinically used to demonstrate slight differences in AHF levels when the concentrations are small When high concentrations of AHF are present the range of variation becomes too large for demonstrating slight differences in the levels

possible (b) AHF utilization—we will not discuss the way in which AHF disappears from the blood—varies greatly between individuals as well as during periods with hemorrhages as compared to periods without bleeding

We have examined AHF in a number of children with hemophilia after transfusions of fresh blood and of fresh plasma during periods with

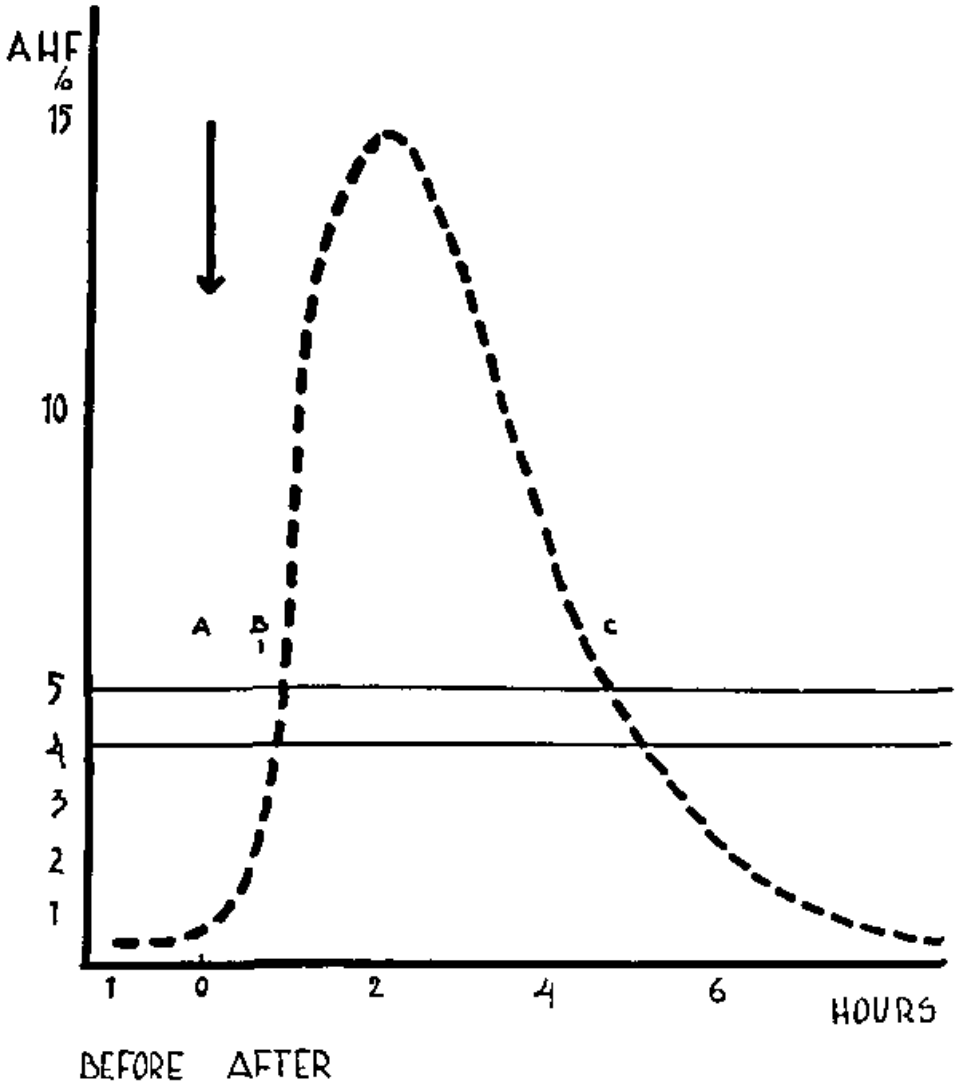


FIG 1 Theoretical Course of AHF Content of Blood After Transfusion of fresh blood or of fresh plasma in a hemophiliac A — B = rate of transfusion B — C = time after termination of transfusion during which the AHF content stays above the critical value Arrow indicates the time of the beginning of transfusion

hemorrhages and during periods when the patients did not suffer from bleeding At certain times the level of AHF of the blood of these children was determined after a carefully measured quantity of fresh blood or of fresh plasma had been administered The rate of transfusion was kept constantly at 100 ml of plasma or blood per hour The results are given in Figs 2 and 3 The patients with hemorrhages were without symptoms of

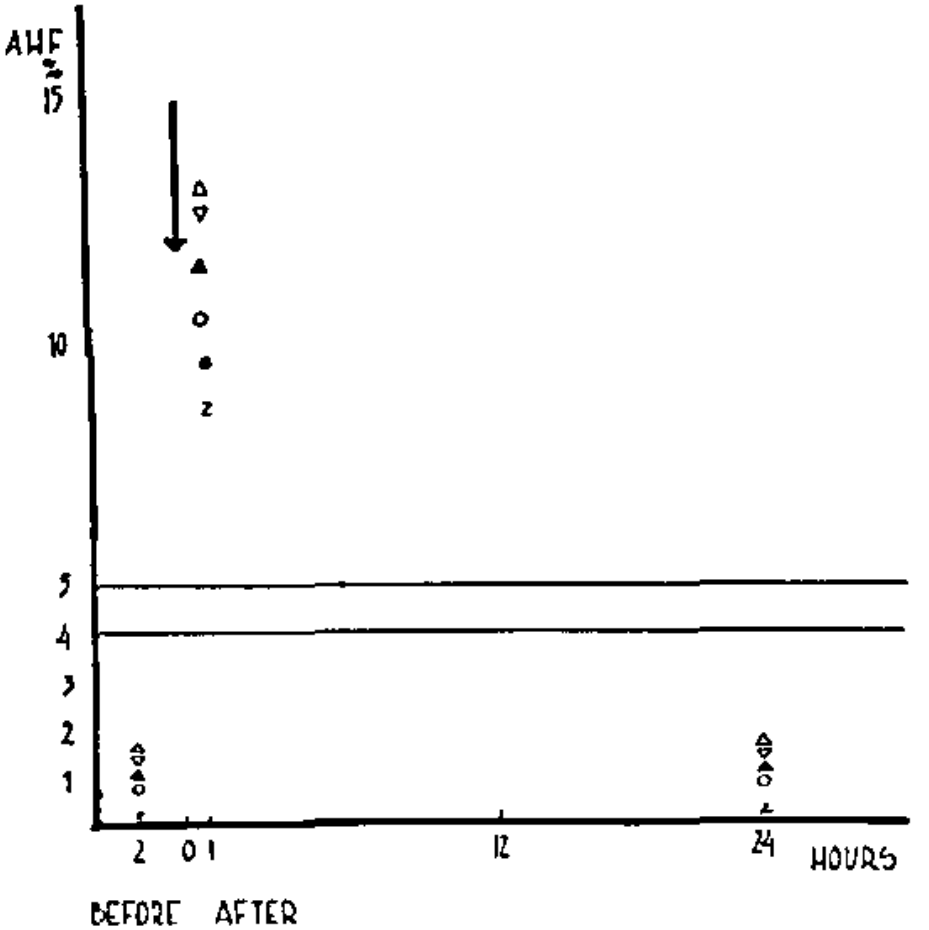


FIG 2 Influence of Transfusion of Fresh Blood and fresh plasma on AHF level after arrest of hemorrhage Arrow indicates the time of beginning of transfusion

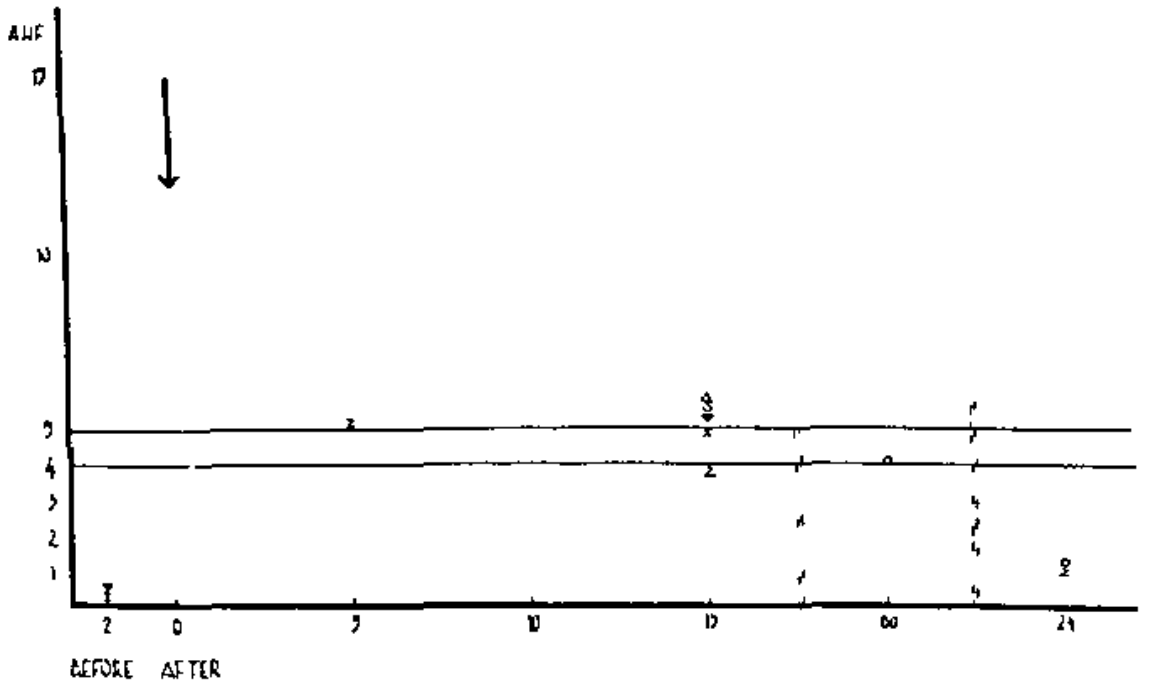


FIG 3 Influence of Transfusion of Fresh Blood and fresh plasma on AHF level during hemorrha e Arrow indicates the time of beginning of transfusion

shock they had moderately severe hemorrhages of joints of gums etc Therefore the rate of transfusions in this group of patients could be kept constant so that comparison of the results of the determination were justified to a certain degree

When severe hemorrhages were present (Fig 3) the AHF level one hour after the transfusion had decreased below the required minimum (5%) while on the other hand when no severe hemorrhages were present this level after one hour was still much higher than the required minimal value (Fig 2)

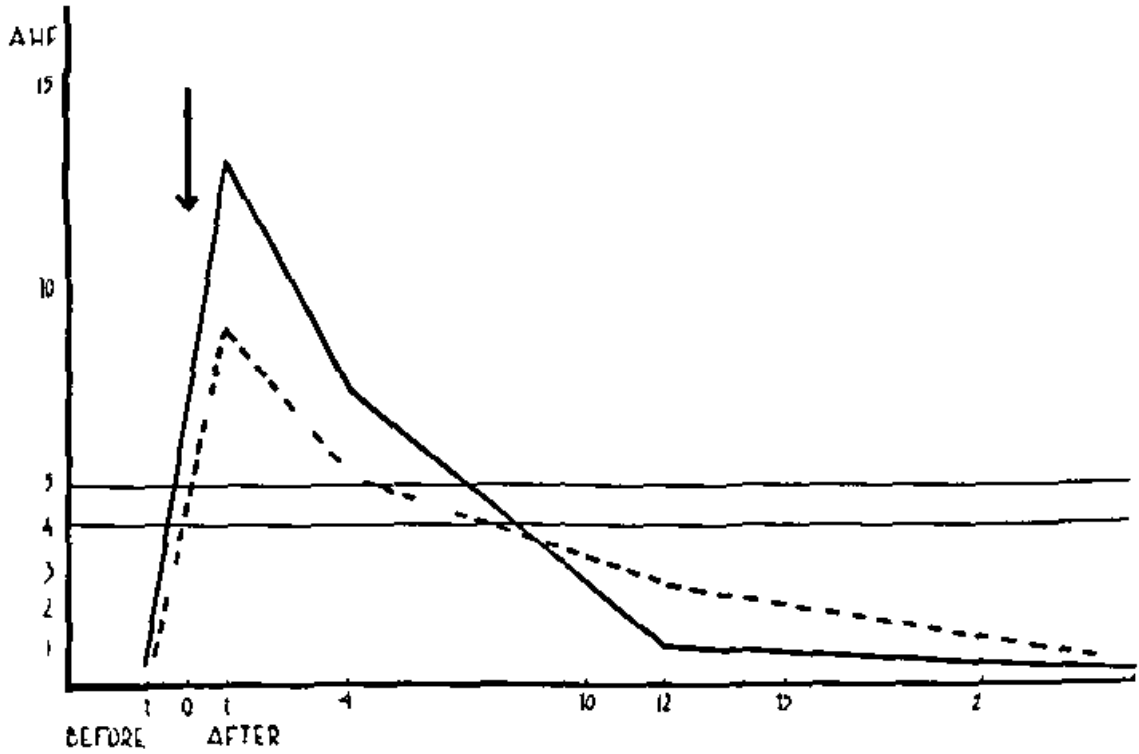


FIG 4 AHF Content of Plasma with Intravenous drip of fresh lyophilized heparinized plasma (200 ml in the course of two hours) Arrow indicates the time of the beginning of transfusion

We then determined in a few patients without hemorrhages how many hours after the end of the transfusion the AHF level was maintained higher than the minimal value (Fig 4) It appeared that after transfusions with a rate of 100 ml per hour the AHF level remained higher than the required minimal value of 4 5% for only 4 to 5 hours

On account of these experiences we take the following stand In dealing with slight bleeding the decision whether a second transfusion is required should be made within 4 hours after the end of the first transfusion (fresh citrated blood or plasma being used in a quantity of 5 ml per kg of body weight with a rate of infusion of 100 ml per hour) If the hemorrhage

has not stopped after the second or even third transfusion there is with great probability a rapid utilization of AHF and we should consider this hemorrhage to be a severe or a rather severe one

In order to combat such a severe condition a continuous drip transfusion is necessary for in such circumstances the AHF level has decreased below the required minimal value one hour after the end of the transfusion It is only of theoretical significance to decide whether under such conditions the transfusions should be given every hour or every 2 3 hours *However it is most important to maintain the AHF level of the blood* The correction of the anemia is secondary if it is not severe To maintain the AHF level fresh citrated plasma or lyophilized heparinized plasma (12) is preferred If in severe or rather severe hemorrhages the bleeding does not stop quickly with continuous transfusions the rate of transfusion must be increased Also concentrated preparations of AHF should be given intravenously All means for maintaining the AHF level must be taken

From these facts we can conclude that severe hemorrhages require an immediate correction of the AHF deficiency by means of continuous administration of fresh blood or fresh plasma (or fresh lyophilized plasma) When symptoms of shock are present this as well will determine the rate of transfusion When the loss of blood is not so severe as to cause shock it is better to begin with intravenous administration of a concentrated AHF preparation if available followed by intravenous blood or plasma infusion

Various AHF preparations have been made from human plasma (fraction I of Cohn (13) obtained by alcohol precipitation fraction I of Kekwick Mackay and Record (14) prepared by ether precipitation) Recently efforts have been made to prepare purified AHF preparations which can be injected Of these we mention here (a) concentrated preparations from bovine plasma made by Bidwell (15 16) and Macfarlane *et al* (17) (b) the AHF preparation of Kekwick and Wolf (18) obtained by modification of the former method of Kekwick *et al* (c) the so-called I O fraction and other fractions prepared by Blomback *et al* (19 20) from human plasma

The problem of preparing a purified AHF preparation has also our special attention Until now none of these preparations have been generally used

We want to illustrate the foregoing by a description of some patients selected from among the more than 100 children with hemophilia A treated by us during the last few years All patients had a greatly prolonged clotting time (more than 30 min)

Patient B a boy aged 5 years had fallen from a scooter and was admitted on account of a hemorrhage in the calf with a distinct swelling and restriction of the movements At intervals of 4 hours he received respectively 250 ml fresh citrated blood and twice 100 ml of fresh heparinized plasma The condition greatly improved the leg became more

supple the painfulness decreased and 5 days after admittance the boy could be mobilized Thus the hemorrhage of this boy was stopped by means of intermittent transfusions of blood plasma

Patient E a boy aged 7 years was admitted on account of a hemorrhage in the right hip The swelling had been observed by the parents two days before after trauma One month earlier the patient had fallen on his right hip resulting in a considerable subcutaneous hematoma At that time he was admitted and treated with 4 transfusions of blood At discharge the movements of the right leg were no longer restricted but the patient dragged one leg a little At the second admission he first got at intervals of 4 hours three transfusions of blood and plasma in the form of a drip transfusion Apparently there was an amelioration the joint was less painful and the patient could again move the leg But rather soon the swelling of the hip increased and any movement of the leg was painful We then changed to a continuous drip transfusion of blood and plasma but in spite of this paralysis of the muscles of lower leg and foot developed Notwithstanding the continuous transfusion the AHF level did not rise above the critical value Then the rate of the transfusion was doubled but still the AHF level did not remain constantly above the critical value Again the rate of transfusion was increased and finally the AHF concentration rose to a mean value of 10% In the meantime there appeared gangrenous spots at the heel and instep By careful treatment these spots have healed but at present four months later the paralytic symptoms have scarcely improved

Could the paralysis and gangrene in the patient have been prevented? Our experience has shown that after due treatment of such cases the com plications only rarely are so serious It is highly probable that it would have been better to give from the beginning a continuous infusion of fresh blood and fresh plasma combined with infusion of a concentrated AHF preparation Our experience has shown how excessively large the AHF consumption can be in such a case and further that an ideal treatment is only possible when in such cases there is a regular control of the AHF level of the blood

Patient F a boy aged 16 years is one of our well known hemophiliacs During a severe hemorrhage in the left hip he had symptoms of an acute abdominal affection so that a serious condition was feared When admitted to the hospital the boy appeared to be very ill with a tense strained abdo- men There were no intestinal murmurs In the last hours he had vomited about one liter of brown bilious fluid The benzidine test was positive On account of his serious condition the patient after an immediate infusion of physiological saline at a high rate was given a continuous infusion of fresh blood and plasma (alternatingly every 6 hours) orally he got nothing After 24 hours there was a marked improvement and the AHF

concentration of the blood was about 5 Now intestinal murmurs could be heard In the lower left part of the abdomen a swelling could be palpated which gradually disappeared The amelioration of the condition continued during the next several days The continuous infusion of alternating fresh blood and plasma went on for another four days Some days later the patient could be discharged

Most probably in this case the extensive hemorrhage in the left psoas had caused the symptoms resembling ileus The continuous administration of blood and plasma caused an immediate improvement

Patient H a boy aged 5 years a *hemophiliac A* was *suffering from* a severe earache on the left side for 14 days he had no fever The practitioner sent him to a specialist who performed paracentesis *and* one hour later the ear started bleeding Four hours later the child was admitted to the Children s Clinic with a considerable bleeding of the ear We gave transfusions of 300 ml of fresh blood alternating with 300 ml of fresh plasma at 4 hour intervals After 72 hours it seemed that the bleeding had stopped but we continued the transfusions In the night after the 4th day the patient swept away the clot from his ear and bleeding started again Next morning (4 hours after the last transfusion of fresh plasma) we withdrew blood for a AHF determination and the continuous intravenous infusion alternating fresh blood and fresh plasma (300 ml of each) was resumed Two days later the bleeding had stopped 24 hours after the beginning of the continuous infusion at the end of a transfusion of blood the AHF concentration amounted to 15 Three days after the beginning of the continuous infusion the therapy was stopped The patient s recovery was uneventful

When extraction of teeth in hemophiliacs is inevitable usually local treatment will be sufficient if the dentist has already had some experience in this field and if the patient is co operative But it is safer to maintain the AHF level by means of alternating blood and plasma transfusions

From the following experience it appears that alternating transfusions of blood and plasma in itself can be sufficient for the hemostasis

Patient I a boy aged 15 years was admitted for extraction of a molar tooth Three hours before extraction a blood transfusion was applied After the extraction the wound was plugged by Spongostan dipped in thrombin Four hours after the extraction the AHF concentration in the blood was about 15% In the meantime the transfusion of blood and plasma was continued for 4 days (alternating at intervals of 4 hours) After 4 days the transfusion was stopped and some days later the patient was discharged

This experience suggests the question of the duration of the continuous transfusion in hemorrhages as a rule we can say for 4 to 6 days However when we have to deal with severe trauma or a serious operation

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in hemophilia then therapy with blood and plasma and also with a concentrated AHF preparation (when in the future this will become possible) should be continued for at least 10 days

When speaking of therapy with fresh plasma usually fresh citrated plasma is meant Sometimes heparinized plasma is used based on our previous experiences (9) Under certain circumstances fresh heparinized plasma when available can be used with even more success as we have shown in some of the cases mentioned in this article

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an experiment an inhibitor (Inhibitor Source Material—ISM) neutralizes the platelet cofactor I activity The quantity of the hypothetical ISM is much higher in hemophilic plasma than in normal plasma and is scarcely found at all in plasma thromboplastin antecedent deficiency (PTA) (4) In plasma thromboplastin component deficiency (hemophilia B) the con centration of ISM seems to be the same as in the normal or average plasma Platelet cofactor I concentrates shorten the clotting time of hemo philic blood (5)

In this laboratory a method for the isolation of platelet cofactor I from bovine plasma (6) was based on the following principle platelet cofactor II and prothrombin were first removed from plasma by adsorption on barium carbonate Platelet cofactor I was then adsorbed on kaolin the adsorbing agent used by Laki and Lorand (7) The kaolin was washed with sodium tetraborate and saline and then the activity was removed from the adsorbant with phosphate buffer at pH 7 5 Further fractionation then involved the use of ammonium sulfate With the description of this procedure the efforts in this laboratory continued without interruption and now the most significant observations of the past two years are described

The chief difficulty with the methods described (6) was found to be inconsistency of results Sometimes the yield and purity were high but often inert protein was obtained The erratic results were obtained when the procedure was apparently carried through exactly as described We accordingly began to make modifications and a few of the important manipulations introduced and observations made are the following (a) the quantity of kaolin used has been reduced (b) the washing of kaolin is better accomplished with physiological saline solution than with sodium tetraborate (c) it is more convenient and less hazardous from the stand point of retaining activity to fractionate with cold alcohol than with ammonium sulfate (d) the ultracentrifuge pattern may remain roughly the same with a tenfold fluctuation in purity and (e) contrary to our previous impression drying from the frozen state is accompanied with much loss in activity In this presentation we describe in detail the technical procedures that are now successfully used in our laboratory The product obtained still does not consist of a single component but represents a valuable concentrate of platelet cofactor I and we can obtain it con sistently

Procedures for the Purification of Platelet Cofactor I

Bovine blood is mixed in a proportion of 9 to 1 with a 1 85% solution of potassium oxalate The plasma is separated by centrifugation This can be used immediately or can be frozen and used within a few days

Washed kaolin 25 gm of kaolin (Baker N F Powder) is twice washed with approximately 500 ml of 0 5 M pH 6 3 phosphate buffer We stir the

5

Further Studies on the Purification of Platelet Cofactor I*

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IN THROMBOSIS and the hemorrhagic diseases the blood coagulation mechanisms are usually involved Accordingly an understanding of these mechanisms may eventually contribute to better management of these afflictions In this laboratory the study of the blood clotting mechanisms begins centrally with isolated prothrombin and extends outward to include other substances One of great interest from that perspective is platelet cofactor I

Platelet cofactor I activity is found in plasma and recognized in tests where purified prothrombin preparations are converted to thrombin In mixtures believed to consist of prothrombin plus Ac globulin plus platelet factor 3 plus calcium ions and platelet cofactor I thrombin activity generates far more rapidly if a little purified thrombin is added at zero time Platelet cofactor I activity is not found in serum (1) but is in the plasma of persons taking Dicumarol for therapeutic purposes (2) The activity disappears from $BaSO_4$ treated plasma upon recalcification if purified prothrombin and a little thrombin are also added (3) Evidently in such

* This study was supported by a research grant (H 20 6 C) from the National Heart Institute of the National Institutes of Health U S Public Health Service Funds for research fellowships in Physiology were provided by Parke Davis and Company Some information obtained after the VIIth International Congress of Hematology in Rome is included in this report

of 1% acetic acid The solution is centrifuged for 10 min at 2 000 g at 0 C

The precipitate is dissolved in 10 ml of acidulated distilled water at a pH of 6 2 and kept cold This is fraction B Fraction B can be kept in the deep freeze for further use on the next day or further purification may follow on the same day Fraction B is placed in centrifuge tubes of the Spinco Ultracentrifuge (Model LS) and centrifuged at 0 C for 15 min at 106 000 g The supernatant solution contains the platelet co factor I activity The activity is assayed and the active products kept in the deep freeze at −20°C or colder

Comments on the Method Described

1) Bovine plasma should be used fresh or kept frozen for only a few days Long time storage of frozen plasma was not followed with good results

2) The barium carbonate adsorption can be made the first day In that case the adsorbed plasma should be kept in the refrigerator overnight after removing the barium carbonate This shortens the procedure for completing the work during the next day

3) We made an extensive study of the way in which to use the kaolin With less than 25 gm there is a loss in yield With more than 25 gm and even up to 250 gm there is no increase in yield but the purity of the product is progressively reduced We determined that a second adsorption with 25 gm does not pick up any consequential amount of activity

4) Washing of the kaolin with 0 9% saline showed the best results of any wash solution that we tried There is practically no loss of activity with 0 9% saline and a large amount of protein is removed from the kaolin Washing with sodium tetraborate resulted in a certain loss of activity washing with 0 5 M pH 6 3 phosphate buffer resulted in elution of activity which was found in both the first and second wash solutions

5) After the elution of the kaolin with 1% ammonium carbonate we cannot remove more activity from the remaining kaolin

6) After the first ethanol fractionation it is important to keep the temperature under 5°C

7) In the adjustment of the pH to 7 0 to get fraction A it is important to be accurate and a perfect working pH meter is the suppo ition The main precipitate should come down in fraction B after the adjustment to pH 6 0 When preparing fraction A it can happen that already at a pH of 6 9 the main precipitate comes out It is then better to keep the pH at 7 1 or 7 2 instead of exactly 7 0 Fraction A should contain a little precipitate and a little activity

kaolin phosphate buffer suspension mechanically for about 15 min during both washing procedures The kaolin is separated by centrifugation at 800 *g* for 5 min

To remove prothrombin and platelet cofactor II from the plasma 5 000 ml of bovine plasma obtained by the above described procedure are mixed with 250 gm of barium carbonate (Baker Powder C P) This mixture is stirred at room temperature for 15 min The barium carbonate is removed by centrifugation for 7 min at 800 *g*

To adsorb the platelet cofactor I activity onto kaolin the plasma is diluted with 4 000 ml of 0 5 M pH 6 2 to 6 3 phosphate buffer 25 gm washed kaolin are added to this mixture and stirred mechanically for 20 min The kaolin is then removed by centrifugation for 7 min at 800 *g*

The kaolin is then washed with 1 000 ml of 0 9% saline A large Waring Blendor is used for mixing the saline and kaolin It is run for exactly 15 sec at the slowest speed of the mixer The wash solution is discarded after centrifugation for 10 min at 800 *g* The kaolin is then again washed with 600 ml of 0 9% saline but without blending in the Waring Blendor The saline is mixed by hand stirring with the use of a glass rod to which a rubber stopper is attached The wash solution is again discarded after centrifugation for 10 min at 800 *g*

The platelet cofactor I activity is then eluted from the kaolin For this purpose the kaolin is placed in the Waring Blendor and mixed with 600 ml of a 1% ammonium carbonate solution and blended at the slowest speed of the mixer for one min The homogeneous mixture is transferred to a beaker and stirred mechanically for 20 min at room temperature The mixture is centrifuged for 20 min at 2 000 *g* at 0°C

The eluate is cooled in an ice salt bath to 4°C and fractionated by adding up to 13% cold (−70°C) 95% ethanol under stirring The mixture is centrifuged for 10 min at 2 000 *g* at 0°C The precipitate which is largely fibrinogen is discarded To collect platelet cofactor I the supernatant solution is chilled to 0°C in the ice salt bath and more alcohol is added up to 33% As before the 95% ethanol is cold (−70°C) and added with stirring The precipitate is centrifuged down for 15 min at 2 000 *g* at 0°C The supernate is discarded

The precipitate is dissolved in 200 ml of cold (4°C) distilled water adjusted to pH 8 0 with 0 1 N NaOH The temperature is kept exactly at 4°C and 20 ml of cold (−70 C) 95% ethanol are added dropwise and with stirring The pH of the solution is adjusted to exactly 7 0 with 1% acetic acid The mixture is centrifuged for 10 min at 2 000 *g* at 0°C The precipitate is dissolved in 10 ml distilled water adjusted to pH of 6 2 This is fraction A The fraction is kept cold

The main fraction of platelet cofactor I is now obtained The supernatant solution is kept at 4°C and the pH is adjusted to exactly 6 0 by addition

thrombin occurs From the amount of thrombin generated the concentration of platelet cofactor I can be inferred A unit was defined in terms of this 2 stage analysis

The 2 stage analysis has many desirable features to recommend it We felt however that errors might be detected if another assay could be run simultaneously and we did indeed frequently spare ourselves the cost of mistaken results by applying two different tests The most recently developed one is a variation of the 1 stage prothrombin time Instead of using tissue procoagulant platelet cofactor I is used alone The test is performed as follows oxalated cow plasma 0 1 ml plus 0 1 ml procoagulant (platelet cofactor I variously diluted) plus 0 1 ml of 0 02 M CaCl in imidazole buffered saline At 37° C and without procoagulant the clotting time was 150 sec As the concentration of procoagulant is increased the clotting time may get as low as 32 sec For an assay we like to work in the range of 32 to 60 seconds This assay procedure is integrated with the 2 stage assay described (6) in such a way that a unit in the 1 stage is a unit in the 2 stage The solution put into the test mixture must contain 600 U/ml to give a 32 sec clotting time (Fig 1)

With our assay procedures our fraction B usually yields material containing 6 000 to 8 000 units per milligram of protein The yield from 5 000 ml of fresh plasma ranges from 1 to 1 5 mg dry weight This is a rather low quantity of material to obtain from so much plasma and perhaps means that the concentration of this factor is low in normal plasma We are however greatly handicapped in the calculation of yield in terms of activity in the starting material Our 2 stage assay does not detect very much cofactor I in the plasma the more marked activity is picked up after a few fractionations

Our purified platelet factor 3 preparations shorten the clotting time of recalcified cow plasma a little less than the platelet cofactor I concentrates A mixture of platelet cofactor I and platelet factor 3 (threone by definition) may bring the clotting time down to 30 sec Incubating the two together for various lengths of time before the test does not alter the result In other words there is no enzyme substrate relationship between platelet cofactor I and platelet factor 3 Dilution of the mixture progressively reduces the procoagulant power in a manner depicted on the chart As a matter of interest platelet factor 3 alone gives an irregular dilution curve

The fact that platelet factor 3 alone or platelet cofactor I alone or the two together do not bring the clotting time much below 30 sec is an outstanding fact to contrast with the much shorter clotting times obtained with tissue extracts such as those from brain or lung The concept plasma thromboplastin compares the activity of ingredients of plasma and platelets with those of tissues The limitations of this concept can be seen quite clearly by any one who considers the nature of purified prothrombin

Fraction B contains most of the activity and inert protein The precipitate does not go completely in solution but gives a suspension The nonsoluble proteins are spun down in the ultracentrifuge and do not contain any platelet cofactor I activity Curiously they have a thrombin like activity and *until this is removed as for instance by ultracentrifugation the I stage assay procedure described below may give misleading results* The platelet cofactor I activity is in the solution

Important is the pH of the distilled water used for dissolving the fractions If the pH is lower than 5 4 the platelet cofactor I activity does not go in solution and after ultracentrifugation appears in the precipitate If *the pH is higher than 6 5 more proteins go in solution and the specific* activity expressed in units per mg protein (ninhydrin) drops

8) When there is no ultracentrifuge available a further purification of fraction B can be achieved on the basis that calcium ions precipitate a protein that accompanies the desired protein The fraction is therefore placed in a water bath at 37°C and an equal volume of 0 02 M calcium chloride pH 7 25 is added This solution is kept for 10 to 15 min in the water bath and then centrifuged 10 min at 2 000 *g* The precipitate is discarded and the supernate is kept overnight in the refrigerator at 4°C The next morning the product is again centrifuged in a refrigerated centrifuge for 10 min at 2 000 *g* and the precipitate discarded The product *has to be dialyzed free of calcium ions against distilled water This is* then the purified platelet cofactor I

Assay of Platelet Cofactor I

The assay previously described (6) used purified prothrombin as a substrate and depended upon its transformation to thrombin according to the following equation

$$\text{Prothrombin} \xrightarrow[\text{Ac globulin, Platelet factor 3, Platelet cofactor I}]{Ca^{++}} \text{Thrombin}$$

An incubation mixture was prepared in a standard manner as follows

(1) Purified prothrombin (with Ac globulin) 3 000 u/ml	1 00 ml
(2) Platelet factor 3 (about 80 u/ml)	0 50 ml
(3) CaCl (0 16 M) in imidazole buffer	0 50 ml
(4) Platelet cofactor I	0 50 ml
(5) Physiological saline	0 50 ml

In such a mixture only a small amount of thrombin forms if the platelet cofactor I concentration is weak If it is strong all of the prothrombin becomes *thrombin* With intermediate concentrations a partial change to

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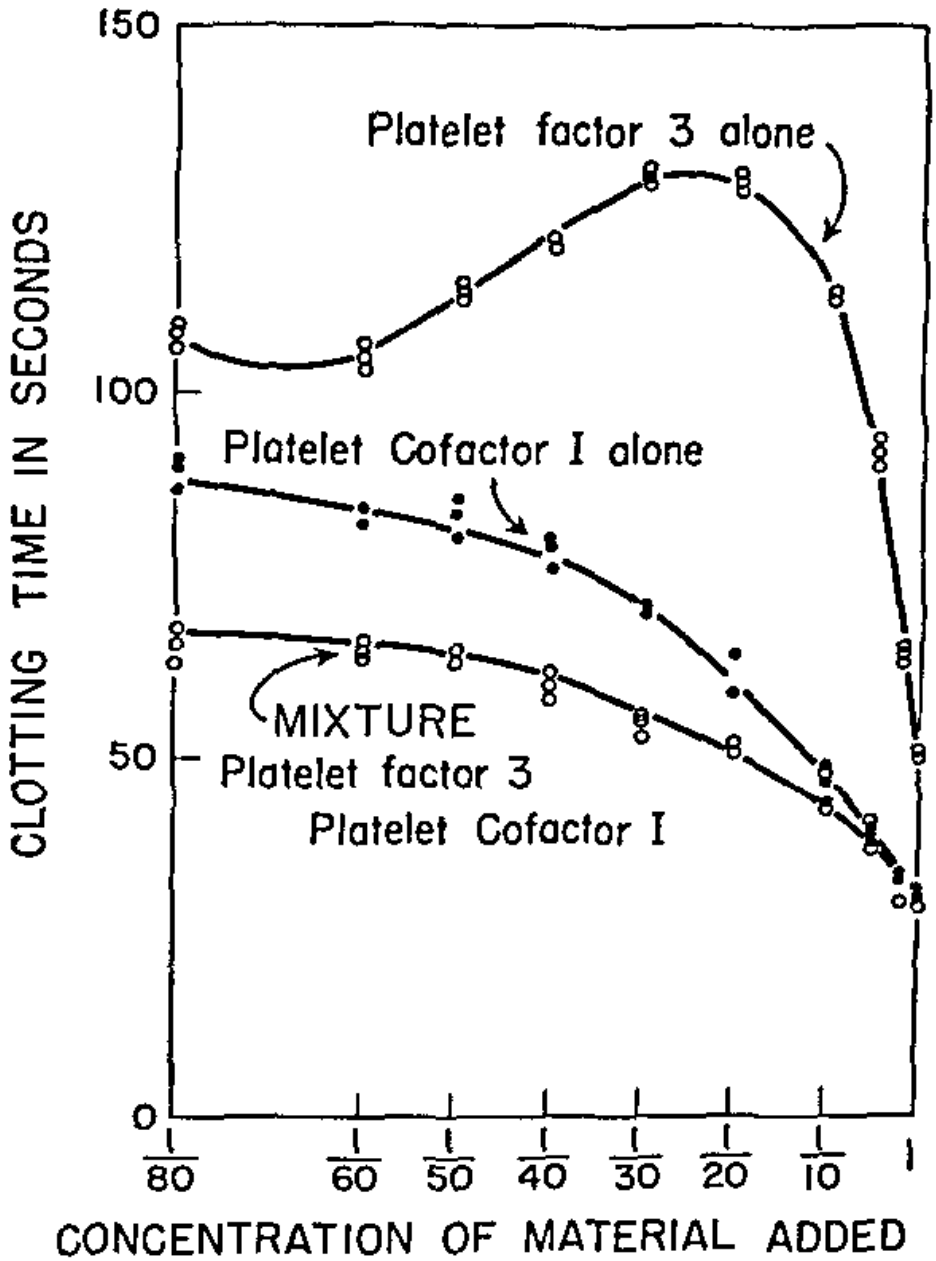


FIG 1 The Clotting Time of Mixed Oxalated Plasma from 6 Different cows was determined by mixing 0 1 ml of plasma with 0 1 ml of pro coagulant and 0 1 ml of 0 02 M CaCl in imidazole buffered saline (pH 7 2) at 37 C The procoagulants were platelet factor 3 (top curve) platelet cofactor I (middle curve) equal volumes of above two procoagulants mixed (lower curve)

The numerals on the abscissa designate the concentration of platelet factor 3 and/or platelet cofactor I The original concentration was 500 U/ml of platelet factor 3 and 600 U/ml of platelet cofactor I Example At 1/40 the concentration was 12 5 U/ml of platelet factor 3 (before putting into the final test mixture) 15 U/ml of platelet co factor I and the mixture contained respectively 6 25 plus 7 5 units

activation in terms of tissue extract material as compared with material obtained from plasma and platelets We have to consider the possibility that we have purified the main ingredients of the plasma thromboplastin concept namely platelet cofactor I platelet factor 3 and thrombin These three plus one of the most important still needed namely Ac globulin provide important tools for exploring prothrombin activation The aspect which we desire to have explored is the activation of Ac globulin with thrombin for that seems to be the key consideration in having plasma thromboplastin as powerful as tissue extracts

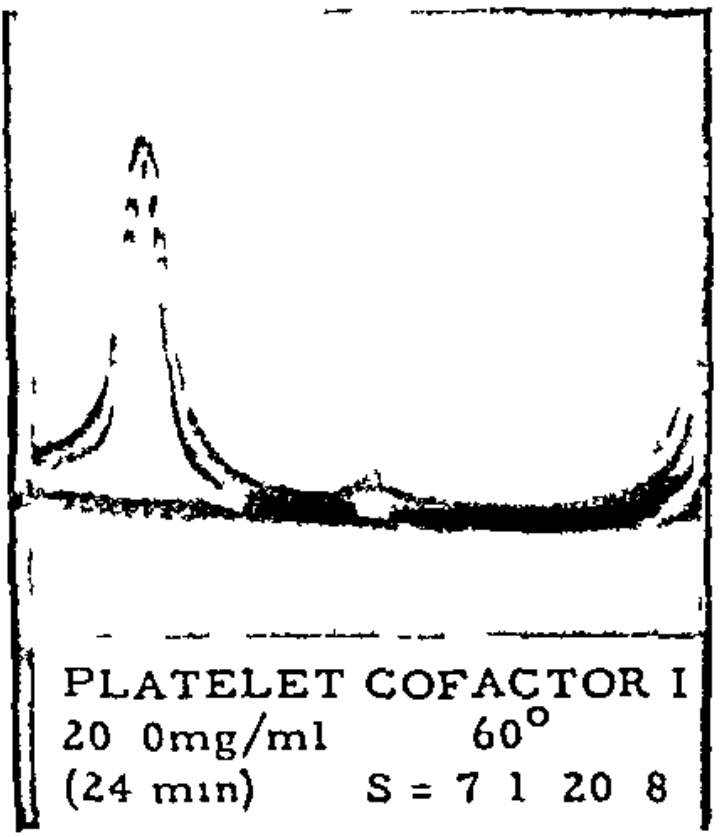


FIG 2 Typical Ultracentrifuge Pattern of a Platelet Cofactor I Concentrate The conditions were bar angle 60° temperature 20 C speed 59 780 r p m Spinco Model E sedimentation time 24 minutes protein concentration 20 0 mg/ml in 0 1 M KCl pH 7 2 During 5 hours of centrifugation no more components were found

Ultracentrifuge Studies

We frequently examined platelet cofactor I concentrates with an analytical ultracentrifuge Sometimes three components were found but usually two A typical pattern is reproduced in Fig 2 The fast moving component (S = 20) was in lowest concentration The other had a sedimentation constant of 7 1 at a concentration of 20 0 mg/ml and a temperature of 20°C At a concentration of 6 mg/ml S = 6 5 We do

not intend anything more to be deduced from these figures than a preliminary orientation regarding the characteristics of platelet cofactor I preparations When prepared by the method previously described (6) the ultracentrifuge pattern was about the same as with the best preparations we now obtain These latter preparations however may be in the neighborhood of ten times the potency of the former Thus we found great disparity between activity and the ultracentrifuge pattern In some further experiments we were able to remove the heavy component(s) with a 5 hour centrifugation in a preparative centrifuge at 106 000 g The supernatant solution then contained only one component (pattern symmetrical 5 hour run concentration approximately 6 mg ml) upon analysis with the analytical ultracentrifuge This same supernatant solution still developed two bands when examined with the use of an agar diffusion plate (Ouchterlony) In these immunochemical examinations the development of two distinct bands thus could be indicative of detecting at least two components of approximately the same sedimentation characteristics and makes it likely that platelet cofactor I is itself a precipitinogen Another possibility is that a second component is present in relatively low concentration and was detected only in the method that was most sensitive for the particular materials we are studying

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6

Effect of Direct Transfusions of Hemophilia A Blood of Various Grades of Severity into Normal and Thrombocytopenic Recipients *

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PATIENTS with the classical clinical picture of hemophilia may owe the defect in their blood to several abnormalities Two entities are now recognized hemophilia A and hemophilia B with combinations of the two defects perhaps being more common than generally realized According to our interpretation (1) the more frequently encountered defect (hemophilia A) results principally from an excessive amount of plasma antithromboplastin while the less common form (hemophilia B) is due chiefly to a decrease in the platelet cofactor in the plasma (plasma thromboplastin component)

If hemophilia A is chiefly due to an excessive amount of an anti coagulant it should be possible to demonstrate this by a transfusion of such blood into a normal recipient A delay in the rate of clotting of the recipient s blood should follow Previous attempts (2 3) to accomplish this failed because of one or more of the following reasons (a) unaware ness of at least two types of hemophilia and of intermediate forms sharing some of the defects of each (b) the existence of grades of hemophilia varying from mild to severe (c) use in the transfusion experiments of highly diluted citrated blood collected in glass containers and (d) meas urement of clotting times with glass tubes and syringes and testing of the plasma in clotting systems in which the concentration of the plasma is

* Aid d by a grant from the U S Public Health Service

greatly reduced by citrate, calcium saline and other solutions (4) The experiments to be described seem to demonstrate that when comparatively small amounts of blood or plasma from patients with moderate and severe grades of hemophilia A are transfused directly and swiftly into normal and thrombocytopenic recipients a lasting prolongation of the rate of coagulation of the recipient s blood follows (5)

Methods

Hemophilia A patients previously characterized as to severity of defect and blood type and having large easily punctured arm veins were selected as donors Table 1 lists the principal criteria employed to determine the grade of the defect With the exception of hemophiliac A C none of the donors had had plasma or blood transfusions for at least one year In most instances, tests of mixing the two bloods in various proportions were

Table 1

CHARACTERISTICS OF THE BLOOD OF THE HEMOPHILIC A DONORS

No	Patient	Glass	Venous Blood Clotting Time (min) Siloe	Plasma Path (Units/ml)	Clot Delay Activity	Activated Activity (Units/ml)	Response to Transf	Grade of Defect†
1	N F	88	>360	330	1 50	62	nil	IV
2	A C	107	>360	338	1 50	65	nil	IV
3	S M	78	220	360	1 20	58	poor	III
4	G M	92	265	315	1 20	45	poor	III
5	H F	65	210	348	1 10	34	fair	II
6	*R P*	*45*	*135*	*345*	*1 10*	*29*	*fair*	*II*
7	J R	19	77	293	1 5	28	good	I
8	E.C	13	67	315	1 5	27	good	I
	Normal Values	8 15	25-40	300-400	—	10 20		

* Minimal amount of plasma (ml) which will significantly delay the rate of clotting of normal plasma For example 1 50 indicates that one part of hemophilic plasma will significantly delay the clotting time of 50 parts of normal plasma

† Mild = Grade I Moderate = Grade II Severe = Grade III Very severe = Grade IV

carried out before the *in vivo* mixtures were attempted The results of these *ex vivo* tests gave an approximate but usually insufficient estimate of the intensity of the clot delaying effect *in vivo* With grade IV hemophilic bloods a clear clot delaying effect as shown in Fig 1 could be demonstrated For these and other procedures only silicone coated syringes and glassware were employed The hemophilic blood was collected *first* and the proper amounts distributed to each tube (13 mm id) *before* the normal group compatible blood was collected in a chilled syringe and added to the hemophilic blood in the proportionate amounts The

tubes were then gently agitated and placed in a water bath at 38° C The venipuncture, collection and distribution of the blood must be swift and faultless

Technique of Transfusion Blood was withdrawn from a fasting hemophilic donor using a 16 gauge needle and a series of sterile siliconized

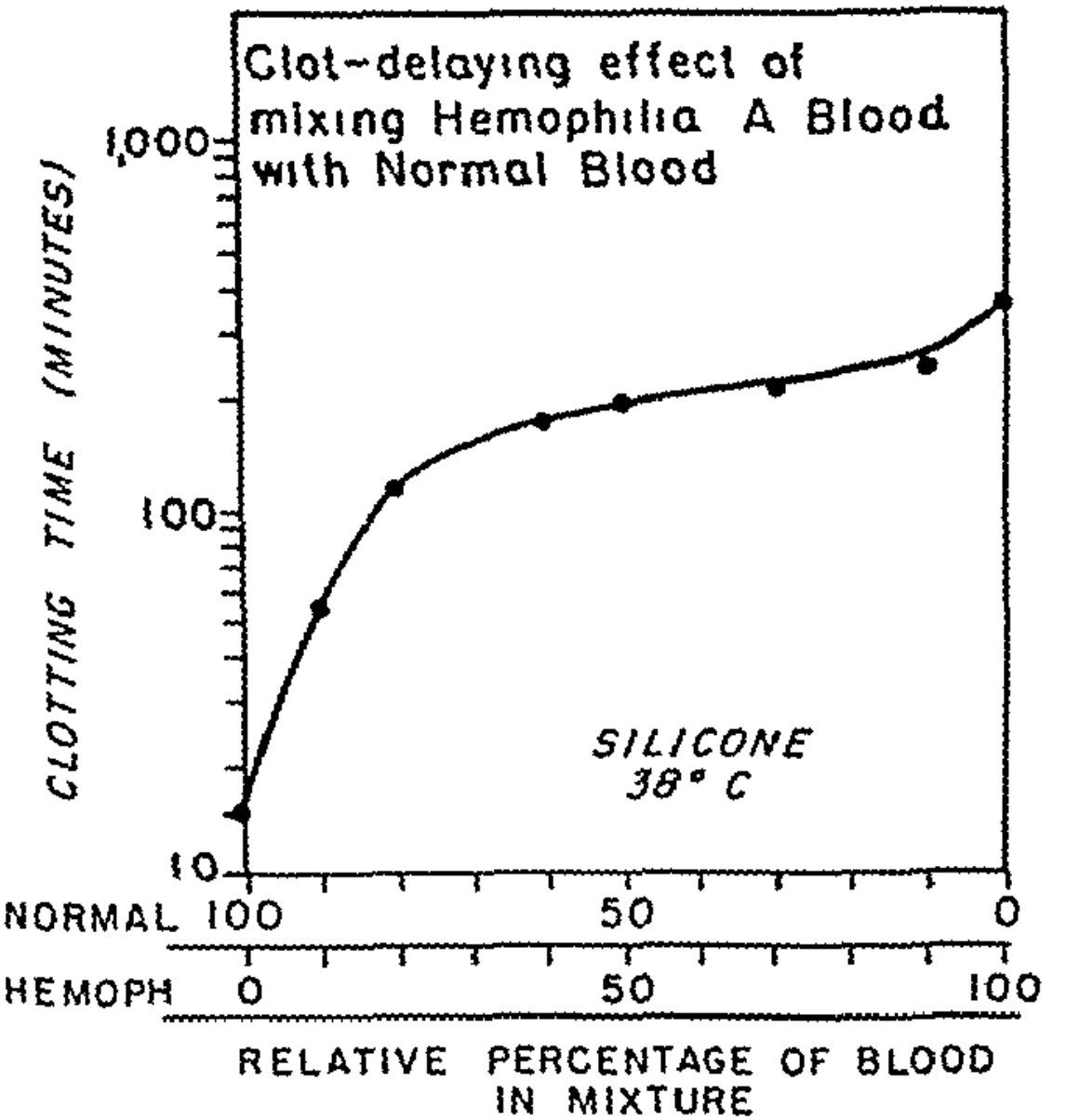


FIG 1

50 ml syringes Each syringe was used only once and after being loaded the blood was injected immediately by another assistant through a 16 gauge needle into the recipient s vein at a rate of 10 15 ml per minute To maintain patency of the recipient s needle a 2 way stopcock was attached one opening of which was connected by means of plastic tubing to a reservoir with 0 85% NaCl The saline solution was infused by gravity between changes of the syringe to prevent backflow of the blood through

the needle and clotting within it If any difficulty was experienced with the puncture of the vein and collection of the blood another vein was used At intervals following the beginning of the transfusion specimens of blood for study were collected from a vein *in the other arm* A fresh puncture was used *each time* and needles no smaller than 18 gauge were employed The blood was collected by means of three siliconized syringes With the first about 1 ml of blood was aspirated this specimen was discarded With the second siliconized syringe 4 ml of blood were aspirated and distributed in 4 tubes One ml was placed in a glass tube another ml in a silicone coated tube 0 6 ml in a silicone coated tube containing 0 4 ml of 0 85% NaCl and 0 3 ml of blood was placed in a siliconized tube containing 0 7 ml of 0 85% NaCl The last two tubes were used to study the effect of simple dilution on the clotting time All observations on clotting were done at 38° C The rate of clotting of the undiluted blood was taken as the index of changes in coagulability after the transfusions The third syringe held a solution of 19% sodium citrate and into it was aspirated sufficient blood to give a proportion of 49 parts of blood to one of the citrate solution The citrated plasma obtained by centrifugation was used for the determinations described in the text

Hemophilia A patients not in an acute phase of bleeding were selected as donors and according to the severity of their defects were classified Grade I (mild) to Grade IV (severe) Two donors were selected from each of these groups The recipients were patients serologically compatible with the donors and either had blood with normal platelet counts and rate of coagulation or were patients with thrombocytopenia and slightly prolonged clotting time of venous blood The following additional studies were performed in both donors and recipients before and after transfusion hemoglobin red cell and platelet counts one stage and two stage prothrombin assays (6) test of effect of dilution on the recipient s plasma clot delaying activity of the recipient s plasma on his own pretransfusion plasma (7) plasma antithrombin (8) fibrinogen (9) and accelerator globulin activity (10)

Grading of the Defect The degree of the hemostatic function defect in hemophilia may be estimated on hematologic clinical or orthopedic grounds There is usually good correspondence between the intensity of the hematologic defect the number of transfusions or hospitalizations required yearly and the extent of involvement of the joints Grading of the hematologic defect provides a means of predicting possible complications of operative procedures number of plasma or blood units required length of hospitalization and other details Surgical procedures which may be risked in a hemophiliac Grade I or II could be extremely hazardous in one with a Grade IV defect Grading rests on the results of the following tests (a) clotting time of venous blood in glass tubes (13 mm i d at 38° C) and (b) in silicone coated tubes (c) clot delaying effect of addition of

platelet poor hemophilic plasma to platelet poor normal plasma in clotting mixtures with 89% final plasma concentration Clot delaying activity is expressed in terms of the ratio of hemophilic to normal plasma necessary to prolong the clotting time of the mixture by at least one third the clotting time of an equal volume of normal plasma Table 2 illustrates results of such mixtures (d) antithromboplastin (anticephalin) activity

Table 2

EFFECT OF ADDITION OF HEMOPHILIC PLASMAS OF VARYING DEGREES OF SEVERITY ON THE RATE OF CLOTTING OF NORMAL PLASMA SILICONE TUBES 38 C

Plasma			*Clotting Time of Normal Plasma to Which was added Hemophilic Plasma of*			
Normal (ml)	*Hemophilic (ml)*	*0.2 M $CaCl_2$ (ml)*	*Grade I (Mild)*	*Grade II (Moderate)*	*Grade III (Severe)*	*Grade IV (Very severe)*
0.50	0.0	0.05	1550	1680	1570	1750
0.49	0.01	0.05	——	1715	1690	11 800
0.475	0.025	0.05	——	1910	2900	No Clot
0.45	0.05	0.05	1670	640	6800	
0.40	0.10	0.05	1800	No Clot	No Clot	
0.30	0.20	0.05	5850			
0.20	0.30	0.05	No Clot			"
0.10	0.40	0.05		"		
0.0	0.50	0.05			" "	"

Table 3

TYPE AND RANGE OF VALUES EMPLOYED IN GRADING THE SEVERITY OF THE DEFECT IN HEMOPHILIA A

Test	*Normal Values*	*Grade I*	*Grade II*	*Grade III*	*Grade IV*
Glass Tube Clotting Time (min.)	8-15	10-20	1-40	41-80	>80
Silicone Tube Clotting Time (min.)	25-40	45-60	60-120	120-240	>240
Clot delaying Titer	——	1:2	1:5	1:10	>1:20
Antithrombopl Activity (units/ml plas.)	10-20	20-30	30-40	40-50	>50
Plasma Clot Time a) 89% concentr	1 000-2 000 (sec.)	3-24 (Hours)	> 4 (Hours)	>24 (Hours)	> 4 (Hours)
b) 33% concentr ** (Ratio to clotting time of normal control)	450-600 (sec.)	1X	2X	3X	>4X

This indicates that 1 part of hemophilic plasma delayed the clotting time of 2 parts of normal plasma by an amount equal to or greater than 1 the clotting time of 3 parts of normal plasma

* 0.5 ml plasma 0.05 ml 0.2 M CaCl
0.17 ml of plasma 0.21 ml buffer 0.85 NaCl 0.71 ml 0.02 M CaCl

performed by a one stage method described in detail elsewhere (11) and (e) effect of dilution on the rate of coagulation of the hemophilic plasma As shown elsewhere (4) dilution with 0 85% NaCl to a final plasma concentration of 10 30% brings the clotting times of both normal and hemophilic plasmas to a level several times shorter than that of the original plasma The ratio of the time obtained in the control plasma at 32% con centration to that of similarly diluted hemophilic plasma provides another estimate of the extent of the defect

In Table 3 are listed the values corresponding to the various grades A blood is considered to be of a certain grade when its values fall in the range of three or more of the criteria for that grade

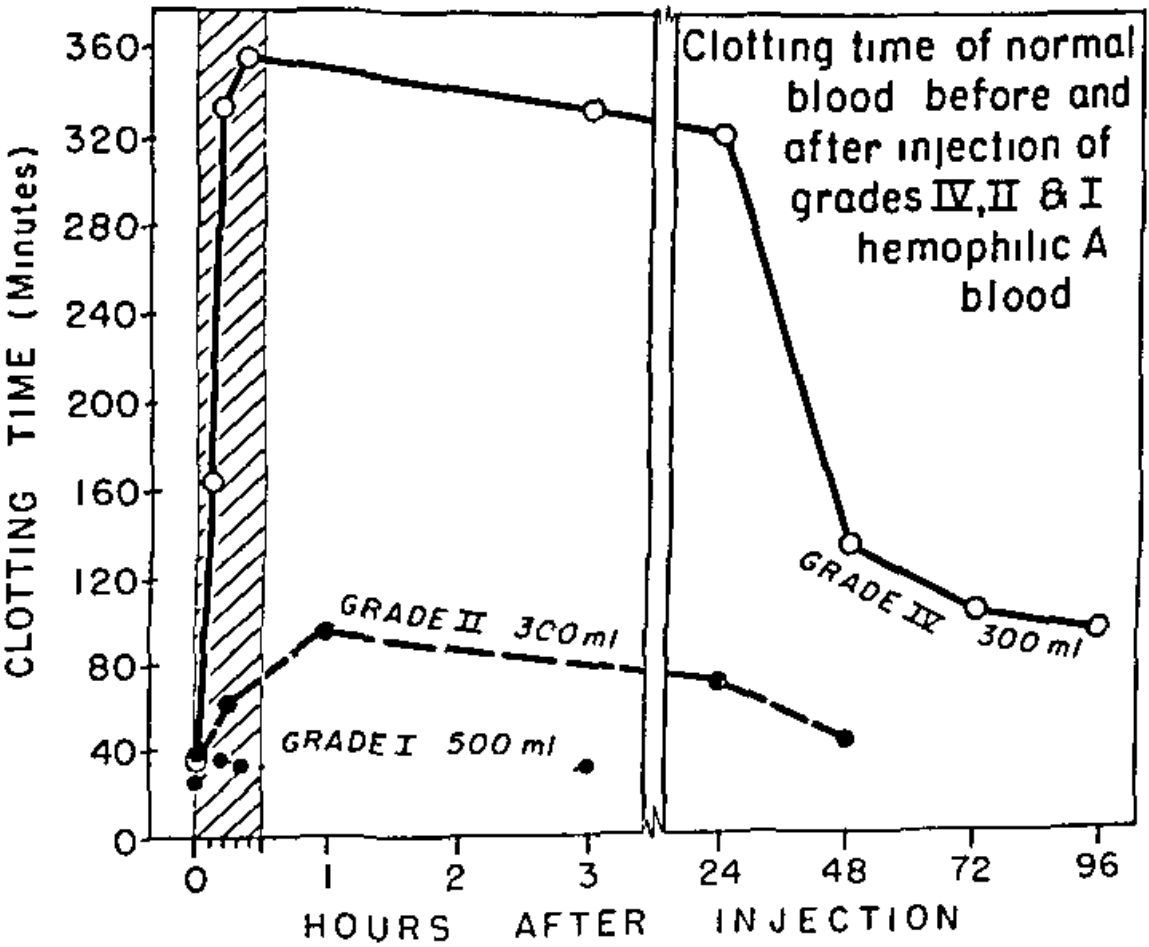


FIG 2

Results

Effect on the Rate of Clotting of Venous Blood Blood withdrawn from a Grade I hemophilic A donor and injected immediately into either a normal or thrombocytopenic recipient produced little or no significant change in the clotting time in either glass or silicone tubes (Fig 2) When a Grade II hemophilic was used as the donor after the injection of 300 ml

of blood a significant delay in the clotting time was observed (Fig 2) A significant delay in the coagulation time (from 36 to 163 min) was observed after the injection of as small a volume as 50 ml of Grade IV hemophilic blood into a normal recipient (Fig 2) After the injection of 300 ml of the same blood the time rose to 357 min it did not return to the pretransfusion level until five days after the initial injection (Fig 2) When a thrombocytopenic patient was used as the recipient the clot delaying effect of an injection of a small volume of blood was even more

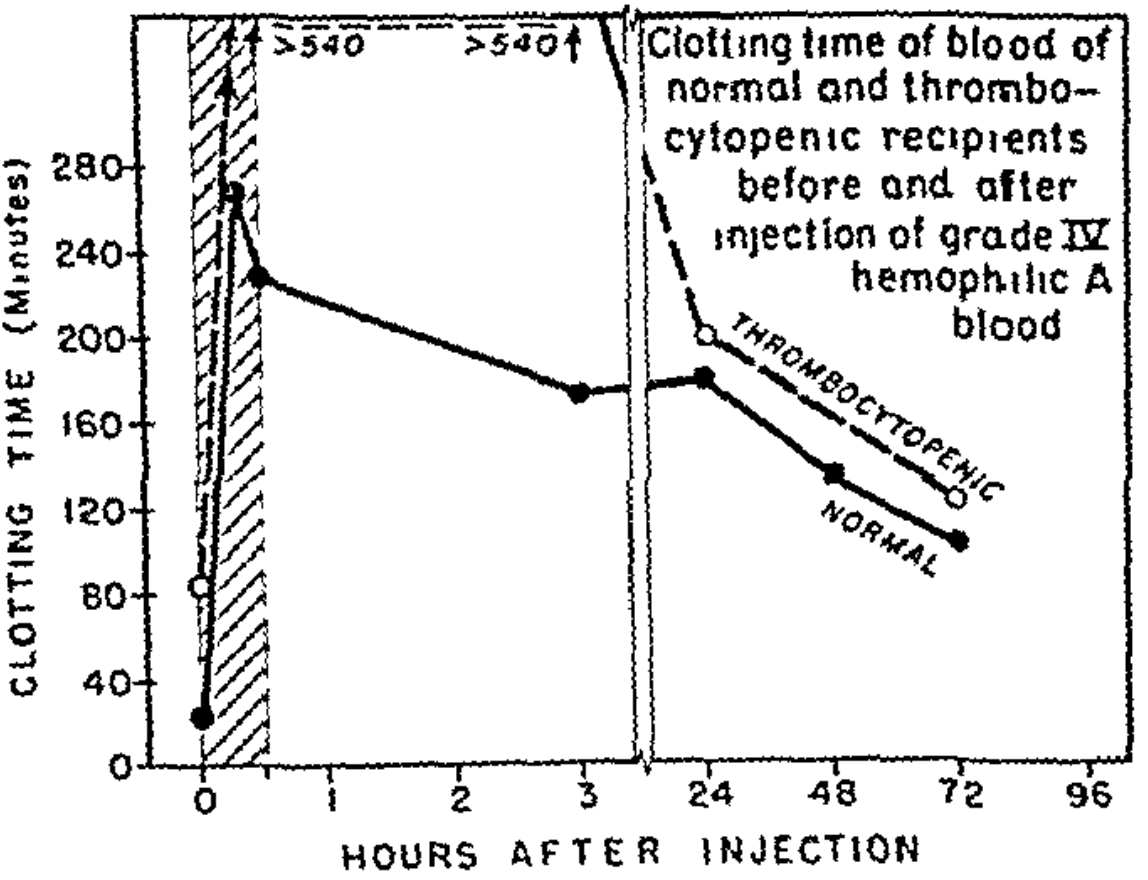


FIG 3

pronounced When 200 ml of the blood of a Grade IV hemophiliac was injected into a thrombocytopenic recipient the clotting time in glass was delayed from 14 min to 223 min and in silicone from 84 min to greater than 9 hours (Fig 3) As one would expect when comparing the effect of the injections on the clotting time the delay was always more pro nounced in silicone than in glass tubes (Fig 4)

It was hoped that the *in vitro* mixtures of hemophilic A blood with normal blood would give an approximate idea of the probable behavior *in vivo* Actually when the blood was injected directly into the recipient a greater effect was often demonstrated than could have been foreseen from the preliminary *in vitro* mixing For example an *in vitro* mixture of one

part of a Grade IV hemophilic blood to nine parts of the recipient s blood produced only a moderate delay in its rate of coagulation (Fig 1) However a much greater prolongation was observed *in vivo* when only one part of the same hemophilic blood was present in thirty p irts of the recipient s blood (Fig 4) The following may account for this (a) difficulties in collecting the normal blood and making the *in vitro* mixtures with the hemophilic blood with sufficient dispatch (b) mechanical trauma of placing the blood into the test tube and the necessity of swirling the tubes

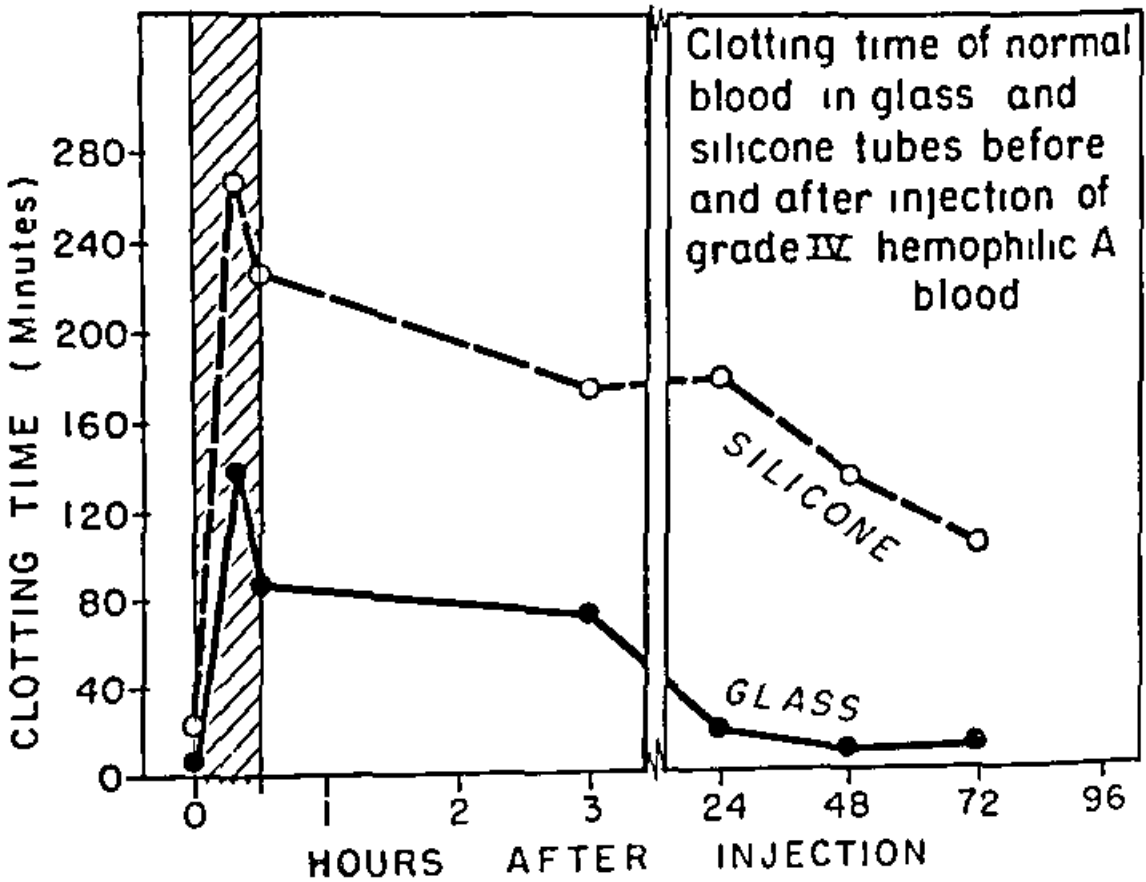


FIG 4

for proper mixing of the bloods (c) the glassware although siliconized does not offer a surface comparable to the endothelial lining of a blood vessel and (d) the larger quantities of blood used in the *in vivo* experiments and the less likelihood of contamination of the blood during its collection by a small quantity of tissue juice

Effect of Dilution on Pre and Posttransfusion Plasma When native or citrated normal or hemophilic A platelet poor plasmas are diluted with 0 85% NaCl clotting is accelerated the dilution effect being generally proportional to the clotting time of the original undiluted plasma (4) A dilution may be reached at which hemophilic plasma with a moderate

defect will clot more rapidly than normal plasma at its original concentration Citrated plasma samples collected from the recipients before and at various intervals after injection of hemophilic blood were diluted and studied as described elsewhere (4) On Fig 5 are shown examples of changes in the dilution curves after injection of Grade I and IV hemophilic blood When 500 ml of the blood of a Grade I hemophiliac were injected there was no significant change in the plasma clotting time or the dilution effect However after 200 ml of Grade IV blood were injected the recipient s plasma at 89% concentration did not clot overnight and on successive dilutions clotting was inhibited until a plasma concentration of 32% was reached when clotting occurred at 4 300 sec After injection of 300 ml of the blood of this same donor the recipient s plasma dilution

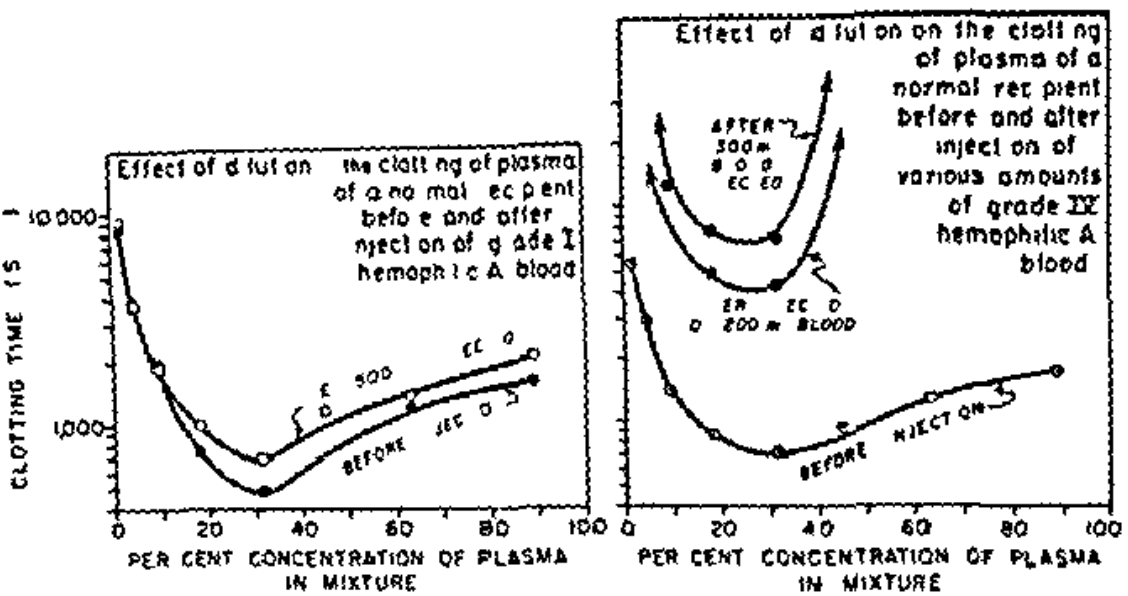


FIG 5

curve was higher clotting being first observed at 7 145 sec in a plasma concentration of 32%

Clot Delaying Activity Addition of relatively small amounts of hemophilic A platelet poor plasma to normal platelet poor plasma followed by proper recalcification will cause a definite measurable delay in the clotting time of the normal plasma provided the tests are carried out under rigidly defined conditions (7) and a high plasma concentration in the clotting mixtures is maintained (Table 2) Plasma obtained from the recipient during and after transfusion of 3 grades of hemophilic blood was added in varying amounts to the recipient s own pretransfusion plasma When 500 ml of the blood of a Grade I hemophiliac were injected into a normal recipient almost equal parts of the posttransfused plasma were required to delay significantly the clotting time of the patient s own plasma

This is the only test that disclosed a clot delaying effect of injecting Grade I hemophilic blood Since the mixtures are done with platelet poor plasma the low concentration of platelets may allow any anticoagulant effect even though slight to manifest itself When 300 ml of Grade III hemophilic blood were used one part of the normal recipient s posttransfusion plasma prolonged the clotting of 9 parts of his own pretransfusion plasma from 1 525 sec to 3 820 sec When 200 ml of blood from a Grade IV hemophilic donor were employed one part of the normal recipient s posttransfusion plasma delayed the clotting of twenty parts of his own pretransfusion plasma from 1 700 sec to 7 350 sec (Table 4)

Table 4

THE CLOT DELAYING EFFECT OF MIXING POSTTRANSFUSION RECIPIENT S PLASMA WITH THE RECIPIENT S OWN PRETRANSFUSION NORMAL PLASMA FOLLOWING INJECTION OF VARIOUS GRADES OF HEMOPHILIC A BLOOD INTO THE NORMAL RECIPIENT SILICONE COATED TUBES 38 C

	Normal Recipient s Plasma			*Clotting Time (Sec) of Mixture After Transfusion of Hemophilic Blood of*		
Tube No	*Pre Transfusion (ml)*	*Post Transfusion (ml)*	*0 2 M CaCl (ml)*	*Grade IV*	*Grade III*	*Grade I*
1	0 5	0 0	0 05	1700	1525	1470
2	0 475	0 025	0 05	7350	——	——
3	0 45	0 05	0 05	>86 000	3820	1550
4	0 40	0 10	0 05	>86 000	10 000	1570
5	0 30	0 20	0 05	>86 000	>86 000	1770
6	0 20	0 3	0 05	>86 000	>86 000	1860
7	0 1	0 4	0 05	>86 000	>86 000	1980
8	0 0	0 5	0 05	>86 000	>86 000	2120
Total amount of blood injected (ml)				300	400	500
Estimated blood volume of recipient (ml)				5 280	6 160	5 440
Approximate dilution of transfused blood by recipient s blood				1 18	1 15	1 10

*Clotting times of substrates (pretransfusion normal plasma)

Other Changes With the exception of fibrinogen there were no other significant changes in the clotting factors after the transfusions (Table 5) When a hemophilic Grade IV donor with a relatively high fibrinogen (530 mgm %) was employed there was a prompt significant rise in the recipient s fibrinogen level which was maintained for at least 3 hours (Fig 6)

There were no immediate or remote systemic reactions observed in the recipients In two of the thrombocytopenic recipients there was moderate bleeding from the gums and hematomas formed at the puncture sites after the needles were removed requiring compression bandages The hemophilic donors themselves felt grateful for the privilege of being able to give blood to the patients most of whom were anemic and needed blood

Table 5

EFFECT OF TRANSFUSION OF HEMOPHILIC BLOOD (VARIOUS GRADES) ON THE CLOTTING COMPONENTS OF THE BLOOD OF NORMAL OR THROMBOCYTOPENIC RECIPIENTS

Period	Platelets (Thous.)		Prothrombin (Units)		Fibrinogen (mgm %)		Antihemophilic globulin (Units)		Antithromboplastin (Units)	
	Hemophilic Donor	Recipient R	Hemophilic Donor	Recipient R	Hemophilic Donor	Recipient R	Hemophilic Donor	Recipient R	Hemophilic Donor	Recipient R
1 Hemophilic (Grade IV) Blood to										
a) Normal Recipient										
Before	290	162	353	338	530	331	15	14	102	96
After		130		311		384		14		92
b) Thrombotope Recipient										
Before	290	62	34	248	510	332	15	11	100	96
After		*56*		*248*		*478*		*11*		*98*
2 Hemophilic (Grade III) Blood to										
a) Normal Recipient										
Before		344	330	375	435	296	15	15	10	100
After		282		375		340		15		100
b) Thrombocyte Recipient										
Before		40	360	263	470	509	15	1 8	110	90
After		30		275		540		13 7		88
3 Hemophilic (Grade I) Blood to										
a) Normal Recipient										
Before	54	190	323	293	348	335	14	13	112	108
After		1 0		311		353		13		108
b) Thrombocytope Recipient										
Before	254	60	323	375	348	508	14	16	112	116
After		40		375		550		15		118

Discussion

The hemophilic donors had not with one exception had any blood transfusions for at least one year Attempts to detect in their blood antibodies against clotting factors yielded negative results (12) Evidence is lacking therefore that acquired abnormal circulating anticoagulants existed in the hemophilic blood It seems more likely that the blood contained an excess of a normally circulating anticoagulant and that the prolongation of the clotting time of the recipient s blood resulted from the addition of this excess The effect was more evident naturally in thrombocytopenic recipients since platelets provide the natural antagonists for the first phase inhibitors In the absence of platelets the anticoagulant becomes the dominant factor in determining the rate of clotting

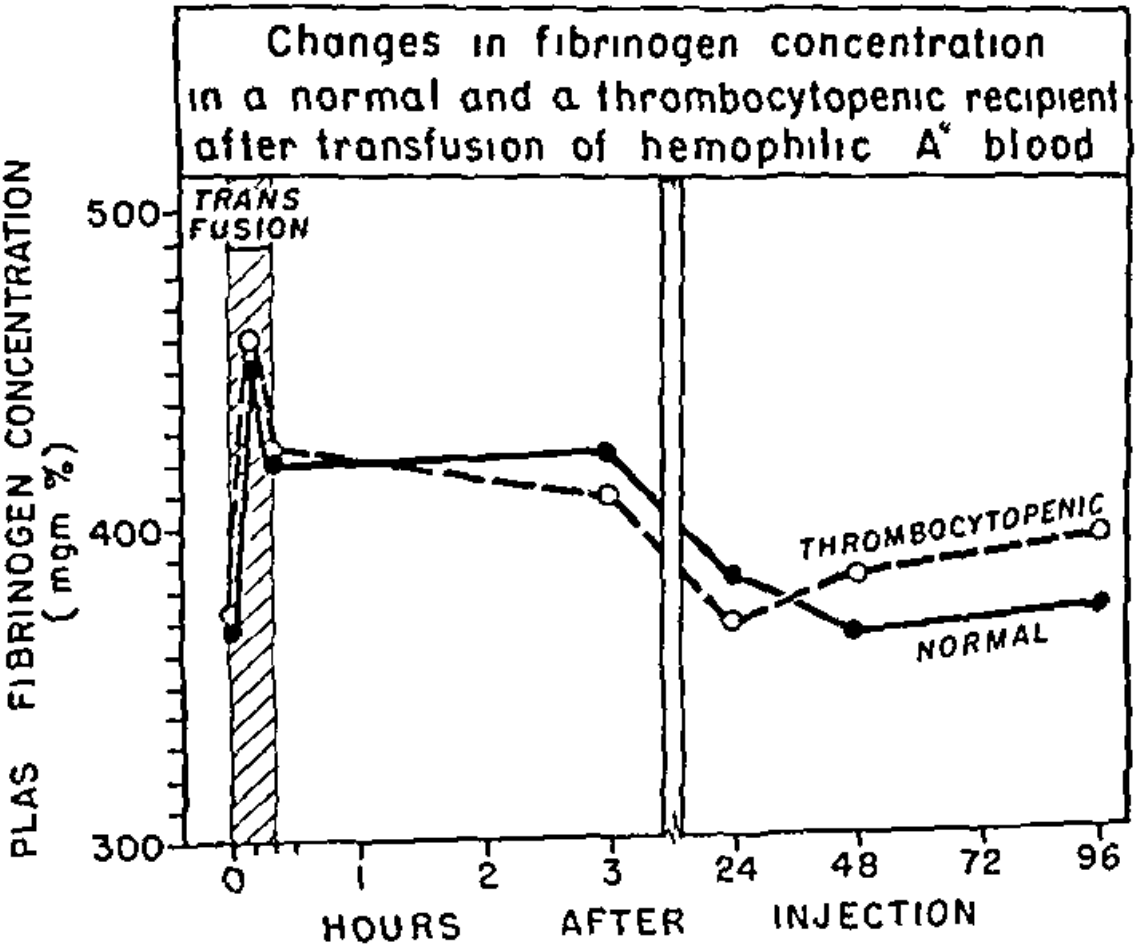


FIG 6

The lasting anticoagulant effect of the transfusions especially in the thrombocytopenic recipients suggests that such transfusions may be tried in the therapy of thromboembolic disorders The source of the first phase inhibitors found in such excess in hemophilia A blood is of course un known Concentrates of this material obtained from tissues such as the brain may prove valuable for this purpose However though a lipid anti thromboplastin obtained from brain (13) blocks the formation of plasma thromboplastin as well as neutralizes it after it is formed there is as yet no evidence that it is the same as or related to the natural plasma anti thromboplastin

It seems needless to emphasize that in order to obtain the results re ported a faultless technique must be employed This applies especially to the collection of blood for transfusion and the samples for study Even with the most severe hemophiliac a clot delaying effect may not be demon strated if the usual conventional methods for blood collection are used Recently we removed 350 ml of blood from one of our Grade IV hemo philiacs through a plastic tube into a siliconized vacuum bottle containing 150 ml of ACD solution The plasma was removed after centrifugation

and placed in a siliconized bottle The entire operation took approximately one hour The clotting time of this plasma was only slightly longer than that of a normal control while the whole blood clotting time measured in a specimen collected independently from another vein was longer than four hours Imperfect collection dilution of blood and plasma frothing and standing of the blood probably accounted for the altered state of the plasma

Summary

Blood from individuals with hemophilia A of varying degrees of severity was injected directly and swiftly into the veins of normal and thrombocytopenic recipients A clear lasting prolongation of the clotting time of the blood of both types of recipients followed such injections the delay in general being directly proportional to the severity of the hemophilia and the amount of blood injected Blood from hemophiliacs with Grade I defect produced no significant changes Grade II blood altered the rate of clotting of the thrombocytopenic recipients but not that of the normal Testing a mixture of posttransfusion recipient s plasma with his own pre transfusion plasma disclosed most clearly the clot delaying effect of the transfusion

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7

The Significance of the So called "Bridge Anticoagulant'

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IT WAS recently reported by English authors (1) that an anticoagulant activity can be detected in the plasma of patients with hemophilia A and B This activity is claimed to be due to a previously unknown factor termed Bridge anticoagulant which is absent in serum and inhibits both thromboplastin generation and its reaction with prothrombin According to the authors (1 2) this inhibitor plays a role in the pathogenesis of hemophilic hemorrhages as well as antihemophilic factor deficiencies

I have re-examined the findings of Nour Eldin and Wilkinson and have tried to understand what they actually mean First of all I have reproduced their experiment with the same simple method which employs the thromboplastin generation test (TGT) A blood thromboplastin that is incomplete for it was generated with deficiencies of antihemophilic factors A or B gives longer clotting times on substrates of hemophilic plasma than on normal plasma (Fig 1) This is true only if the hemophilic plasma lacks the same factor that is deficient in that thromboplastin (Fig 2) If thromboplastin is deficient of both antihemophilic factors A and B the clotting time of plasma substrates from both A and B hemophilic patients is prolonged (Fig 3)

It is important to establish whether this phenomenon is due actually to the presence of an inhibitor in hemophilic plasma or it may bear an alternative interpretation

It can be ascertained in a preliminary way that no anticoagulant activity can be detected when $BaSO_4$ treated hemophilic plasma of A or

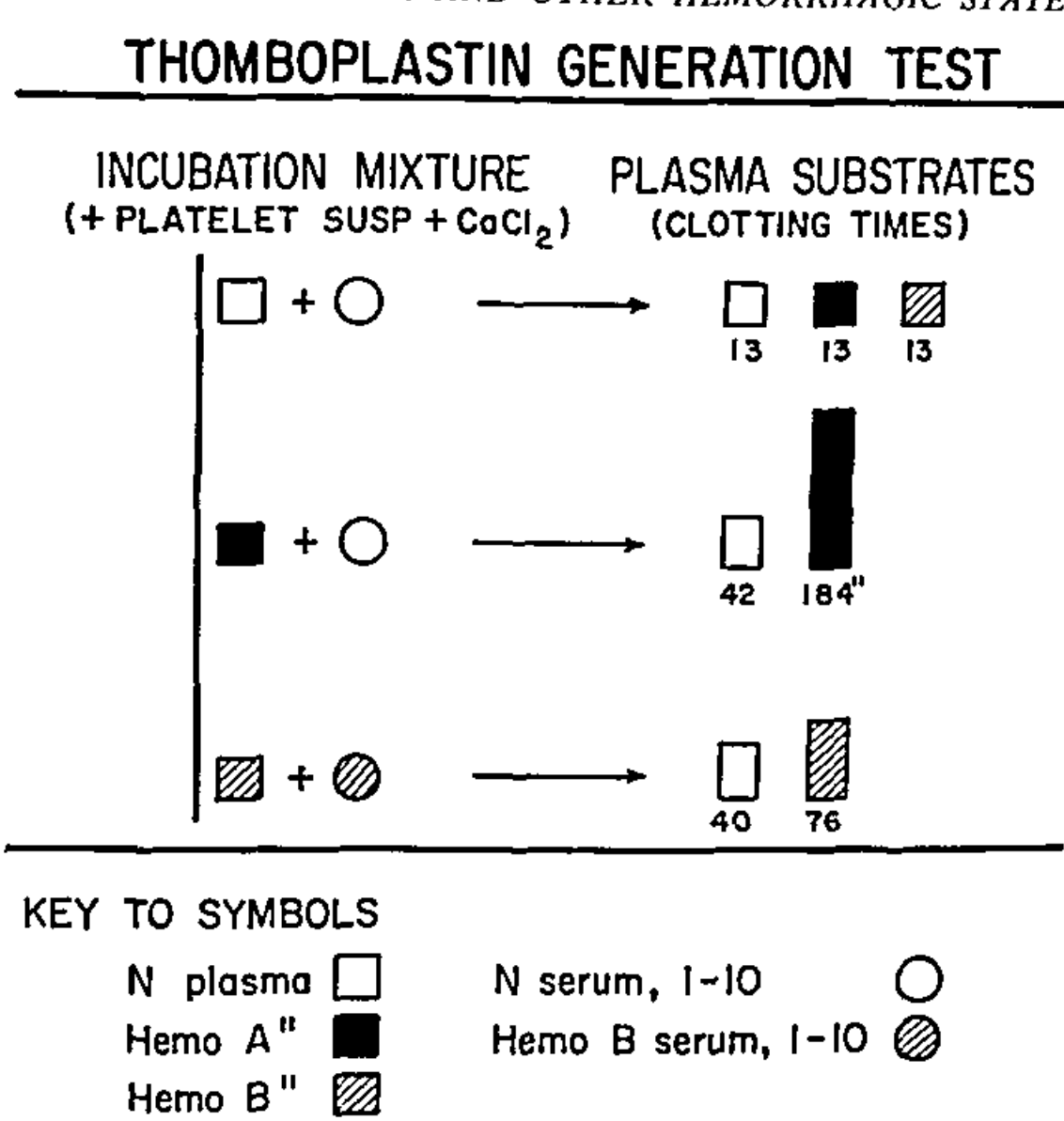


FIG 1 Blood Thromboplastin Generated with Deficiencies of Anti hemophilic factors A or B in the incubation mixture of a TGT clots a *substrate of hemophilic plasma in a longer time than normal plasma* Normal and hemophilic plasma in the incubation mixture is diluted 1 5 the plasma of substrates is undiluted Symbols apply to Figs 1 10 incl

B type is added to an incubation mixture complete with all normal reagents in a TGT (Fig 4)

Moreover when the experiment of Nour Eldin and Wilkinson is re peated using as a substrate a mixture of two plasmas each deficient in a different antihemophilic factor the clotting time of this mixture is the same as of normal plasma even if thromboplastin was generated with a deficiency of both antihemophilic factors (Fig 5) Such a finding could not be obviously obtained if any anticoagulant was in hemophilic plasma

The phenomenon disappears also if normal plasma or hemophilic plasma of a different type is added either to the incubation mixture or to the substrates of hemophilic plasma provided that a concentration of 20%

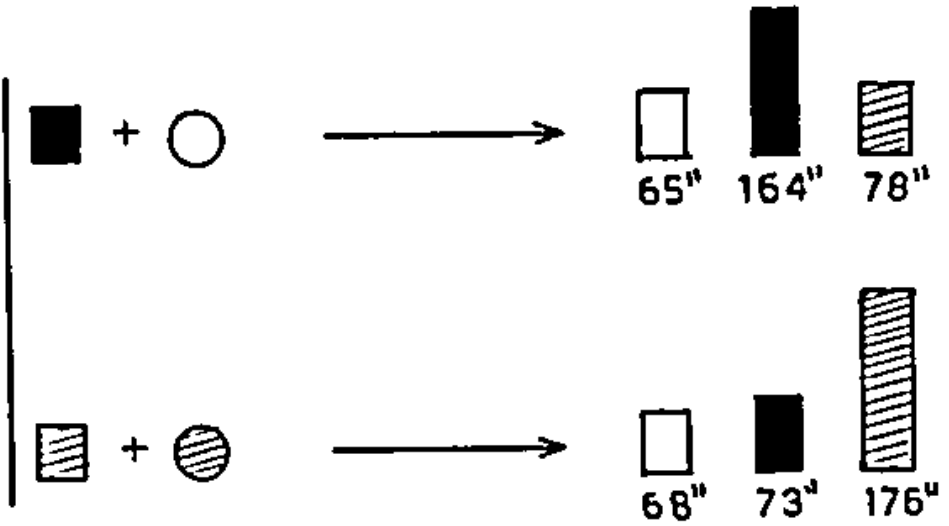


FIG 2 An Incomplete Thromboplastin Clots a Substrate of Hemophilic plasma in a longer time than normal plasma only when the substrate lacks the same antihemophilic factor that is deficient in the incubation mixture of TGT if thromboplastin lacks antihemophilic factor A (AHG) the CT of hemophilia A plasma is prolonged and that of hemophilia B plasma is normal On the contrary if thromboplastin was generated without AHF B (PTC) it clots hemophilia A plasma in the same time as normal plasma but hemophilia B plasma in a much longer time

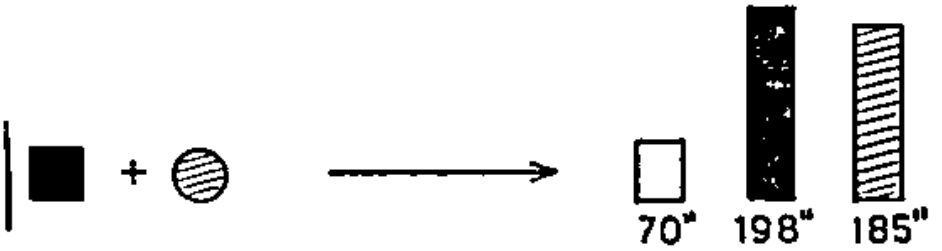


FIG 3 When the Incubation Mixture of a TGT is Deficient of both A and B antihemophilic factors the CT of substrates of both A or B hemophilic plasma is prolonged

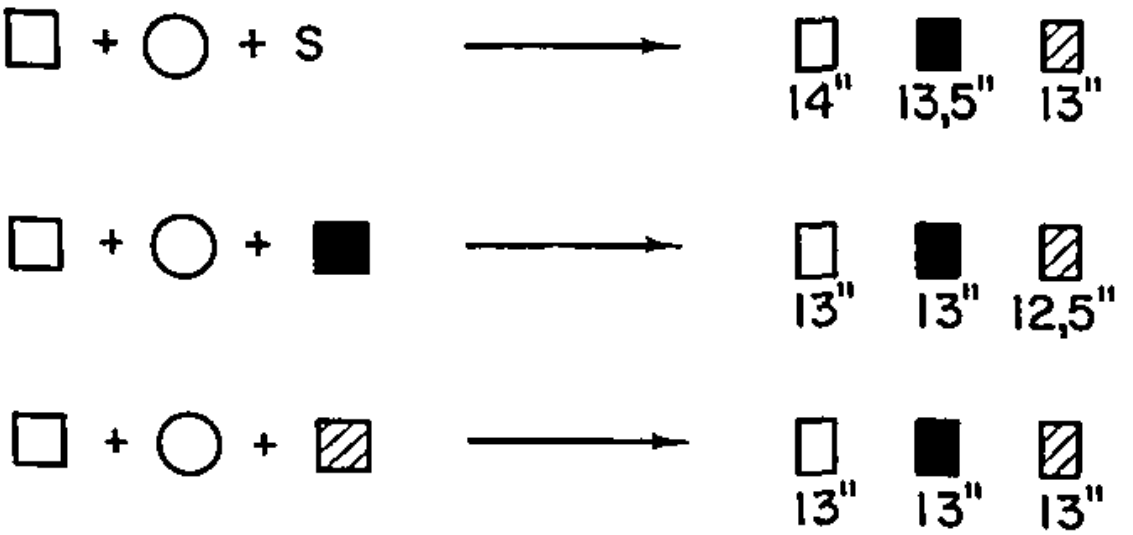


FIG 4 The CT of Plasma Substrates of Hemophilia A or B is Normal when $BaSO_4$ treated hemophilic plasma is added to an incubation mixture of a TGT containing all normal reagents (S = saline)

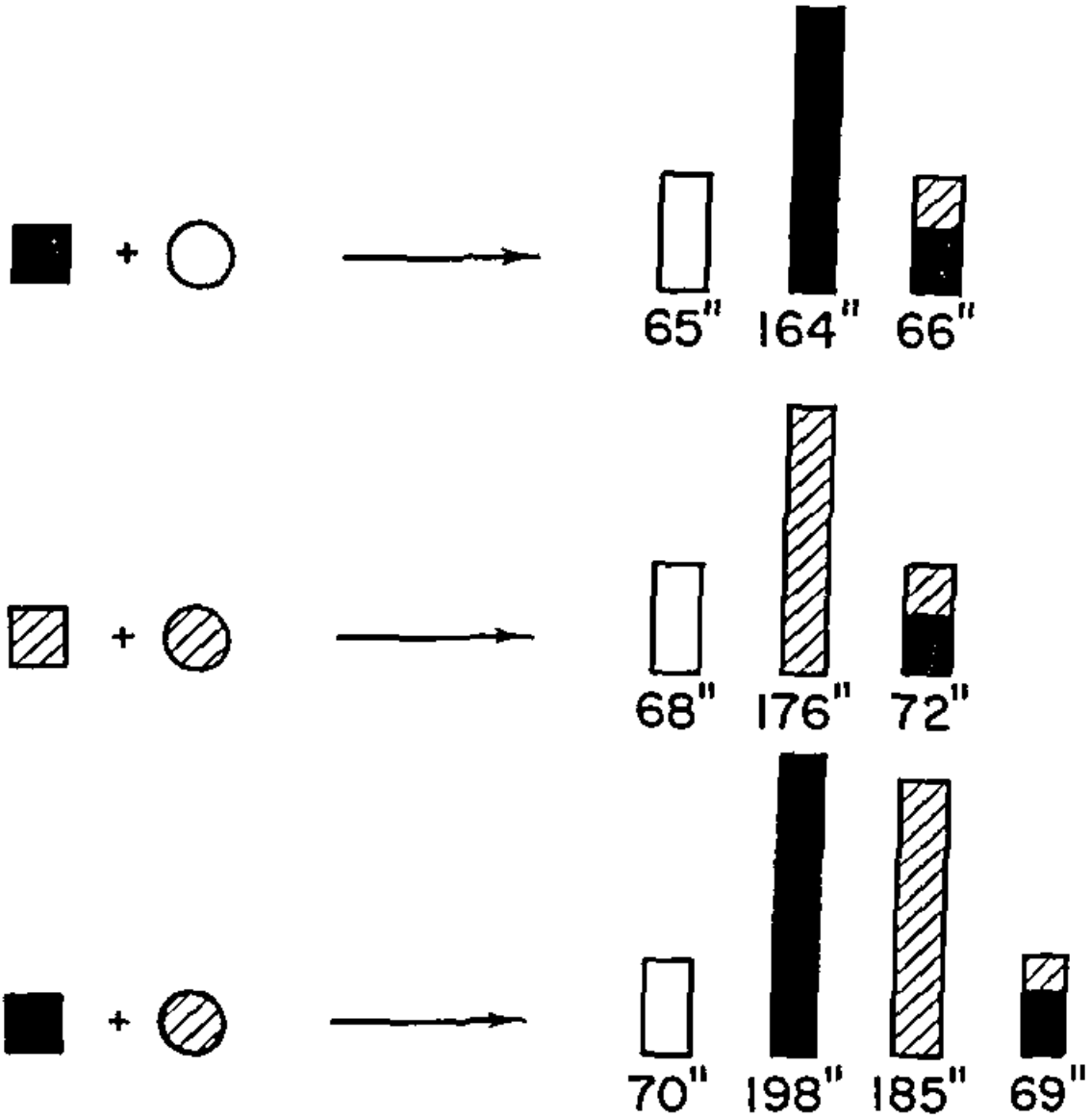


FIG 5 An Incomplete Thromboplastin Clots a Mixture (1 to 1) of *hemophilic A and B plasmas in the same time as normal plasma also* when the incubation mixture of TGT lacks both antihemophilic factors

of the plasma containing the antihemophilic factors either in the mixture or in the substrates is reached (Figs 6 7)

According to Nour Eldin further evidence of the presence of Bridge anticoagulant in hemophilic plasma should be supported by the following experiment when undiluted hemophilic plasma is substituted for the 1 5 diluted plasma in the incubation mixture of a TGT a greater inhibition of thromboplastin occurs (*Fig 8) But this experiment is not probatory* according to our findings The inhibitory effect of undiluted hemophilic plasma depends on platelet concentration in the incubation mixture and disappears if a concentrated platelet suspension is used with a weak platelet concentration even an undiluted normal plasma exerts some inhibi tory effect (Table 1) If undiluted $BaSO_4$ treated normal plasma is substi

Table I

INHIBITORY EFFECT ON THROMBOPLASTIN GENERATION OF UNDILUTED NORMAL OR HEMOPHILIC PLASMA WITH CONCENTRATED OR DILUTED PLATELET SUSPENSIONS THROMBOPLASTIN GENERATION TEST

Platelet Suspension Used	*$BaSO_4$ Treated Plasma in the Incubation Mixture*			
	of Hemophilia A Patients		*Normal*	
	Diluted 1 to 5	*Undiluted*	*Diluted 1 to 5*	*Undiluted*
	Shortest Clotting Time of Normal Plasma Substrates (sec)			
Concentrated	19	19 5	12	17 7
Diluted 1 to 20	32 7	48	17 9	21

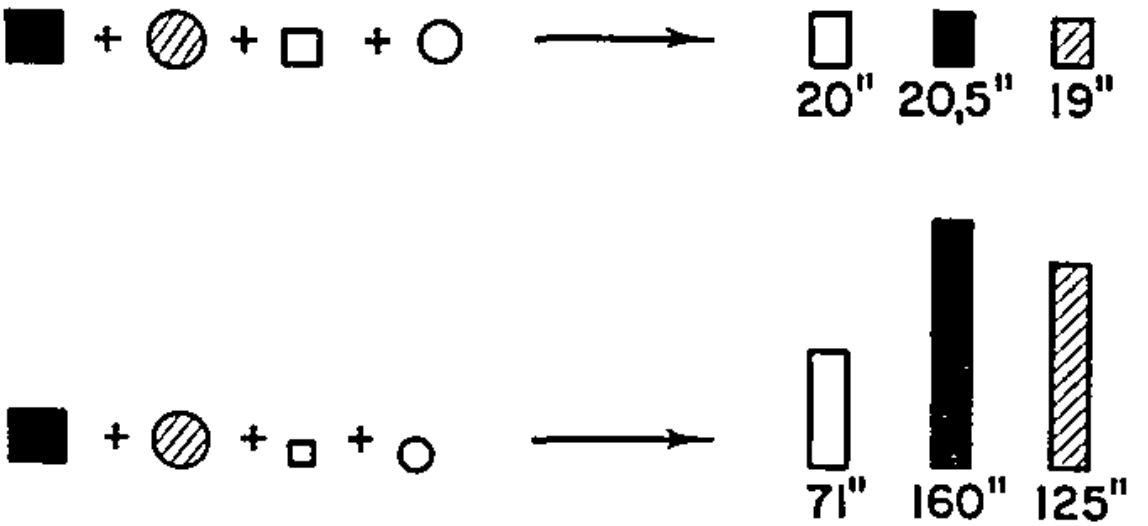


FIG 6 The CT of Hemophilic Plasma is the Same as Normal Plasma when normal plasma and/or serum is added to the incomplete thromboplastin provided that a 1 to 4 ratio between normal and hemophilic plasma and/or serum in the incubation mixture is reached (upper figure) When the normal reagents are added to the incubation mixture in a lesser ratio the difference between the CT of the hemophilic plasma substrates and that of normal plasma is still evident

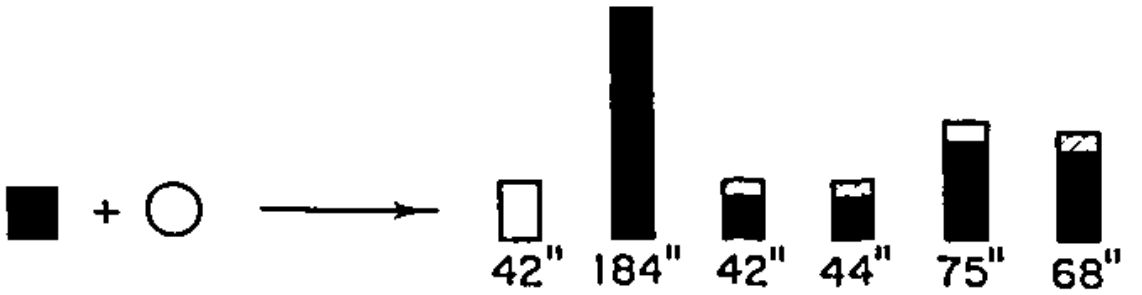


FIG 7 When Normal Plasma or Hemophilic Plasma of a Different type is added to a substrate of hemophilic plasma in a ratio of 1 to 4 or higher an incomplete thromboplastin clots the substrate in a normal time if the level of AHF in the substrate is 10 of normal plasma the CT of the substrate is slightly prolonged

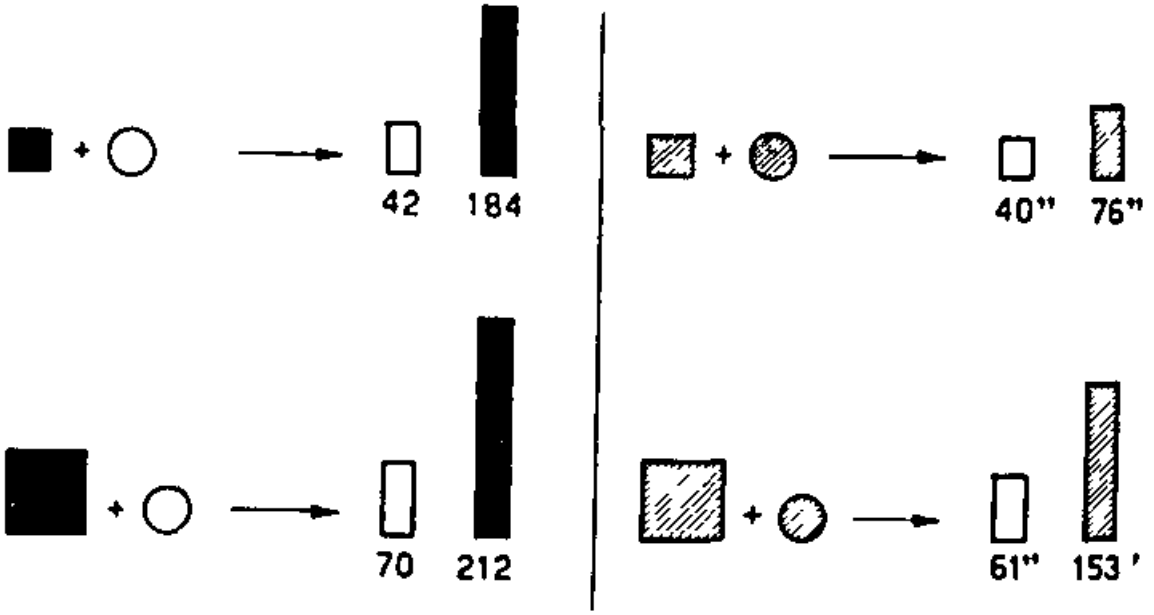


FIG 8 When Undiluted Hemophilic Plasma Is Substituted for the 1 to 5 diluted hemophilic plasma in an incubation mixture the CT of a hemophilic substrate is more prolonged

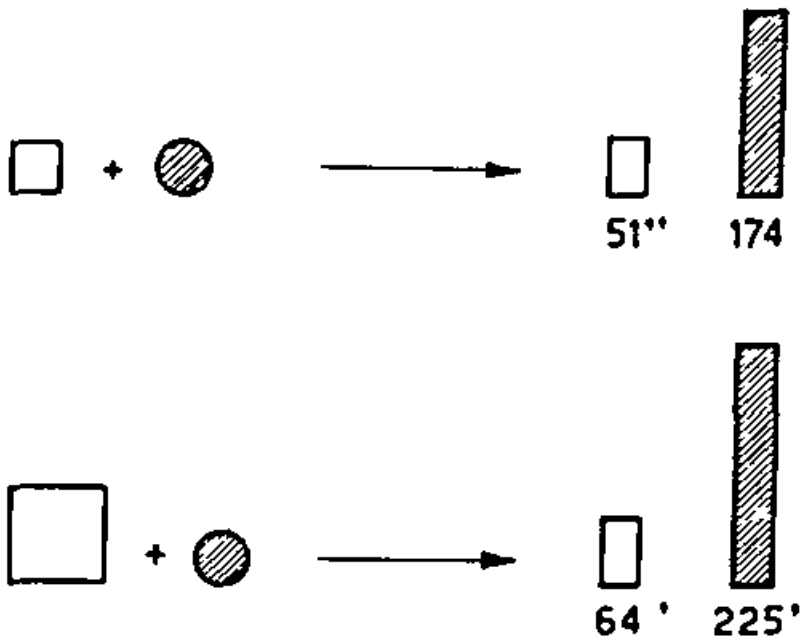


FIG 9 The CT of a Substrate of Hemophilia B Plasma markedly increases when undiluted normal plasma is sub stituted for the diluted plasma in the incubation mixture

tuted for diluted plasma in an incubation mixture lacking PTC the clotting time of the substrates is lengthened still more (Fig 9) As Biggs (3) showed a marked inhibition occurs when $BaSO_4$ treated normal serum is added to the incubation mixture of the TGT (Fig 10)

It follows from these data that even the thromboplastin inhibition by undiluted hemophilic plasma is not due to any anticoagulant but probably

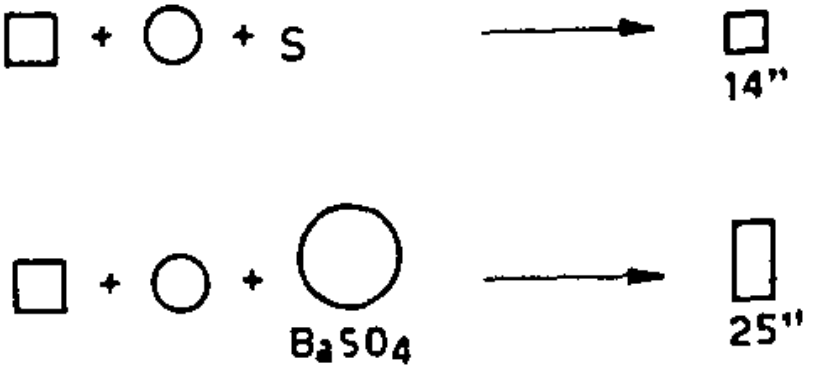


FIG 10 The Inhibitory Effect on Thromboplastin Generation of undiluted $BaSO_4$ treated normal serum added to the incubation mixture of a TGT (S = saline)

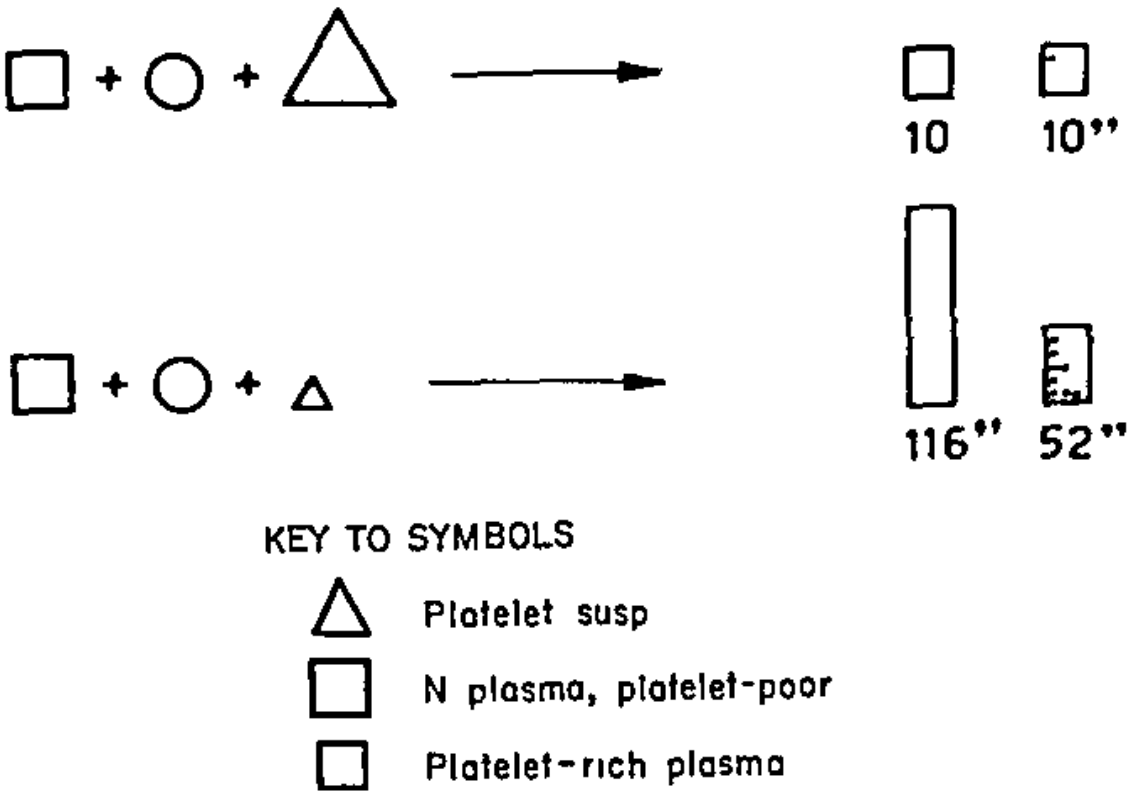


FIG 11 An Incomplete Thromboplastin Generated with a deficiency of platelet factor clots platelet poor plasma in a longer time than platelet rich plasma

to a change of the optimum ratio between the different thromboplastic factors chiefly between the protein and phospholipid constituents of thromboplastin (4 5)

Consequently it appears that a different and more likely interpretation of the phenomenon reported by Nour Eldin and Wilkinson may be taken into consideration An incomplete thromboplastin causes any plasma containing the factor(s) that it lacks to clot in a shorter time than plasma

lacking the same factor because in the former condition thromboplastin can be completed and further activated by the substrate plasma Such a phenomenon concerns not only the deficiency of antihemophilic factors but even that of other thromboplastic factors Indeed it can be proved that when thromboplastin is generated with a deficiency of platelet factor it clots platelet rich plasma in a shorter time than platelet poor plasma (Fig 11) This point of view is supported also by the research of Hougie (6)

In conclusion the phenomenon reported by Nour Eldin and Wilkinson reflects peculiar experimental conditions of in vitro testing for thrombo plastin generation but it cannot provide any reliable information on the pathogenesis of hemophilia No conclusive evidence of the presence of any inhibitor in hemophilic plasma appears to be available on the contrary the results of the present paper and even the findings of the English authors are actually in accord with the deficiency theory

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8

Female Hemophilia

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WE WISH to report two cases of female hemophilia observed by us amongst 135 cases of hemophilia followed during the past 26 years These cases can be added to the female hemophiliacs observed by Merskey (1) Israels (2) Pinninger and Franks (3) Quick and Hussey (4 5) and Pavlovsky (6 7) (reference to one of the cases) as well as Biggs and Macfarlane (8) for hemophilia B The remarkable studies of Brinkhous and colleagues on female hemophilia in dogs (9 10) led to the acceptance of the possibility of its existence in humans Doubts as to the veracity of the cases reported in the past are understandable since the clinical observations of kindreds of hemophiliacs have led to the conclusion that only the males could inherit the tendency to bleed Also it was believed that female hemophiliacs could not exist because the double dose of the hemophilic gene which would occur in the female bleeder would be lethal (11)

Macklin in 1928 established the mode of transmission of hemophilia as accepted at present demonstrating the possibility of the existence of female bleeders (12) [well summed up recently by Quick (13)]

Pallard Jeune and Revol (14) of the Lyonnaise school have made a critical review of the reported cases of female hemophilia Weil believes that only a few of the cases can be considered as this form of hemophilia (15) The rareness of these cases is explained by the fact that the disease can only exist in daughters of hemophilic males and female carriers both contributing an X chromosome containing the hemophilic gene Thus in

the studies of Skold on hemophilia in Sweden (16) of van Creveld on 99 hemophiliacs in Holland (17) and of Moor Jankowsky and colleagues on the Tenna kindred (hemophilia B) (18) no female cases are reported although these authors admit theoretically its existence Delcroix in 187 cases studied has not observed any female hemophiliacs but has 4 cases of female angiohemophilia (19) Stefanini and Dameshek have not seen any cases (20) Possibly in the future the observation of female bleeders will be more frequent due to the better care and treatment of the hemo philiacs More of them will live to adult age in good physical condition and will marry (7 21)

We have amongst our patients two cases of female bleeders daughters of male hemophiliacs A The mothers in both cases have no family history of a hemorrhagic tendency we therefore suppose they are carriers by mutation

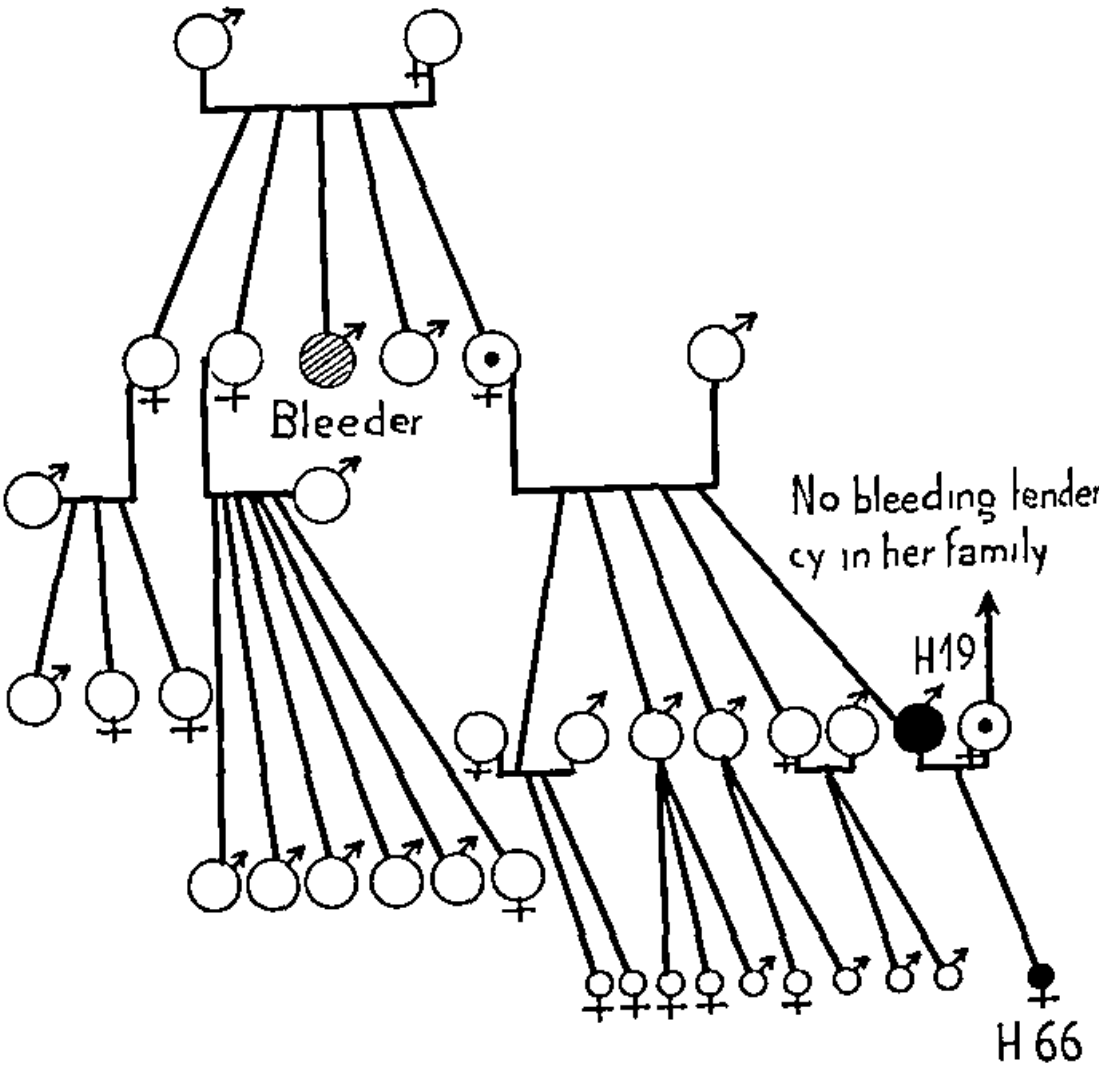


FIG 1 Pedigree of Case No 1

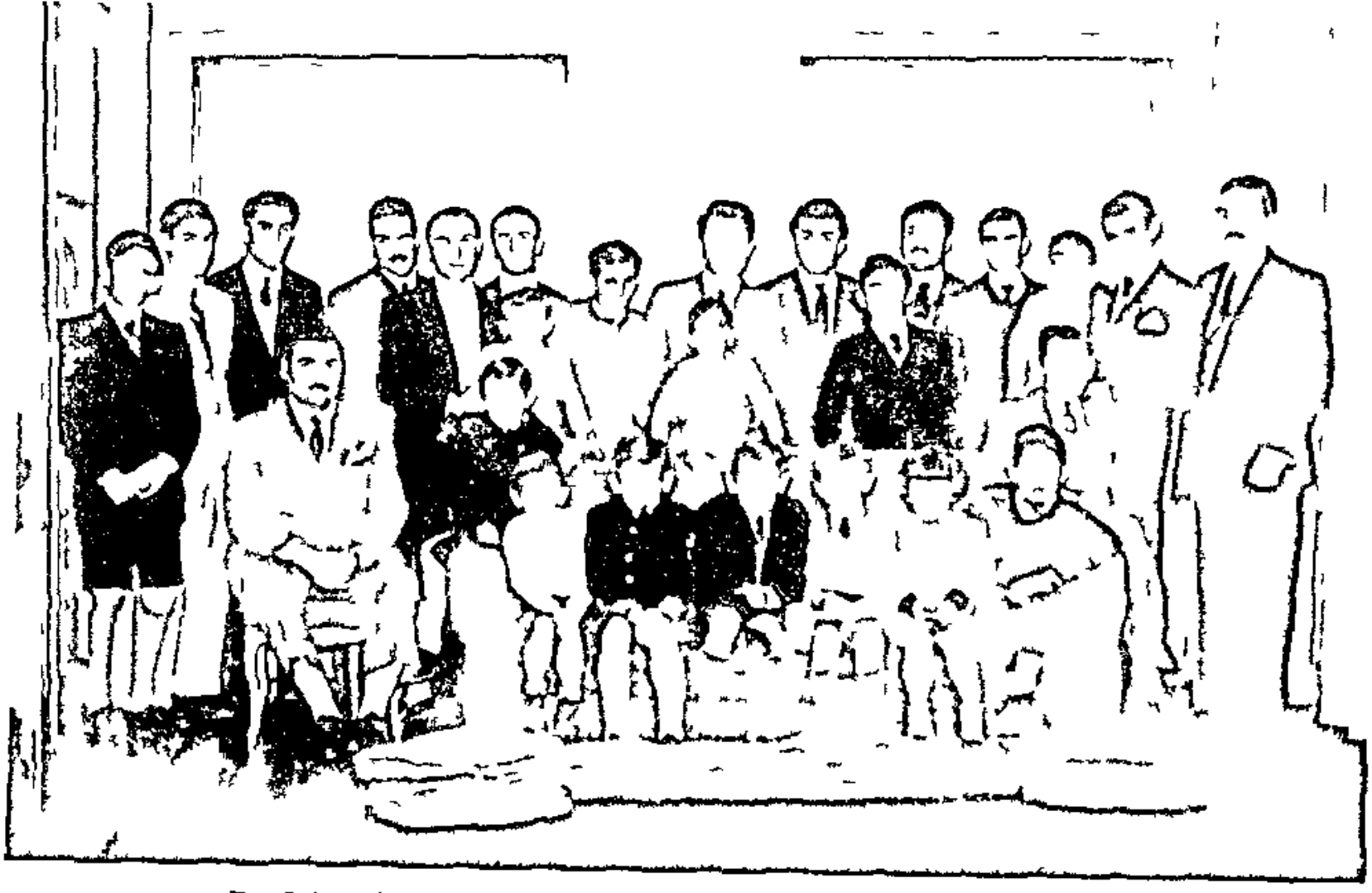


FIG 2 Snapshot of Some of Our Hemophiliacs Presented to the Conference on Hemorrhagic Diseases (Marquette University Prof Quick) May 21 1954 Among them is our female hemophiliac Case 1 H66 when she was 4 years old

Case Studies

Case No 1—H66 Name C R female age 8½ years

Family History Her father is a hemophiliac (A) Her mother is normal with no hemorrhagic family history (See Figs 1 and 2)

Clinical History The patient is an only child born after a normal pregnancy and delivery When she was 6 months old hematomas developed in one arm followed by others in her four limbs At the age of 15 months hemorrhage from the frenum of the upper lip and a month later a hematoma on her head developed When she was 2 years old a severe blood effusion in the floor of the mouth and tongue developed and spread to the tonsils neck and supraclavicular fossa As a consequence of this hematoma she experienced difficulty in eating and in speaking At this time she was given one blood transfusion (150 ml) In 1954 (4 years old) hemarthrosis occurred in her left knee In October 1955 bronchitis petechiae and hematuria were observed In April 1956 an effusion in her left knee was accompanied by pain She was given Prednisone 5 ml a day for 3 months As a consequence of the synovial tumefaction she could not extend her leg completely for 3 months The synovial tumefaction persisted for another 5 months After that the recuperation of her knee was complete In December 1956 effusion occurred in her left foot In May 1957 a new effusion developed in the calf of her left leg In 1958 bloody effusions occurred in ankles knees and right thigh along with hematuria After treatment with autovaccines she experienced clinical improvement Coagulation tests are shown in Table 1 and Fig 3

Table 1

COAGULATION TESTS

	H66 (Daughter)	*H19 (Father)*
Bleeding time	2 min	2 min 30 sec
Tourniquet test	Neg	Neg
Platelets	240 000 per mm	210 000 per mm
Plat morphology	Normal	Normal
Clot retraction	Good	Good
Clotting time	14 min Varied since 1951 58 from 11 to 28 min	16 min Varied since 1946 58 from 14 to 28 min
Prothrombin time	100%	98%
Prothrombin consumption test	7 sec	
Howell time	7 min	
Dosage of AHF—Factor A (VIII)	0 5%	0
—Factor B (IX)	84%	87%
Classification	Hemophilia A	

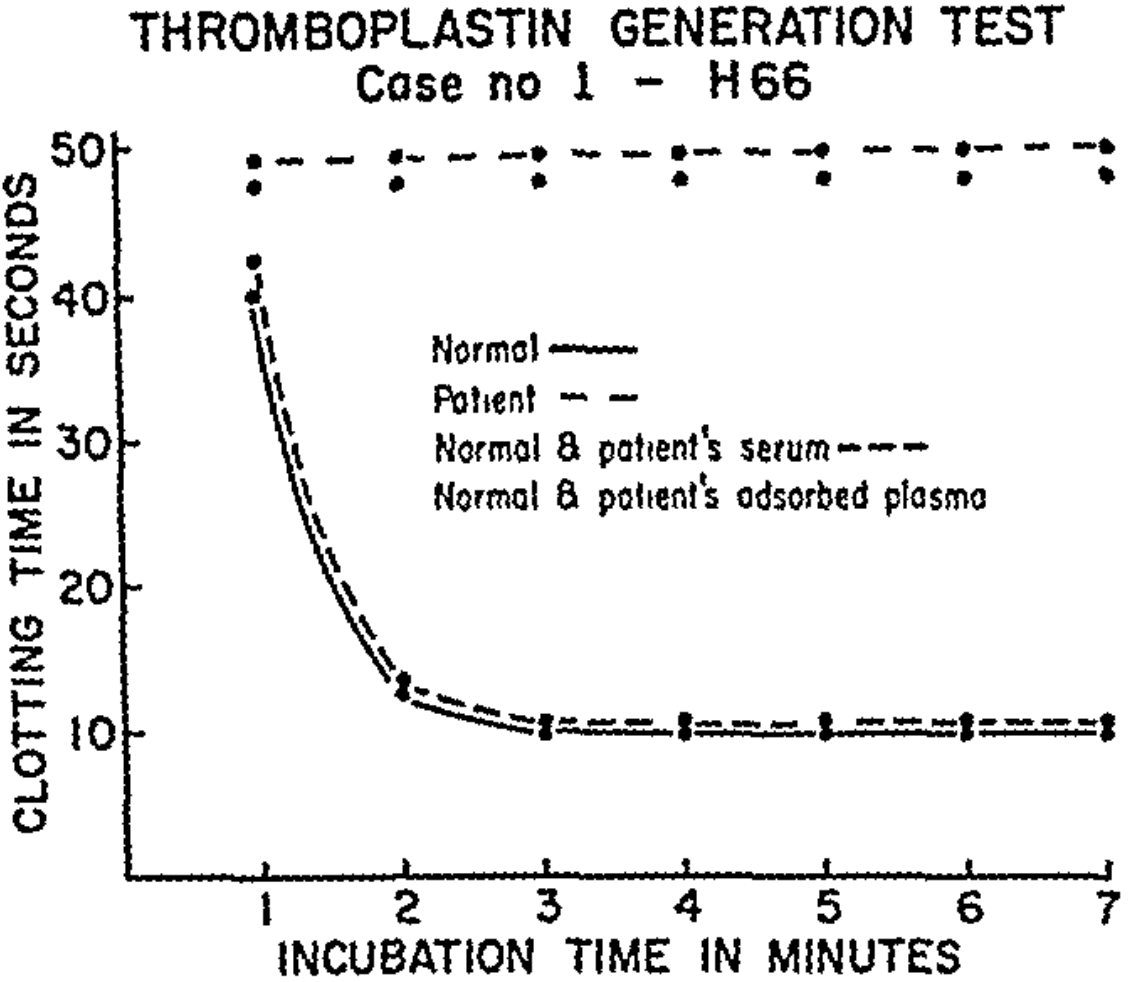
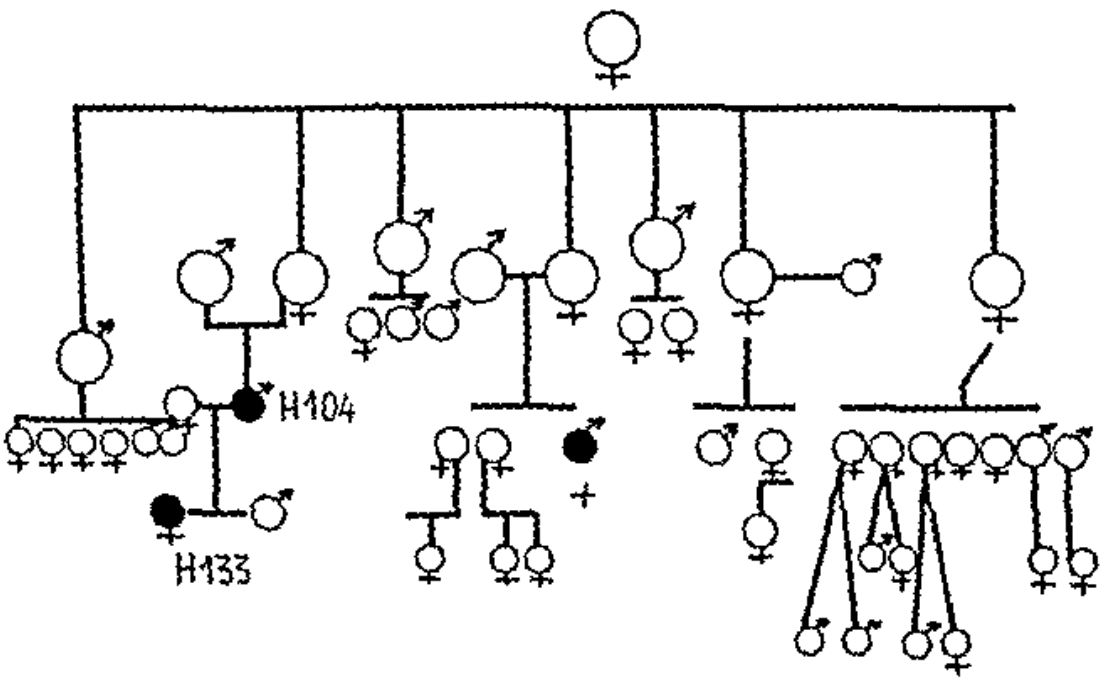


Fig 3 TGT on Female Hemophiliac Case No 1 In Addition results with all patient s components using cephalin instead of pa tient s platelets were the same as patient curve in graph above



No data could be obtain further back of 2 generations

Fig 4 Pedig ee of Ca e No 2

We consider that the hemorrhagic manifestations of father and daughter are similar They are both classified as hemophilia A medium severity We conclude that she has inherited the type of hemophilia from her father (the same variety A and the same degree of intensity)

Case No 2 —H133 Name O del R G female age 11 years

Family History (See family pedigree Fig 4) Her father is a hemophiliac H104 A first cousin of her father (by his mother) died of hemophilia Her mother was normal with no bleeding tendency in her family The patient has a brother 9 years old who has not had any hemorrhagic problems

Clinical History She was born normally No bleeding occurred from umbilical cord or during dentition Her first traumatic hematomas appeared when she began to walk Periodically hematomas have occurred since then After a tooth extraction when she was 5 years old she suffered a profuse hemorrhage which caused a severe anemia No blood transfusion was given After two other tooth extractions she again had severe hemorrhages which stopped with local treatment Recently a large traumatic hematoma developed in the wall of the abdomen Coagulation tests are shown in Table 2 and Fig 5

Table 2

COAGULATION TESTS

	H133 (Daughter)	*H104 (Father)*
Bleeding time	1 min	7 min (r e) 1 min 20 sec (l e)
Tourniquet test	Neg	Neg
Platelets	135 000 per mm	200 000 per mm
Clot retraction	Normal	Normal
Clotting time	18 min	11 min
Prothrombin time	100%	100%
Prothrombin consumption test	8 sec	14 sec
Howell time	3 min	3 min 40 sec
Dosage of AHF—Factor A	7%	0 2%
—Factor B	76%	55%
Classification	Hemophilia A	Hemophilia A

The father of the patient H104 36 years old has the classical form of hemophilia considered as moderately severe with many blood effusions into the right elbow left knee and both ankles Before the diagnosis of hemophilia was made (in 1957) he was treated as rheumatic arthritis Since 1948 he has had several gastrointestinal hemorrhages and bouts of hematuria In the coagulation tests note that his BT (7 min) is prolonged in the right ear but it is normal in his daughter

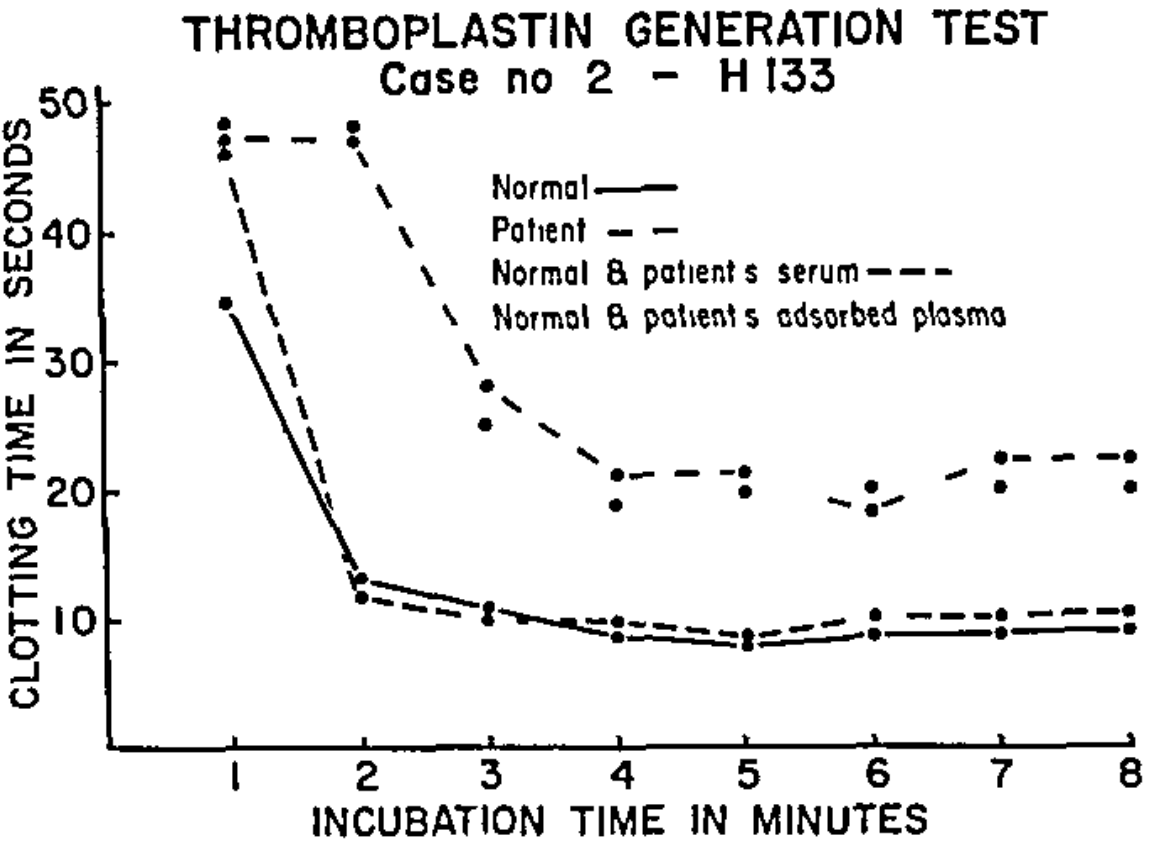


FIG 5

Discussion and Conclusions

The acceptance of the existence of true hemophilia in females has led to new studies to clarify older concepts on inheritance Logically these cases will be observed more frequently in the future as hemophilic males attain adult age in good physical condition and can therefore marry Some of the cases reported seem to transgress the classical laws of inheritance In the cases of Merskey for instance the disease seems to have been transmitted directly from male to male in the first generations In one of our families all the members of the first generation (H1 H2 another hemophilic male who died and a female carrier) were affected In the second generation all the males i e sons of the female carrier are hemo philiacs Of course this could be due to chance

Also there are increasing numbers of sporadic cases reported in the literature which leads to the conclusion that the number of carriers due to mutation is higher than suspected

The fact that the two cases reported in this paper are daughters of two of our hemophilic males (most of them are just arriving at adult age) who have married carriers by mutation leads to the suspicion that the laws of inheritance of this disease are not operating exactly as we believe they should However it can be a mere coincidence

In the cases of angiohemophilia (the association of mild hemophilia with a prolonged BT) we see that the transmission differs from classical hemo

philia as both sexes can inherit the disease Weil had already described this type of transmission in cases of hemophilo hemogenie The study of these cases such as the patients reported by Willebrand in the Åland Islands shows a moderate deficiency of AHF (Jorpes) that differs from classical hemophilia In other cases reported the deficiency of AHF is greater A case studied by us (which will be published separately) female angiohemophilia with the clinical picture of typical hemophilia (severe hemorrhages and hemarthrosis) has a severe deficiency of AHF

We see that although there are slight differences in the mentioned cases some have fixed laws of inheritance (hemophilia) and the others have different characteristics in the hereditary transmission These facts lead to the following reasoning must we accept inflexibly the laws of inheritance of hemophilia—in which case we have to accept mutations as being more frequent than we thought? Or do we have to revise some of the classical concepts of the hereditary transmission? I believe that these questions will be clarified with the study of the descendants of the present generation of hemophiliacs since many will marry

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9

A Simple Way of Distinguishing Between Hemophilia A and B

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THE TGT generally used and suggested by Biggs and Macfarlane is relatively complicated and requires much time The test can be replaced by a simpler and faster method described below First we want to make some theoretical remarks

It was a pure coincidence that our first 10 cases of hemophilia all happened to be cases of hemophilia B (Christmas disease) Since the publication in 1935 of our first paper dealing with blood clotting we have described in our hemophilic cases the stability of the plasmatic clotting precursor which we have named prothrombokinin (prothromboplastinogen according to Quick) Later in 1943 we related the delayed coagulation to the lack of a clotting factor until then unknown and always present in normal blood We called this factor thrombokatalysin because it brings about the transformation of relatively stable plasmatic prothrombokinin to active thrombokinin

It was then our view that blood clotting is initiated not by the platelets but by a plasmatic element Not until a certain amount of thrombin has formed do the blood cells also deliver their secondary clotting elements thus starting the well known thrombin avalanche This opinion has been misunderstood by most of the authors (Owren Deutsch etc) and yet *in hemophilia A* (according to the actual nomenclature) we find tangible evidence for the mentioned opinion for in this disease in spite of the presence of thrombokatalysin the blood cells are unable to start and end coagulation in the normal time because the plasmatic clotting precursor is missing Instead if added to fresh and immediately centrifuged (cell

free) normal citrated plasma they will fully normalize the formation of thrombin once the initial plasmatic coagulation has started

We can therefore compare the plasmatic clotting precursor prothrombokinin with the role played by the percussion cap of a cartridge whilst the blood cells would represent the powder which explodes only after a certain initial temperature is achieved

The following experiment speaks very much in favor of this opinion if one part of fresh hemophilic blood is hemolyzed by adding 4 parts of distilled water clotting time remains practically unchanged In other words although the blood cells are greatly damaged in the hypotonic environment they are by no means capable of initiating blood clotting sooner This proves that they do not deliver the primary clotting precursor

The 2 following simple tests enable us to distinguish between hemophilia A and B 1 The hemophilic blood is citrated immediately after being drawn from the patient The following two test tubes are prepared in the water bath at 20 C Tube 1 1 cc of citrated blood + 1/10 part of saline solution + 1 drop of 5% CaCl Tube 2 1 cc of citrated blood + 0 1 cc of normal serum (24 hr) + 1 drop of 5% CaCl The moment calcium is added the chronometer is started and the beginning of coagulation noted It is indicated by the first appearance of little clots adhering to the oblique wall of the test tube which must be periodically rotated slowly around its longitudinal axis Thirty seconds later there is added to the tube 4 cc of distilled water and a drop of a 5% solution of CaCl and the tube is shaken for a short moment About every 2 minutes the tube is shaken 3 or 4 times Immediately after each shaking (i e every 2 minutes) 0 2 cc of the content of the tube are added to 0 2 cc of normal citrated blood and the onset of coagulation in the continuously and slowly rotating oblique test tube is noted This is continued until the shortest possible clotting time is determined The latter depends directly upon the amount of thrombin formed Blood from hemophilia A as well as B will show a prolonged clotting time and deficient thrombin values in test 1 (with saline) while in test 2 (with 1/10 of its volume containing normal serum) hemophilia B (Christmas disease) will not only show a normal clotting time but also normal amounts of thrombin In contrast hemophilia A (Factor VIII deficiency) though giving an approximately normal clotting time in test 2 (with 1/10 of its volume being normal serum) shows instead a greatly diminished amount of thrombin and a definite delay in its formation (Table 1)

II For a rapid and approximate differentiation of the two types of hemophilia we also suggest the following *defibrination* test two test tubes containing 2 cc of hemophilic citrated blood are placed in the water bath at 37° C 0 2 cc of saline solution are added to the first test and 0 2 cc of stored normal serum to the second sample Immediately afterwards 2 drops of a 5% solution of CaCl are added to both tubes and the

Table 1

A SIMPLE TEST TO DISTINGUISH HEMOPHILIA A AND CHRISTMAS DISEASE

	With 1/10 Phys Saline				*With 1/10 Volume of Normal Serum (24 hr)*			
		Thrombin				*Thrombin*		
	Clotting Time	*sec*	*units*	*Time from Start*	*Clotting Time*	*sec*	*units*	*Time from Start*
Normal citrated blood (15 tests)	5	13	29	15	3 50	13	29	15
Hemophilia B (Factor IX deficiency) (4 tests)	27	21	16	60	5 16	12	32	14
Hemophilia A (Factor VIII deficiency) (7 tests)	20	34	9 2	66	9 10″	29	11	48

onset of clotting is determined in each Once clotting has started the tubes are shaken 3 or 4 times vigorously every 20 sec This leads each time to temporary defibrination The time is noted when complete defibrination has occurred This is indicated when the test mixture remains fluid after waiting 1 2 minutes or after adding a little thrombin to a portion of the test With this method we found normal clotting time to vary between 80 110 sec and defibrination time between 40 70 sec Hemophilia B offers the same results when normal serum is added while hemophilia A invariably shows in spite of an almost normalized clotting time a greatly prolonged defibrination time Without normal serum however the clotting time as well as defibrination time are distinctly increased in both hemophilia A and B (Table 2)

Table 2

DEFIBRINATION TIME IN HEMOPHILIA A AND CHRISTMAS DISEASE

	With Physiol Saline		*With 1/10 Vol of Normal Serum (24 hr)*	
	Clotting Time	*Defibrination Time in sec*	*Clotting Time*	*Defibrination Time in sec*
Normal citrated blood	1 2	40 70	1 2	40 70
Hemophilia B (Factor IX deficiency) (2 tests)	10'20	480	2 10	95
Hemophilia A (Factor VIII deficiency) (5 tests)	9'24″	306	4 55	260

10

The Early Recognition of Mild Hemophilia Diagnostic and Therapeutic Implications

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THE existence of mild hemophilic syndromes was recently ascertained and widely confirmed both for the Factor VIII (AHF) and for the Factor IX (PTC) deficiency Their clinical importance is substantiated by the fact that hemorrhagic manifestations may occur (a) in the absence of history (sporadic cases) (b) in the presence of a normal whole blood coagulation time and sometimes of a normal prothrombin utilization and (c) in the absence of typical localizations (hemophilic arthropathy) In the past the early recognition of such forms was difficult or not possible insofar as no reliable method was available for the assay of Factor VIII and several cases were possibly misinterpreted as hemorrhagic syndromes on thrombopathic or vascular basis The systematic determination of Factor VIII made it possible (a) to identify exactly and early the mild hemophilic syndromes which previously escaped all controls (b) to establish a correlation between Factor VIII level and severity of the syndrome and (c) to evaluate the effectiveness of the substitutive therapy with respect to the so-called hemorrhagic level of Factor VIII This is also true within limits for Factor IX

In our experience the early recognition of the hemophilic syndromes proved to be particularly important when the hemorrhages occurred in adult patients after dental extractions or after the incision of superficial abscesses or in the gastrointestinal tract thus suggesting the existence of ulcerative processes of the stomach or duodenum In some cases these manifestations were particularly severe and could not be controlled by the usual hemostatic measures The Factor VIII assay revealed the pres

ence of a rather marked deficiency between 2 and 5% i e within the limits of the so called mild hemophilia The other tests were normal or slightly altered Such a behavior can be visualized in Figs 1 3 in which the whole blood coagulation time the recalcification time and the pro thrombin utilization are compared with the Factor VIII levels The values refer also to some cases of classical hemophilia in which the Factor VIII level was below 1% and to a number of assays which were performed in the course of transfusion therapy with blood or plasma Similar results were obtained by taking into consideration the thrombelastographic values

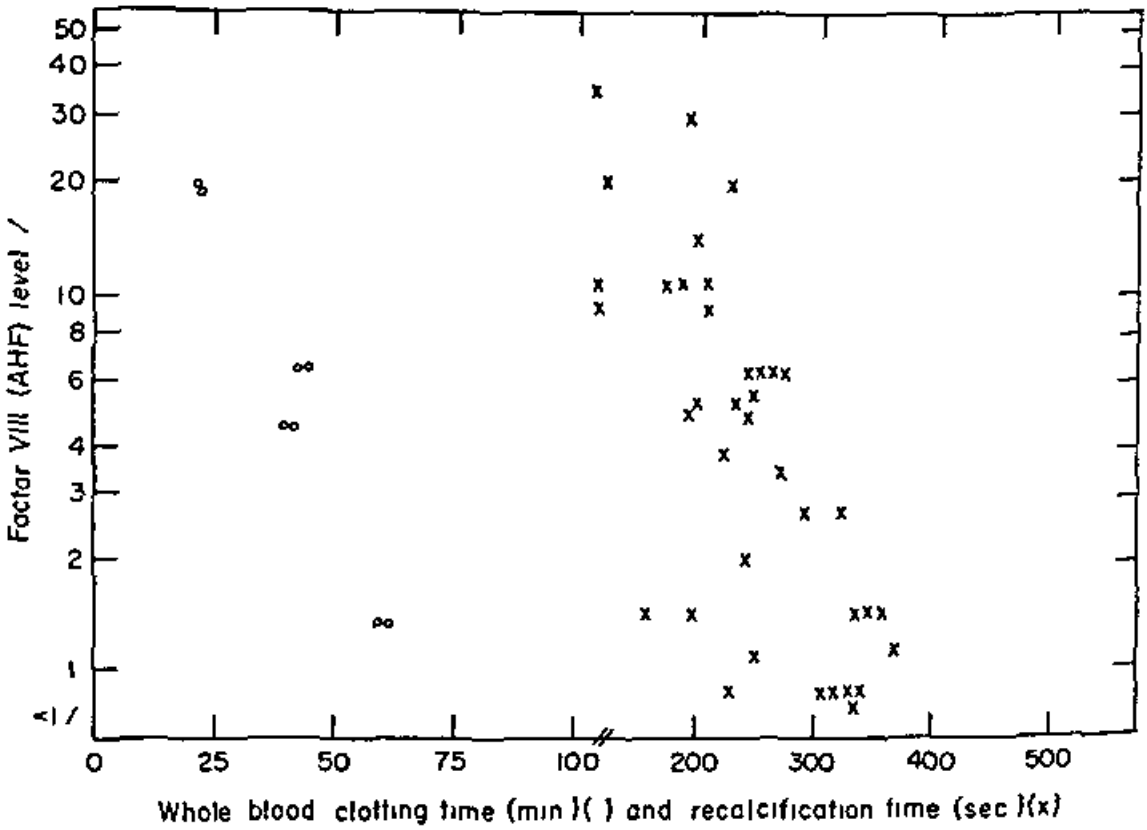


FIG 1 Correlation between Factor VIII (AHF) Level Whole blood clotting time and recalcification time in a group of hemo philiacs

In some instances there was a definite correlation between the Factor VIII level and the onset or the arrest of hemorrhages On the basis of such observations the Factor VIII level of 15 20% seemed to be adequate to insure hemostatic efficiency in the course of dental extractions These levels can be reached by repeatedly and generously administering suitable preparations of lyophilized plasma These supplied by the I S I proved to be safe and active for the correction of Factor VIII deficiencies If higher Factor VIII levels are required for a safe performance of major surgery the use of purified Factor VIII preparations seems to be indispensable

In conclusion the early recognition of mild hemophilia represents today

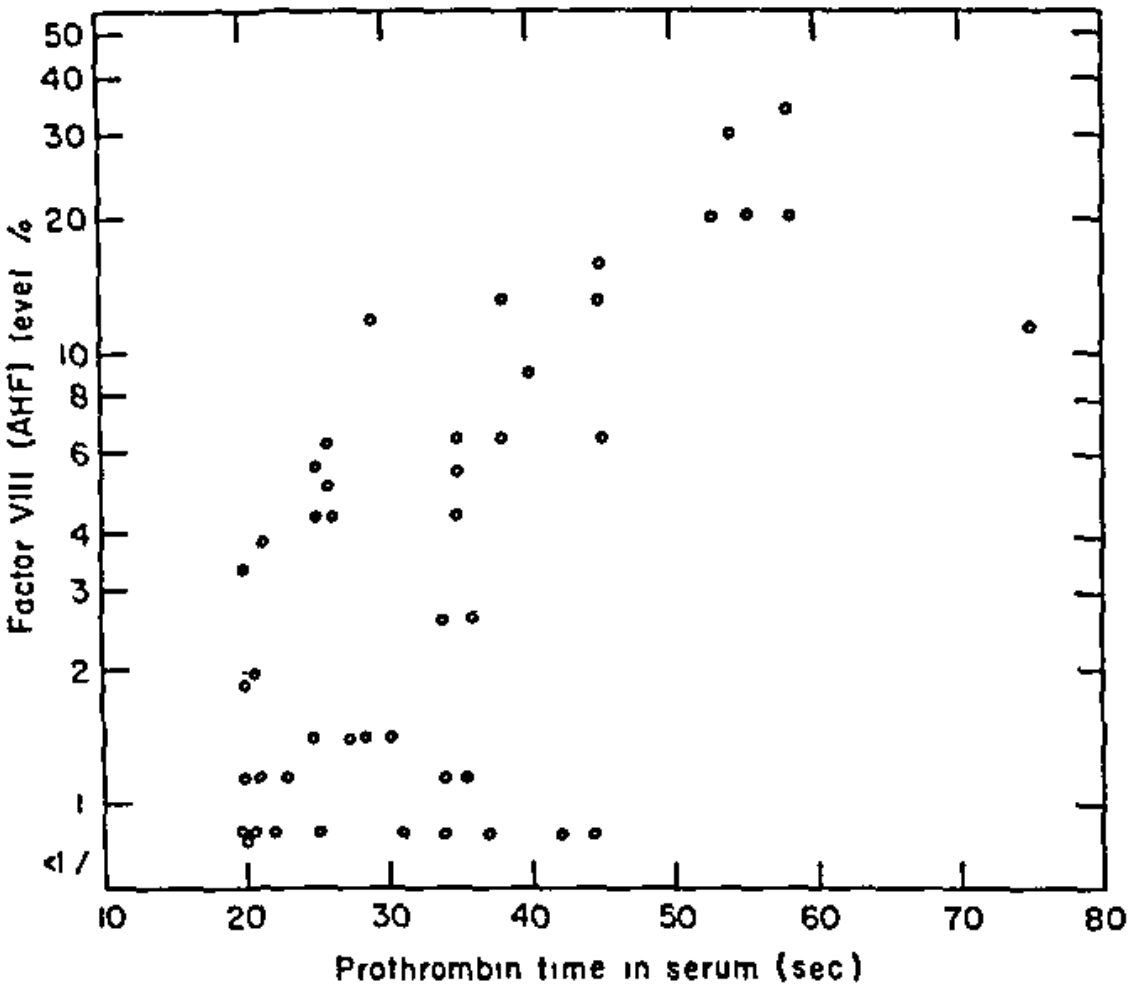


Fig 2 Correlation between Factor VIII (AHF) Level and prothrombin time in serum in a group of hemophiliacs

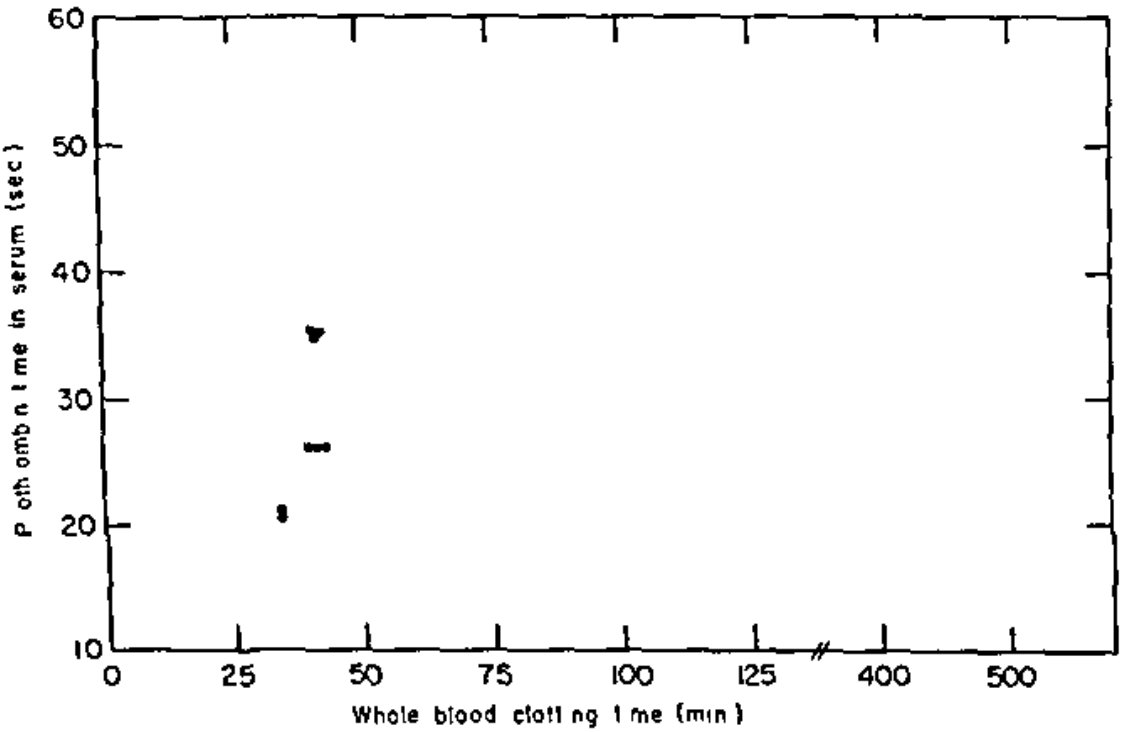


Fig 3 Correlation between Prothrombin Time in Serum and whole blood clotting time in a group of hemophiliacs

a problem of clinical importance It can be faced from the diagnostic point of view by employing adequate methods for the quantitative determination of the AHF and from the therapeutic point of view by routinely controlling the level of the deficient factor during the substitutive treatment in case of spontaneous hemorrhages or before and after surgical interventions

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Part 2

VASCULAR HEMOPHILIA

11

Pseudohemophilias A and B *

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MUCH confusion still exists concerning a group of hereditary hemorrhagic diseases which have in common a consistently prolonged bleeding time Prior to von Willebrand s report of a new bleeding disease found on the Islands of Aland (1) only three bleeding states—hemophilia thrombocytopenic purpura and telangiectasia—were generally recognized Von Willebrand named this new disease which was characterized by a prolonged BT normal platelet count good clot retraction and a dominant hereditary pattern pseudohemophilia Many investigators who found other cases resembling the Aland disease likewise employed this name Later however von Willebrand with Jurgens (2) renamed the condition constitutional thrombopathy because they believed it was caused by defective platelets The new designation however gained little acceptance outside of continental Europe Gradually the noncommittal name von Willebrand s disease has been gaining favor

Only after the development of the prothrombin consumption time (3) did it become possible to restudy more critically the group of hereditary bleeding diseases that have a prolonged bleeding time as a distinguishing characteristic Further assistance came from another test the thromboplastinogen activity time (TAT) (4) The basic prothrombin consumption time does not distinguish between a platelet and a plasma defect in the production of intrinsic thromboplastin whereas the TAT is low only

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when a plasma factor deficiency exists By means of these two tests it is possible to differentiate the thrombopathic type from pseudohemophilia

Several families have been found in our studies (5) whose affected members have a bleeding state due to defective platelet function as measured by the prothrombin consumption test Morphologically the platelets appear normal and clot retraction is not impaired The defective consumption of prothrombin is corrected by the addition of normal platelets to the blood or native plasma The thromboplastin generation test (6) has been found less sensitive and reliable for establishing this platelet defect Since the condition is transmitted as an autosomal dominant it can easily be mistaken as pseudohemophilia especially if the methods for establishing the platelet defect are not carefully selected and supervised

Pseudohemophilia A

Although the term pseudohemophilia is as illogical as the name hemophilia itself its use has been fairly widely and firmly established For nearly three decades the name has been applied by many authors and reviewers to a non thrombocytopenic state characterized by a prolonged BT and simulating the disease found on the Islands of Åland There are advantages therefore in retaining the name for a specific clinical entity which can be defined by a certain type of clinical picture by its hereditary pattern and by its laboratory findings The availability of patients from six families with the disease which corresponds in general to the entity designated as pseudohemophilia has made it possible to formulate diagnostic criteria

Clinical Picture The bleeding is purpuric in type—spontaneous mucosal and cutaneous hemorrhages are common Ecchymoses epistaxes gingival bleeding and menorrhagia are frequent and prolonged bleeding from small cuts is often noted Bleeding does not follow any set pattern In one instance the mother suffered primarily from excessive menstrual bleeding but never had epistaxes whereas her daughter has only had severe nosebleeds Deep intramuscular bleeding is rare and the occurrence of hemarthroses is even more uncommon Neither type of bleeding has been observed in the present series The incidence of gastrointestinal bleeding appears to be greater than that of hematuria while in hemophilia the reverse is true

Hereditary Pattern Even in the mild case the pattern is that of an autosomal dominant Both male and female can have the disease and the distribution is about even The original claim that the Åland disease is inherited through a dominant sex linked character residing in the X chromosome (7) has not been substantiated and probably is erroneous In each patient of our series the disease could be traced through several generations

Laboratory Findings A prolonged BT is the most important and often the only abnormality In most of our patients it is generally less than 10 minutes and at times is normal It can vary greatly in the same individual In one patient the range during five years was from 4½ min which is one min over the upper normal limit to over 30 min at which time the bleeding was stopped mechanically by pressure The prothrombin consumption time the TAT and the TGT were rarely found to be significantly abnormal When a defect was noted it was localized in the plasma suggesting that a factor similar or identical to thromboplastinogen or AHG is in some manner related to the prolonged bleeding time The platelet count in all cases was found to be normal as was the clot retraction

The clinical picture of the patients in the present series is remarkably similar to that described by von Willebrand (7) and also by Minot (8) Interestingly in one of the families on Aland both parents were bleeders and of their offspring five were severe bleeders two were mild bleeders and five were normal This suggests that the heterozygote is apt to be a mild bleeder while the homozygote has the disease in a severe form This may account for the differences in findings concerning such tests as the prothrombin consumption time In the mild bleeder the values are usually normal

Pseudohemophilia B

A bleeding state characterized by a prolonged BT and a reduced antihemophilic or thromboplastinogenic activity was reported independently in 1953 by Alexander and Goldstein (9) Larrieu and Soulier (10) and Quick and Hussey (11) Singer and Ramot (12) suggested that the disease be named pseudohemophilia B Other investigators later proposed the name vascular hemophilia or angiohemophilia

If it is recognized that pseudohemophilia B and vascular hemophilia are synonymous terms the decision which name should be adopted is academic It will depend largely on the interpretation of the term hemophilia If on the one hand the hereditary pattern is regarded as the most characteristic feature of the disease the name vascular hemophilia seems unsuited for an entity not inherited as a sex linked recessive If on the other hand the deficiency of thromboplastinogen is considered the most specific character of hemophilia some justification for the name vascular hemophilia can be seen but one must not overlook the possibility that even in hemophilia a vascular defect may be present Pseudohemophilia B is a noncommittal term The one possible objection as Soulier (13) has pointed out is the possible danger of implying that the condition is in some manner related to hemophilia B If however the pseudohemophilias are regarded as a class entirely unrelated to the hemophilias no confusion should result The name pseudohemophilia B has been tentatively retained in this presentation

Clinical Picture The findings are based on the history of five patients ranging in age from 5 to 36 years some of whom have been studied repeat edly for the past five years Unlike the type of bleeding observed in p eudo hemophilia A which is purpuric and spontaneous the hemorrhages in this condition are partly purpuric and partly like those seen in hemophilia Epistaxis and other forms of mucous membrane bleeding are frequent Gastrointestinal hemorrhage is a particularly serious type of bleeding One of the patients in the series had a spontaneous cerebral hemorrhage prob ably of the type commonly seen in thrombocytopenic purpura Excessive bleeding from small cuts and bleeding of gums from brushing teeth have been frequent findings As to types of hemorrhages which are also common in hemophilia hematomas following contusions should be men ioned first because these are rarely seen in pseudohemophilia A Deep intramuscular hemorrhages occur and hemarthrosis may be as disabling as in hemophilia Curiously this form of hemorrhage appears to be more common in males than in females Hematuria has occurred in several of the patients

Interestingly the patients with pseudohemophilia B despite their seem ingly dual hemostatic defect do not appear to be as severe bleeders as hemophiliacs Apparently the basic faulty hemostasis may not manifest itself in active bleeding for long periods of time and hemorrhage from trauma does not appear to be as pronounced as in hemophilias A and B Serious bleeding has been observed when toxic factors such as produced by infection or by certain drugs were superimposed on the inherited hemo static defect In one patient measles was complicated by what appeared to be bleeding from the lung vomiting and coughing of blood presented an alarming situation from which she fortunately recovered

Hereditary Pattern The defect in pseudohemophilia is transmitted as an autosomal recessive Only individuals who are homozygotes are bleeders therefore both parents must be carriers It has been possible to establish by laboratory tests an abnormality in the heterozygote For this purpose the thromboplastin generation test has been found the most sensitive Since pseudohemophilia A is inherited as a dominant the two types can readily be differentiated by the hereditary history

Laboratory Findings The BT was markedly prolonged in the six patients studied In each the bleeding had to be terminated by pressure The platelet count and clot retraction were found to be consistently normal The clotting time was usually normal or only slightly prolonged The tourniquet test on one adult patient was strongly positive The prothrombin time in each was normal A low prothrombin consumption was obtained when measured by the basic one stage procedure (Quick) and by the TAT test The TGT was abnormal and the defect was localized in the plasma Thus it seems fairly certain that one characteristic defect is lack of thromboplastinogen On assaying the plasmas o four of these patients values ranging from 0 5 1% of normal have been found The lack of thromboplastinogen does

not explain the prolonged BT for in true hemophilia this test is normal Occasionally however a temporarily prolonged bleeding time has been found in established cases of hemophilias A and B

The Bleeding Time

The key to the approach of the pseudohemophilias is the bleeding time Unfortunately this simple test is still poorly understood It is certain that lack of platelets causes a prolonged BT In severe thrombocytopenic purpura this test is usually strongly positive In both congenital and acquired thrombasthenia diagnosed by poor prothrombin consumption the BT likewise tends to be prolonged Because of such findings any condition with a prolonged BT was regarded as a thrombopathy In the pseudohemophilias however the BT is also prolonged but no evidence can be found that the platelets are defective Since a deficiency of a plasma constituent is found the possibility that a normal BT requires both a platelet and a plasma factor must be considered At present such an assumption is based on meager experimental data but since it may offer a clue to a better understanding of the hemostatic mechanism it deserves consideration and further study

Summary

At least three hereditary hemorrhagic diseases are known in which the BT is prolonged In one a qualitative defect of the platelets can be demonstrated by the prothrombin consumption test and the blood can be corrected by the addition of normal platelets In the other two diseases no defect in the platelets can be established The latter two have been designated as pseudohemophilias A and B In the A type the bleeding is primarily purpuric in nature and the hereditary pattern is that of an autosomal dominant In type B the bleeding is partly purpuric and partly deep bleeding such as is observed in hemophilia The hereditary pattern is that of an autosomal recessive and only the homozygote is a bleeder

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12

The Pathogenesis and Treatment of v Willebrand's Disease

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IN 1956 we (1 2) gave a report on a 17 year-old girl with AHG deficiency and prolonged bleeding time (BT) in which case it was possible to correct not only the coagulation defect but also the prolonged BT by injection of AHG-containing human fraction I O prepared aseptically by the glycine method of Blomback and Blomback (3) Using preparations of fraction I O hysterectomy was performed without any abnormal bleeding In 1957 we (4 5) gave an account of 13 patients from 10 different Swedish families with an inherited autosomal dominant hemorrhagic diathesis characterized by deficient AHG (1 10% of normal) and prolonged BT Platelet factor 3 was normal Human fraction I O with AHG was administered to six further cases and the effect on the coagulation defect and on the prolonged BT was as satisfactory as in the first case Fraction I O without AHG activity also controlled the BT while fibrinogen had no effect These findings indicated that the prolonged BT in this type of hemorrhagic disorder was due to lack of a plasma factor present in normal fraction I O and not to a disturbance in the capillary wall or the platelet function as believed earlier

As the clinical features of the patients in Sweden closely resembled v Willebrand's disease we investigated 15 subjects from the Islands of Åland affected with v Willebrand's disease (6) We found decreased AHG values and a normal function of the platelets in respect to platelet factor 3 One of the patients from Aland was given human fraction I O which controlled the coagulation defect and the BT

In view of these results we consider the inherited hemorrhagic disorder

with AHG deficiency and prolonged bleeding time observed in Sweden to be closely related to or identical with v Willebrand s disease

Thus far 20 patients in Sweden (9 males and 11 females in 16 families) with AHG deficiency and prolonged BT i e v Willebrand s disease have been studied (Table 1) Sixteen of the patients had had severe clinical bleeding symptoms and in these cases the BT was more than 60 min and the AHG level ranged between 1 10% of normal The remaining 4 cases with mild bleeding symptoms had only a slight prolongation of the BT and an AHG level of 10 40% of normal

Table 1

COAGULATION STUDIES IN 20 PATIENTS (9 MALES AND 11 FEMALES) FROM 16 DIFFERENT FAMILIES IN SWEDEN WITH V WILLEBRAND S DISEASE

	16 Cases with Severe Bleeding Symptoms	*4 Cases with Mild Bleeding Symptoms*
Coagulation time	Slightly prolonged	Normal
Bleeding time	>60 min	10 15 min
Capillary fragility test	Negative 10 cases Positive 6 cases	Negative
AHG		
1 Test on hemophilic A plasma (6)	1 10%	10 37%
2 Method of Biggs *et al* (10)	2 15%	17-43%
PTC (test on hemophilic B plasma)	70 125%	96 120%
Platelet count	Normal	Normal
Platelet factors 1 3 and 4	Normal	Normal
Other coagulation factors (Prothrombin and Proconvertin Factor V Fibrinogen)	Normal	Normal
Circ anticoagulants	0	0
Fibrinolysis	0	0

* The coagulation tests were performed in the manner described by Nilsson Blom back and v Francken (4) Nilsson *et al* (6) and Blomback and Nilsson (7)

AHG-containing human fraction I O prepared by the glycine method of Blomback and Blomback (3 7 8 9) was given to 9 of these patients in connection with hysterectomy tooth extractions and bleeding episodes such as intra abdominal bleedings large hematomas and epistaxis (Table 2) Usually one dose of fraction I O prepared from 1500 ml of fresh normal plasma was administered on each occasion The bleedings were arrested and surgical procedures could be performed without any abnormal bleeding The BT became normal and the AHG increased usually from 5 10% of normal before the injection to 40 60% after The effect of one dose of fraction I O lasted for 24 48 hours Seven of the patients obtained repeated doses of fraction I O and the response was equally good on every occasion No side effects were seen

Since fraction I-O contains fibrinogen and AHG 2 patients obtained

Table 2

Diagnosis	Number of Cases Treated	Preparation	Effect of the Preparation		
			AHG Level	Bleeding Time	Fibrinogen Level
v. Willebrand's disease	9 (45 *)	Fraction I-O from normal plasma	+	Normalized	+
Hemophilia A	14 (79 *)	— —	+	—	+
Thrombocytopenia	1	— —	+	None	+
Macroglobulinemia Waldenstrom	1 (? *)	— —	+	None	+
v. Willebrand's disease	3	Sterile filtered fraction I-O without AHG from normal plasma	0	Normalized	+
— —	1	Fraction I-O from hemophilia A plasma	0 (+)	Normalized	+
— —	1	Fraction I-O from plasma of v. Willebrand patients	0	None	+
Hemophilia A	1	Purified AHG separated from normal fraction I-O	+	—	0
v. Willebrand's disease	1 (2 *)	— —	+	None	0
— —	1	Fibrinogen separated from normal fraction I-O	0	None	+
— —	3	Purified fibrinogen from outdated blood	0	None	+
— —	1	Platelet suspension	0	None	0
— —		1350 ml. of fresh blood	+	Almost normalized	0

The preparations compared were derived from corresponding amounts of plasma

* Total number of administrations

fibrinogen from outdated blood to investigate whether purified fibrinogen in itself had any effect on the BT It was found to have no effect on the BT and the plasma AHG content

Three patients received doses of fraction I O in which the AHG activity had been lost during sterile filtration These fractions still had a normalizing effect on the BT although the plasma AHG did not increase

Fig 1 demonstrates the effect of various forms of therapy in a 10 year old girl with a severe form of v Willebrand s disease The patient received two doses of ordinary fraction I O from normal plasma The plasma AHG

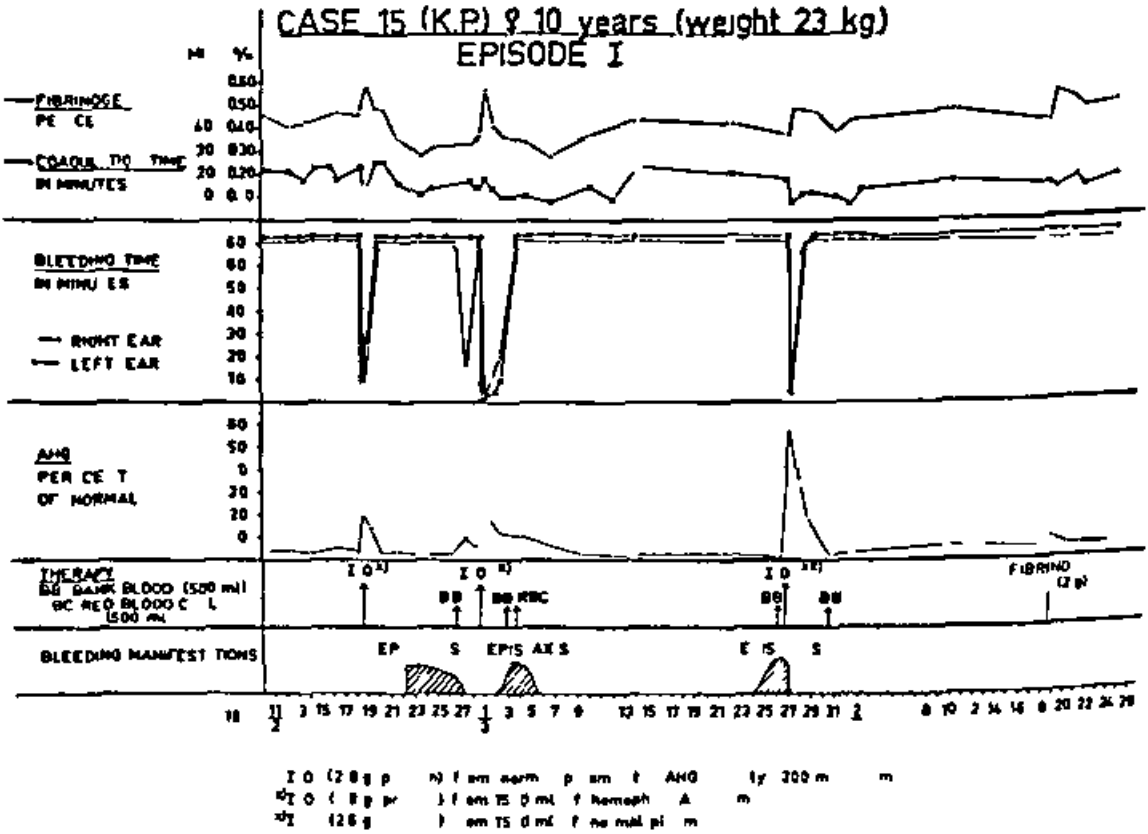
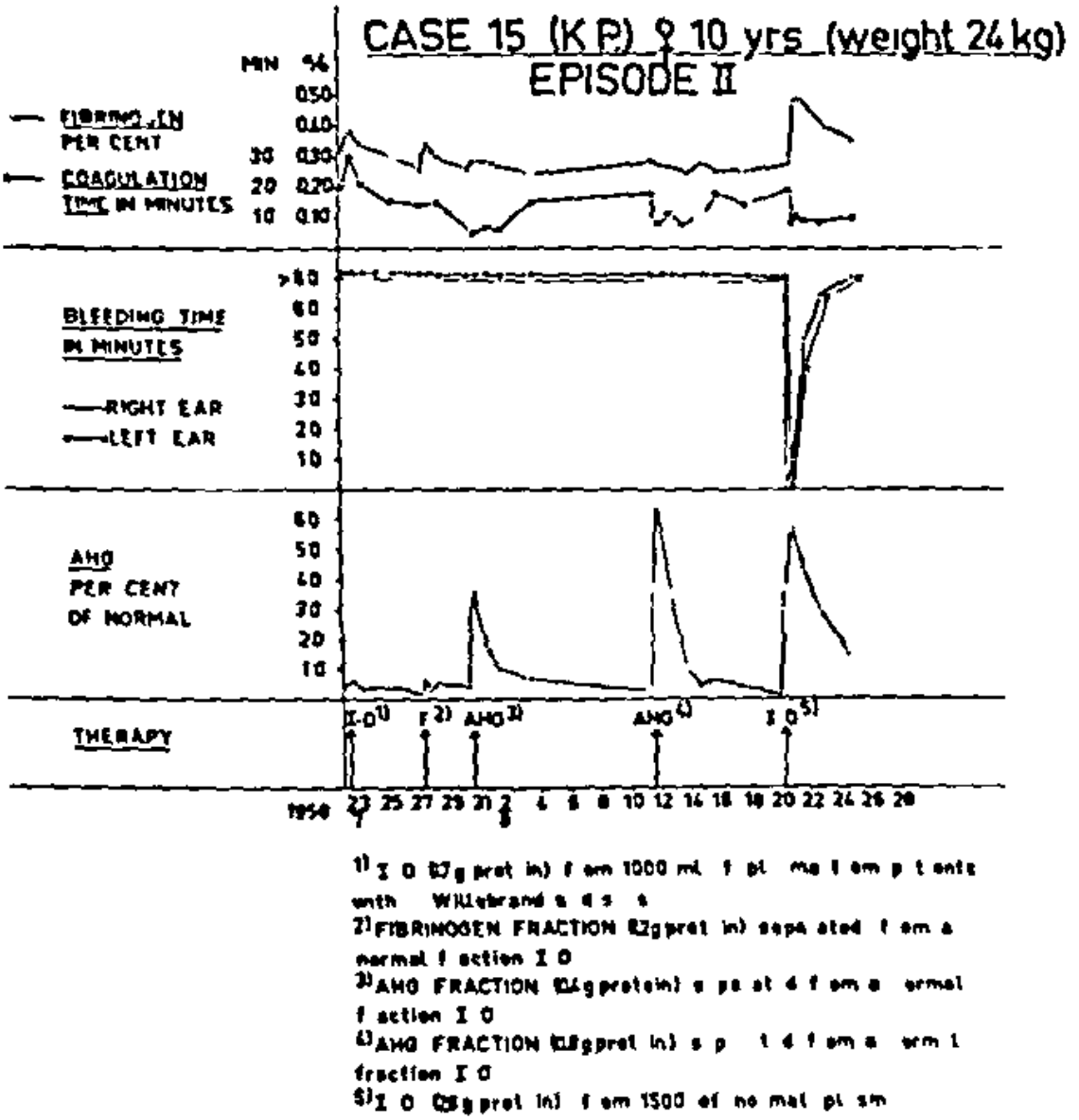


Fig 1 Use of Various Fractions I O in a case of severe v Willebrand s disease

increased and the BT became normal As can be seen from the figure highly purified fibrinogen had no effect on this bleeding disorder This patient was given a preparation of fraction I O from hemophilic blood This hemophilic fraction I O was derived from 1500 ml of plasma from 8 hemophiliacs with an AHG content ranging from less than 1 to 2% of normal Tested *in vitro* the AHG activity of the fraction corresponded only to that of 4 ml of normal plasma The effect on the BT of the hemophilic fraction I O was not only pronounced but also of longer duration than that of ordinary fraction I O Two hours after the administration the AHG level had risen from 6 to 12% of normal It is noteworthy that the AHG level 24 hours later had increased to 19% of normal It is evident that

the factor correcting the BT in v Willebrand's disease is present not only in normal blood but also in hemophilia A blood Fraction I O was also prepared from fresh plasma of patients with v Willebrand's disease and given to this case (Fig 2) It had no effect on the BT nor on the AHG content It has been suggested that an artefact produced during the prepara



1) I O (17 g prot in) from 1000 ml fresh normal plasma with Willebrand's factor
2) FIBRINOGEN FRACTION (12 g prot in) separated from a normal fraction I O
3) AHG FRACTION (1.4 g protein) separated from a normal fraction I O
4) AHG FRACTION (1.8 g prot in) separated from a normal fraction I O
5) I O (2.8 g prot in) from 1500 ml normal plasma

Fig 2 Use of Various Fractions I O and Subfractions in a case of severe v Willebrand's disease

tion of fraction I O might be responsible for its correcting effect on the BT The failure of the fraction I O derived from v Willebrand plasma to correct the BT argues strongly against such an assumption

Furthermore in this case it proved possible by giving further purified AHG preparations to increase the AHG to as much as 65% of normal without any effect on the BT The AHG had been separated from an ordi

fibrinogen from outdated blood to investigate whether purified fibrinogen in itself had any effect on the BT It was found to have no effect on the BT and the plasma AHG content

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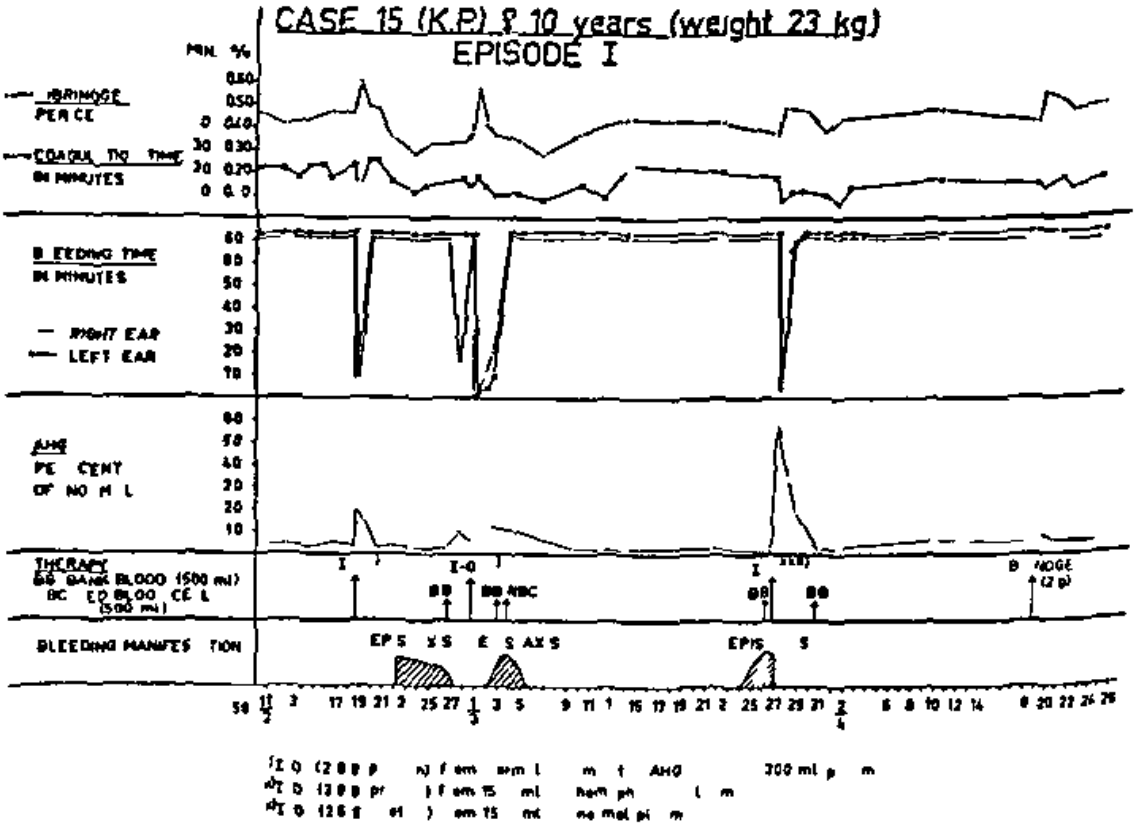


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disease is concerned with the production or the activation of AHG If this is true and if the primary defect in v Willebrand's disease is just the lack of the plasma factor correcting the BT it would explain why there is a combined defect of prolonged bleeding time and AHG deficiency in this disease

To summarize it has been clearly established that AHG-containing human fraction I O from normal plasma corrects the coagulation defect and the prolonged BT and controls the bleedings in v Willebrand's disease This implies that specific therapy is now available in v Willebrand's disease Our experiments have provided further evidence that the prolonged BT in v Willebrand's disease is due to lack of a plasma factor present in normal plasma as well as in hemophilia A plasma The plasma factor correcting the BT can be recovered in fraction I O and it is not identical with AHG fibrinogen or platelets

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nary fraction I O by precipitation at low ionic strength and low ethanol concentration in the manner outlined by Blomback (8) The fibrinogen remaining in the supernate after the precipitation of the AHG was recov ered from it by raising the ethanol concentration This fibrinogen fraction increased the fibrinogen level but had no effect on the BT and the AHG content in this case (Fig 2) These observations indicate that the plasma factor correcting the BT remained in the supernate after the precipitation of the fibrinogen Experiments are in progress to prepare this supernate for clinical use After these fractions the patient obtained an ordinary preparation of fraction I O in order to be sure that the response of the BT was still satisfactory (Fig 2) In addition it appears from Fig 2 that the AHG disappeared more rapidly from the plasma after injection of the separated AHG fraction than after injection of the ordinary frac tion I O

Ordinary fraction I O was prepared from platelet rich and from platelet poor normal plasma The effect on the BT was one and the same One of our patients was given a transfusion of platelets but it had no effect (Table 2) One dose of fraction I O was administered to a patient with thrombocytopenia No effect was seen on the BT and the bleedings were not arrested One female with macroglobulinemia Waldenstrom and with prolonged BT also received fraction I O The BT was more than 60 min both before and after administration and the bleedings continued It appears obvious that the correcting effect of fraction I O on the BT is not attributable to platelets or platelet derivatives The failure of thrombo cytopenia and macroglobulinemia to respond to fraction I O argues against the effect of fraction I O in v Willebrand s disease being nonspecific Some of our patients were treated with ACTH cortisone progesterone and premarine but in none did these control the defects

From these experiments it appears evident that the ordinary fraction I O contains a plasma factor which is able to correct the prolonged BT in v Willebrand s disease and which is not identical with AHG fibrinogen or platelets

On comparison between hemophilia A and v Willebrand s disease we have found the AHG of fraction I O to be consumed appreciably slower in v Willebrand s disease than in hemophilia A (7) In some cases of v Willebrand s disease we observed that the AHG content was higher some hours or even a day after the administration of fraction I O than immediately after it As already mentioned in one case (Figs 1 and 2) we found a moderate increase of the AHG level after injection of fraction I O from hemophilic blood but not after injection of fraction I O from v Willebrand blood In this patient we also observed that the AHG of the separated AHG fraction I O was consumed more rapidly than the AHG of an ordinary fraction I O The reason for this is not known It might be that the plasma factor correcting the BT and lacking in v Willebrand s

Nr	Name	Sex	Age	Dat	Plat 1st Con t (1 thous ml)	Trom bd Pl t	C pil l ary Pl t	Pro throw in-Concentration- I	Plasma Prot sec	Serum-Prothr sec	Antihaemophilic Globulin (F r VIII) Pa%-Pl ma Con ration Nf	ml	sec	Bleeding Time 5	10	15	20	Remark
	Normal val				350	350		<20	>30		Normal		to 5	6-12				within 0-3½ min. 85% of all normal persons within 0- 7 min 100% of all normal persons
											Mild hemophilia		5	12 18				
											Severe		5	18-32				
1	Ba P m	60	V	58	( ) 275			17	54 4	(1)	o	5	12 4					
			VI	56	293							4	16 2					
2	P	21	IV	58	179			17 o	18 5			4	21,8					
3	U	17	V	58	( ) 190			27 2	29 8	(2)	o	6	15 2					
4	H	53	II	57	500			18 2	9			3	26 2					
5	K	16	II	57	( ) 400			24 1	39 6			3	15 5					
6	Kn P	57	II	58	134			19	76 3	(3)	o	3	20 4					wild bleeding
7		22	II	58	186			18	17 7	(4)	o	9	19 4					cystorectom.
8	LO P m	58	V	57	251			20 4	26 4			6	17 6					
9	P	35	VI	57	179			28 8	28 5			4	14 4					
10	M L	9	IV	57	214			70	8 3			4	19 1					
11	P P	38	VII	57	248			18 6	22 9			5	10 5					wild bleeding
12	Ed P	17	VI	57	192			19 4	17 3			7	15 7					severe bl eding
			III	58	174					(5)	o	10	19 3					
13	Ha S	6	II	58	274				14 6	(6)		7	23 9					ver re bleeding
14	Lo O	41	VI	57	146			20 3	45 6			4	18 6					F tal l ten bleeding

FIG 1 Correlation of Bleeding Tendency BT and AHG in Angiohemophilia

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Angiohemophilia (Vascular Hemophilia)

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ANGIOHEMOPHILIA A is a well defined clinical entity presenting many problems Therefore one must separate clearly facts and assumptions

The facts are as follows (1) Bleeding is of 3 main types traumatic bleeding especially after tooth extraction spontaneous bleeding from mucous membranes especially epistaxis and metrorrhagia and superficial hematomas and occasional hemarthrosis (2) The disease is familial and is inherited as a dominant (3) The bleeding time (BT) is prolonged and the tourniquet test is usually pathological (both tests vary) (4) There is a plasmatic clotting defect identical with that found in hemophilia A

These facts are well recognized by all authors In addition no platelet defect was found in our cases by the methods applied shape and viscous metamorphosis studied by phase microscopy platelet coagulation activity as demonstrated by the prothrombin consumption test platelet factor 3 estimated by the TGT and platelet adhesivity and retraction according to Wright s and Fonio s methods respectively

Some of the problems are indicated below What correlations exist between the severity of the bleeding tendency the BT and the AHG level? The results are shown in Fig 1 All severe bleeders had a marked AHG deficiency a markedly prolonged BT and a pathological tourniquet test The 2 mothers of the female bleeders No 7 and 12 showed no bleeding tend ency except easy bruising They had a normal BT and a normal AHG level but a pathological tourniquet test and a pathological prothrombin consumption test In another family showing a moderate bleeding tendency a strong correlation between prolonged BT and AHG level could not

be found Fig 2 shows 6 curves (TGT pat $BaSO_4$ plasma N serum N platelets) from 6 different angiohemophiliacs in comparison to a severe hemophiliac (curve 7) In curves 1 3 the patients had a mild bleeding tendency a mild AHG deficiency (1 2) or a normal AHG level (3) respectively Patients No 1 and 3 had a normal and patient No 2 a greatly prolonged BT Curves 4 6 show the AHG level of 3 severe bleeders with a greatly prolonged BT A 41 year old woman who died from severe intestinal bleeding had a moderately decreased AHG level and a strongly prolonged BT In summary a strong correlation was found in nearly all cases between the severity of the bleeding tendency and the grade of AHG deficiency but there are exceptions from this rule There is also a strong correlation between the bleeding tendency and the prolongation of the BT In some cases there is less correlation between the AHG level and the bleeding time

Fig 3 illustrates that Cohn fraction I normalizes the pathological hemostasis tests (BT tourniquet test AHG level prothrombin consumption test) in a case of angiohemophilia An important difference between angiohemophilia and hemophilia should be emphasized In severe hemophilia there is commonly a nearly total lack of AHG an AHG level of about 5 10 is usually associated with a mild bleeding tendency in hemophilia In angiohemophilia the same AHG level will be combined with severe sometimes fatal hemorrhages from mucous membranes In angiohemophilia A it is more evident than it is in hemophilia that the bleeding tendency depends on an unknown factor probably acting with AHG to normalize the clotting defect There is no evidence that any well defined platelet function acts as this unknown factor

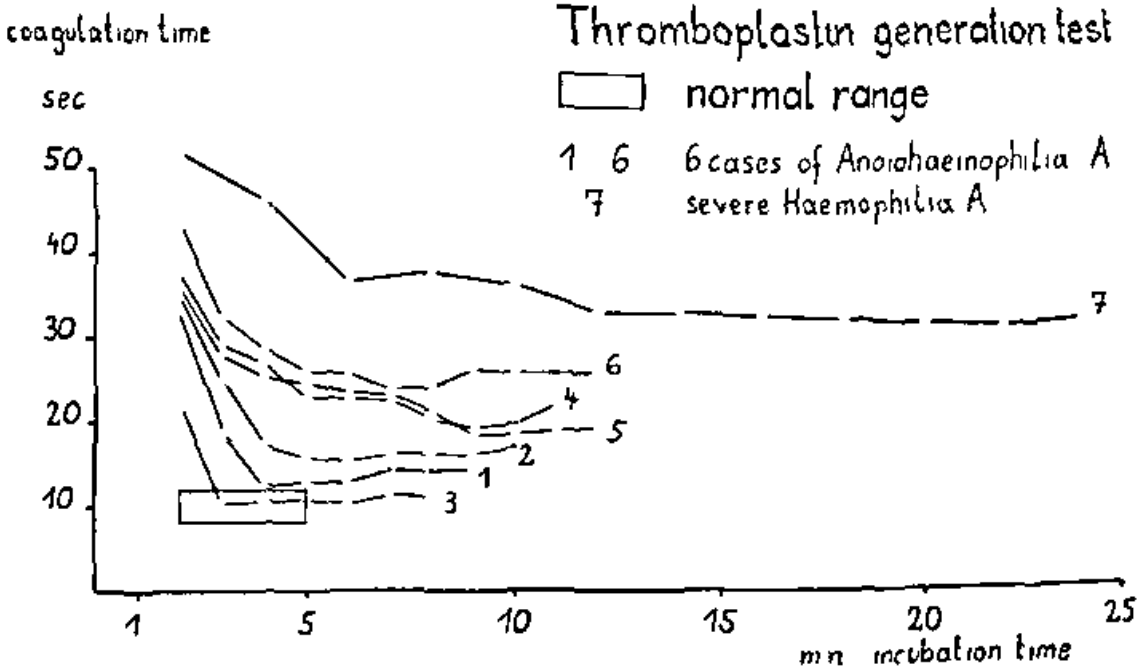


FIG 2 TGT in 6 Cases of Angiohemophilia A and in 1 case of hemophilia A

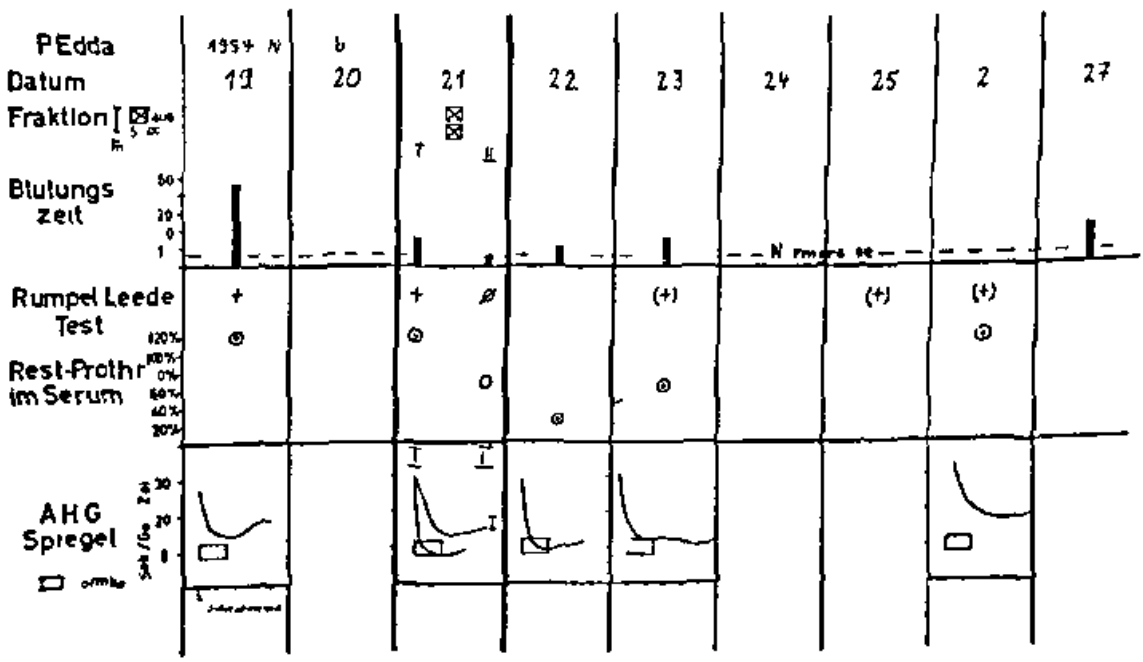


FIG 3 Effect of Cohn Fraction I in Angiohemophilia

great differences in methods and interpretation Fig 2 shows such a compilation from nearly 100 papers with about 300 cases The number of pathological findings within all categories could be explained by a mixture of different diseases Therefore I would like to stress that we as well as other

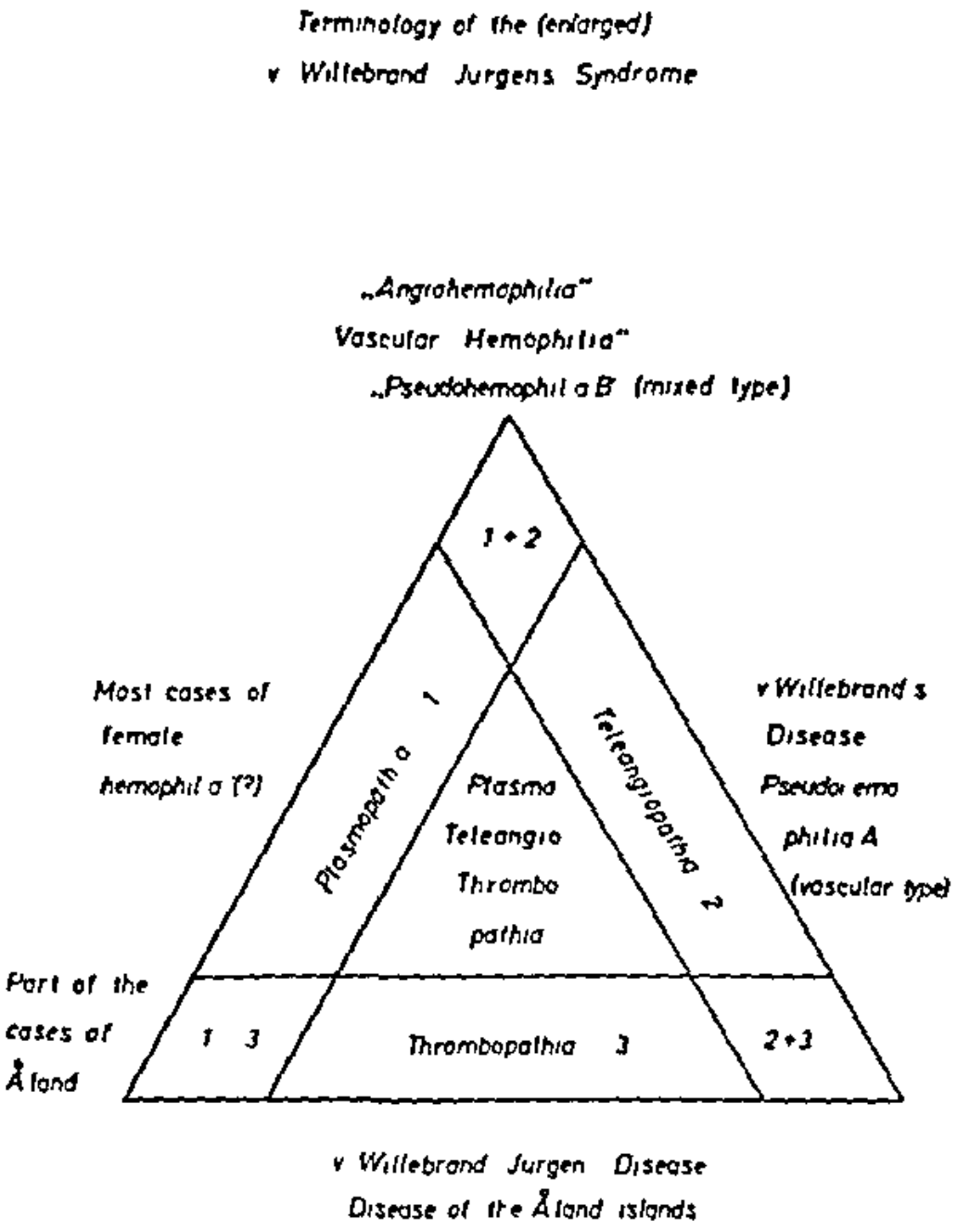


FIG 1

examiners saw a combination of different platelet disorders with lowered plasmatic Factors VIII or IX

The main arguments in favor of a connection between so-called angiohemophilia and some thrombocytopathias are the observations made last year in the Åland Islands by Jurgens and coll and Nilsson Blomback and Francken Furthermore Bruster saw morphological disorders in platelets in

14

Angiohemophilia and Thrombopathia v Willebrand Jurgens

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SOME months ago it was stressed (1) that in our opinion the so-called angiohemophilia vascular hemophilia von Willebrand s disease and thrombopathia of v Willebrand Jurgens are varieties of the same disease Similar conclusions were drawn in the meantime among others by Beller and Brockhaus We proposed to use the term syndrome *of v Willebrand and Jurgens for the whole group and to characterize the* individual findings by the suffixes teleangiopathia thrombopathia or plasmopathia as used by Quattrin Fig 1 shows the possible combinations of the 3 basic disturbances and the terminology from our point of view We no longer believe that angiohemophilia exists as a separate nosological entity But the combination of defects which led to the terms vascular hemophilia or angiohemophilia seems to be especially frequent within the Willebrand Jurgens syndrome

We believe that the term pseudohemophilia should be avoided because it has been used for at least 8 different kinds of hemorrhagic diatheses *in the past [Gross and Mammen (1)] and because classical hemophilia is* quite different from this kind of hemorrhagic tendency

Our opinion is based on personal examinations of 2 families one with a defect in Factor IX (PTC) and 4 single cases Table 1 shows the hematological data of our cases which manifest bleeding Although tests for overall coagulability are usually not altered as much as for example in classical hemophilia with the same AHG values the more sensitive tests easily uncover the *disturbance Because of the naturally limited experience of one* author with the disease an even better understanding of the findings may be obtained by summarizing the data from the literature disregarding the

H matolog col f d g th f t g d) W ll brand J gen y d om

facco d g t dat s of 96 p blic t o s)

C t o	Cas s to be sed	P tag of path l g al f d gs	
Port of femal s	256		50
Bl d g t m	269		84
To q t t sts	216		1
C g lo t m	242		33
Recalc f t m	66		47
Prothrombine consump	150		65
D cy f V	59		8
f VII	58		12
f VIII	78		7
f IX	67		12
Thrombocytop ia	234		8
Morphol regul ty	100		37
D f pl t l t f 3	64		39
Clot t ct	188		14

FIG 2

Addendum

In the meantime in collaboration with Drs Lohr Waller Vogell (Marburg) and Achenbach (Koln) we found platelet anomalies by electron microscopy and in their energy metabolism even in a case of pure tele angioplasmopathia (angiohemophilia A) i e in a case without any detectable disorder of the hemostatic functions of the platelets (unpublished observations)

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a case of (in his opinion) typical angiohemophilia using electron microscopy (See Addendum p 107) Finally our own observations [Gross Mammen and Illig (2)] with capillary microscopy revealed a localization of spontaneous petechial hemorrhages in the same segment of capillary bed as can be seen in thrombocytopenia With this method as well as histologically the fine blood effusions are clearly different from both the Osler group of hemorrhages and from anaphylactic purpura

Table 1

HEMATOLOGICAL OBSERVATIONS IN 7 CASES OF V WILLEBRAND JURGENS SYNDROME WITH HEMORRHAGES

Symptoms	*Number of Pathol Findings*
Clinically	
Petechiae	2
Sugill and suffus	6
Blood vessels	
Bleeding time	4
Sign of Rump Leede	5
Suction tests	6
Blood coagulation	
Lee White	1
Tolerance of heparin	4
TEG (r + k)	7
Thromboplastin Time	0
Prothrombin consumpt	6
Factor VIII (AHG)	5
Factor IX (PTC)	2
Thrombocytes	
Number	0
Platl f 3	0
Clot retraction	4
TEG m	4

From a genetic point of view it is difficult to understand the manifestation in one single hemorrhagic diathesis of 3 different disturbances of hemostasis in the blood vessel wall in the thrombocytes and in plasma But first we have to look at the facts and then to search for explanations Differing symptoms from patient to patient even within the same kin are more frequent in this than in other hereditary hemorrhagic diseases The disorders of the blood vessels are the most constant and most important findings Without the typical dominant transmission prolonged bleeding time may be regarded as a condition *sine qua non*

In summary the so-called angiohemophilia is clinically and genetically clearly distinguished from classic hemophilia but not from thrombocytopathia of the Willebrand and Jurgens type We should therefore avoid all designations containing the term hemophilia By extending the meaning of Willebrand Jurgens disease beyond the pure thrombocytopathias the many different interpretations could be easily brought together

H mat log l t d gs h f l g d) W ll brand J rgens sy drom

(accord g to dat s of 96 p bl cat o)

C t	Cos s to b sed	P c t g of path log cal f d gs
Part o of f mat s	256	50
Bl d g t m	269	84
To q t t sts	216	61
C g l t t m	242	33
Rec lc f c t m	65	47
Prothrombine consump	150	65
D cy f V	59	8
f VII	58	12
f VIII	78	7
f IX	67	12
Thrombocytop nia	234	8
Morphol regula ty	100	37
D fic plat l t f 3	64	39
Clot t act	188	14

FIG 2.

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15

The Problem of Differentiating Pseudohemophilias

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ARE all of the pseudohemophilias with their characteristic symptoms of prolonged bleeding time (BT) normal number of platelets and in complete dominant heredity *one* hereditary disorder or does the term pseudohemophilia include anomalies and/or disease entities resulting from different genes? Is it in accord with available data to include all these diseases under the terms vascular hemophilia (1) or angiohemophilia (2) or (expanded) (3) von Willebrand Jurgens syndrome (4) or teleangio plasmothrombopathies (5)? It seems that this is one of the burning questions emerging from the study of the hemorrhagic diseases with the above mentioned symptom triad Adapting ourselves to prevailing Anglo Saxon *usage we will use the name pseudohemophilia or pseudohemophilia* syndrome for these conditions not that we have any preference for this term which has already been defined in many different ways (3) but rather we employ it for lack of a more appropriate and widely used term

In the complex of hemorrhagic diseases with prolonged BT and normal platelet count with dominant heredity there is the group of thrombopathies or functional thrombocytopathies After thorough investigations (6) there seems to be no doubt that there are hereditary thrombocytogenic anomalies In individual cases they can be combined constitutionally or transitorily with plasmatic or coagulation disorders Although in the case of the Naegeli thrombopathy (Naegeli Glanzmann thrombasthenia) (7) *there are pri*mary cytogenic disorders of adhesion agglomeration and clot retraction our cases of this disease did not show any distinct functional or morpho*logical anomalies of the nail bed capillaries*

Until 1953 it was permissible to make the diagnosis of *von Willebrand*

Jurgens thrombopathy using the nomenclature of the German speaking nations if the following characteristics of the bleeding anomaly were observed (a) incomplete dominant heredity (b) generally a prolonged BT (Duke) (c) somewhat prolonged coagulation time (CT) *in vitro* or in the thrombelastogram using venous blood (d) usually a normal platelet count (e) frequently an impaired prothrombin consumption (f) inconstant disorder of platelet agglomeration in the capillary blood smear (g) inconstantly decreased capillary resistance (h) normalization of prothrombin utilization after addition of isolated platelets and (i) normal clot retraction and essentially normal platelet morphology in the stained capillary blood smear

After introduction of the TGT (8) in 1953 the following characteristics were added in 1954 (a) normal TGT with patient's plasma but with normal platelets or platelet homogenates and (b) pathological TGT if platelet suspension or platelet homogenate of the patient is used

Experiences gained during the past four years showed the following

A Dominant heredity is found in those diseases which belong in the pseudohemophilia syndrome With the TGT and the Gross Schwick modification (9) of the Deutsch Johnson Seegers test not all groups have shown discernible platelet anomalies or platelet factor 3 (4) anomalies

B The prolonged BT can be completely or partially normalized by infusion of plasma fractions This may possibly indicate the absence of a plasma component controlling the bleeding time [antibleeding factor (10) capillary factor]

C Prothrombin consumption may be deceiving This becomes evident if one makes 3 to 5 parallel determinations

D The methods for determining platelet factor 3 are far from perfect and errors are rather easily made These errors are due to a number of things including adsorption of clotting factors onto platelets errors in counting platelets because of platelet fragments differing volumes of platelets blood group differences between platelets and test plasmas and damage done to the platelets while being obtained for the test These potential errors in the determination of platelet factor 3 activity make it imperative to improve the methods for both intact and disintegrated platelets Among these procedures are Johnson's method with Seegers prothrombin (11) use of boiled platelets (12) measurement of prothrombin consumption and thrombin generation after adding a very small number of optimally washed platelets to platelet free plasma and platelet factor 3 extraction False positive results and especially false negative results for factor 3 activity of platelets in native blood may be encountered This is particularly true when plasma coagulation factors are normal and prothrombin consumption is significantly pathological and when coagulation times are (possibly) prolonged and platelet factor 3 dysfunction is possibly masked because of damage to the platelets during isolation and washing

E With the TGT errors can arise in regard to plasma coagulation factors For example in determining Factors VIII and IX by the PTT methods these factors may be decreased while the TGT turns out normal due perhaps to the fact that the serum retained some prothrombin

Apart from these numerous potential errors our experiences as well as that of other authors (R Jurgens Braunsteiner J Jurgens Ulutin Frank Johnson Rebuck Koch Schultze Schwick) indicate that there are symp tomatic thrombopathies with dominant inheritance, which are due to primary thrombocyte factors particularly decreased platelet factor 3 ac tivity They might be listed as a special group of pseudohemophilias as well as of thrombocytopathies Some of them are perhaps *formes frustes* of an over all hereditary disorder affecting blood vessels plasma and platelets This possibility cannot be denied in view of the results obtained by Koch Schultze and Schwick (13) and Beller (4)

Of the hereditary hemorrhagic diatheses with a prolonged BT combined with a normal platelet count the observations first made by Alexander and Goldstein (14) Quick and Hussey (15) Marx (16) and van Creveld Jordan and Punt (17 18) delineated a subgroup showing a decrease of Factor VIII combined with prolonged BT and with no discernible platelet function and factor disorder In 1957 a parallel group with an hereditary deficiency of Factor IX a normal platelet status and a prolonged BT was found by Klesper and Achenbach (2) and Soulier and Larrieu (19) Based on the hypothesis th it the prolonged BT resulted from a primary vascular anomaly Klesper and Achenbach suggested the name of angiohemophilia A or B for this subgroup of pseudohemophilias Schulman and co workers (1) in 1957 suggested the name vascular hemophilia for this group since they considered the vascular anomaly the essential factor as had Macfarlane (20) in 1941 They studied seven cases from five different families of unascertained hematological heredity six of which showed pro longed BT normal platelet counts Factor VIII deficiency and patholog cally shaped capillary vessels in the nail bed and the conjunctiva bulbi One case merely showed morphologically pathological capillary vessels with prolonged BT Klesper and Achenbach thought it justifiable to use alter nately the Latin Greek term vascular hemophilia and their own purely Greek term angiohemophilia

It appears advisable at least for the time being to be careful in in terpreting the prolonged BT as a vascular disorder The two cases examined by Alexander and Goldstein and by van Creveld and co-workers respec tively showed morphological capillary anomalies while some cases which we observed showed no morphologically pathological capillary vessels in the nail bed (observed in several fingers) One case with a hereditary Factor VIII deficiency and prolonged BT but without perceptible anom alies of the capillary picture had a normal BT for some hours after infusion of one ampule of Cohn s fraction I Another hereditary case with the same

kind of symptoms and BT of 80 min [according to Marx Ressel (21) maximum normal values up to 5 min] had a BT of 69 min after the infusion In our opinion this is a Cohn negative reaction considering the range of values with this method Therefore it might be better to speak of vascular hemophilias and angiohemophilias only when *hereditary* anomalies in form reaction and resistance or hyperpermeability of capillaries have actually been found It must be remembered that capillary changes simulating primary anomalies may develop in a number of diseases After Nilsson and co workers in Sweden succeeded in normalizing the BT of a pseudohemophilia type patient by administering fibrinogen free and Factor VIII free Cohn s fraction I the problem of the existence of a discrete antibleeding factor without coagulation effect (humoral capillary factor) remains to be cleared up since vascular dysfunction indirectly dependent on coagulation factors is well imaginable

If the investigation of *several* cases in *one* family offers no evidence of a primary vascular or platelet anomaly but rather points to a disorder of an early phase of coagulation with a prolonged BT this anomaly could be interpreted tentatively as a genuine plasmopathy or as dominant hereditary hemophilia with prolonged BT

In our experience there are cases of hemophilia B with a normal BT combined with very distinct capillary anomalies However in individual cases with indistinct heredity we are unable to determine whether one is dealing with angiohemophilia with normal BT (repeated determination) or recessive hemophilia with a concomitant (possibly acquired) capillary anomaly (22) (Fig 1) We observed two patients with dominant hereditary deficiency of Factors VIII and IX combined with acquired liver disease possibly affecting the capillaries (23) (pseudo Osler cases)

Using the comparative qualitative method developed by Marx Koppel Kreiter (24) and Ibrom (25) we investigated some cases of pseudohemophilia with prolonged BT normal platelet count and platelet function (retraction agglomeration me in the TEG adhesion to glass and zapon lac and platelet factor 3 activity) We found a pathological platelet metamorphogram in citrate blood Only one patient among 50 persons without hemorrhagic diathesis showed a disorder of platelet metamorphosis on the zapon lac foil when similarly investigated This patient had a very mild case of allergic diathesis (25) On the basis of these investigations it is not yet possible to distinguish a primary thrombocytogenic (but rather a secondary plasmogenic and/or thrombocytogenic) disorder of platelet metamorphosis from the disorder of platelet metamorphosis observed in citrate blood Plasma abnormalities may also cause such alterations in metamorphosis under standardized conditions Nevertheless even with this improved method for determining the qualitative condition of platelets the question arises as to the homogeneity of pseudohemophilias

There appear to be other inherited platelet anomalies with inconstantly

prolonged BT which are very difficult to classify because of the incomplete penetrance and expressivity of the anomaly This is indicated by the family study detailed below

The family consisted of 2 sisters one brother hospitalized because of *paramyeloblastic leukemia* and one brother who had bled to death The hereditary hemorrhagic diathesis was also manifested by epistaxis and bleeding after tooth extraction One sister showed a decrease of platelet adhesiveness to zapon lac and glass in a capillary system (repeated four times) combined with a normal BT normal platelet count (262 000)



FIG 1 Pathologic Capillaries (Candelabra Form) of hemophiliac B with normal bleeding time

and an insignificantly prolonged venous blood CT TEG values were r 14½ min k_1 8 min and ma 98 PT and TGT were normal The other sister showed a BT of 10½ min combined with normal platelet and plasma coagulation factor values

Only people who have painstakingly worked in this field for many years are aware of the difficulties of thorough hemostatic investigations of families, particularly since the methods especially those of capillary vessel analysis are far from being perfect

Methods Used for Platelet Functions and Platelet Factors (a) Agglomerogram in the capillary blood smear (Rovatti) (b) determination of platelet adhesion to glass and standardized zapon lac surfaces in capillary glass tubes (Marx and Derlath) (c) determination of platelet alterations on

zapon lac-coated Neubauer counting chambers (Marx Koppel and Kreiter) (d) platelet count with the phase contrast microscope (Feissly Luedin Derlath s modification) (f) assessment of the me value in the TEG (Hartert) (g) prothrombin consumption in glass tubes (Soulier) (h) determination of platelet factor 3 activity in lightly washed and carefully isolated platelets in the TGT using 50 000 and 30 000 platelets/cc determination of platelet factor 3 activity in platelets isolated from citrate plasma (two washings) [Gross Schwick modification Deutsch Johnson Seegers principle (system prothrombin Factor VIII and calcium)] determination of the platelet factor 3 activity in eight times optimally washed platelets (Deutsch Johnson Seegers test) (i) determination of antiheparin activity in platelet homogenates compared with parallel dilutions of a normal platelet homogenate Platelets are frozen three times and macerated mechanically in a plastic homogenizer Prior to homogenization the platelet count/cc is balanced

Some of our patients from 24 families with the symptom triad of pseudohemophilia appear noteworthy and have furnished part of the background for the classification of hereditary pseudohemophilias suggested below

Hereditary Pseudohemophilias

I von Willebrand Jurgens syndrome

1 Vascular hemophilias or angiohemophilias i e deficiencies of Factor VIII *and/or* IX with normal platelets but hereditary pathological capillaries incomplete dominant heredity and (inconstantly) prolonged BT

a *Cohn s fraction I positive*

b Cohn s fraction I negative?

2 Teleangioplasmothrombopathies (term after Quattrin) or plasmothrombopathies i e combined vascular platelet and plasma (coagulation) disorders or plasma coagulation and platelet factor disorders and platelet functional disorders or teleangiothrombopathies with incomplete dominant heredity and *(inconstantly) prolonged BT*

II Genuine plasmopathies ? or dominant hereditary deficiencies of Factor VIII *and/or* IX with prolonged BT without capillary and platelet disorders (dominant hemophilias with prolonged BT)

1 Those with temporarily normalized BT after administration of (the correct ? dose of) Cohn s fraction I (as plasma bleeding factor deficiencies ?)

2 *Those not responding*

III Genuine thrombopathies without indication of plasma coagulation disorders with normal capillary status (morphology resistance reaction to stimulus permeability) with varying expressivity and prolonged BT

IV Genuine teleangiopathies and purely vascular pseudohemophilias with prolonged BT and without coagulation and platelet disorders

1 Primary constitutional vascular disorders

2 Secondary teleangiopathies caused by lack of humoral factors or by pathological plasma factors

Should electron microscopic studies using the ultrafine slide technic show a significant and specific alteration in platelet structure in all vascular hemophilias the two subgroups I 1 and I 2 would be placed in a single category There seems to be no evidence for the assumption that group I 1 is necessarily a *forme fruste* of group I 2 with its broader genetic disturbance It could as well be assumed that the two genetic disorders are independent and different from each other At any rate group I 2 has an incomplete dominant heredity and a varying expressivity

If it were possible to produce evidence that the families of group II actually have a primary vascular deficiency there would be no objection to including them with the von Willebrand Jurgens syndrome For the time being however a primary plasmatic factor disorder appears more probable

There also seems to be insufficient evidence for the assumption that all hemorrhagic diseases classified as purely vascular pseudohemophilias are merely *formes frustes* of subgroups I 1 and I 2 although two sisters were seen who had a prolonged BT one had Factor IX and platelet adhesion deficiencies while the other showed only prolonged BT without other disturbances (family Gr)

In view of the present state of knowledge elimination of the terms thrombopathy and angiohemophilia (4) would in my opinion be going too far Also I do not think it necessary to exclude the term hemophilia which is familiar in many combinations (such as parahemophilia circulatory anticoagulant (Hemmkorper) hemophilia etc) in connection with diseases with prolonged BT and normal platelet count The designations angiohemophilia or dominant hemophilia (with prolonged BT) seem appropriate for a good many cases

Family and Case Studies

"Vascular Hemophilia (angiohemophilia A von Willebrand Jurgens syndrome pseudohemophilia) Family G (Fig 2 3 4) had a dominant hemorrhagic diathesis with excessively prolonged BT (patients No 2 4 5 6 and 7 more thoroughly examined) normal platelet status (platelet agglomeration in the capillary blood smear normal No 4 5 6) and moderate Factor VIII deficiency (26) (5 11%) The main bleeder (No 6) of this family was found to have no platelet anomalies [electron microscope studies by Marx and Koppel (27)] and normal retraction There were pathological

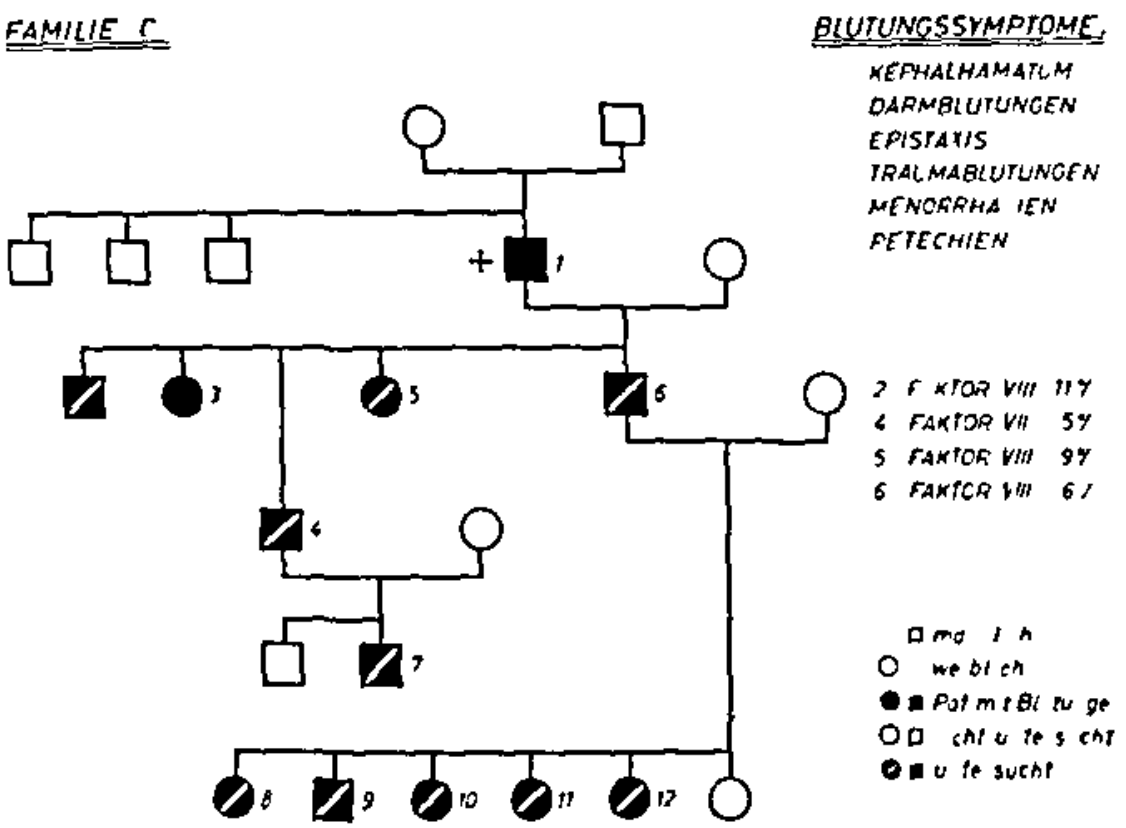


FIG 2 Pedigree of Family G



FIG 3 Pathologic Capillaries in the Nail Bed Patient No 6 Family G (Vascular Hemophilia A)

nail bed capillaries and constantly decreased capillary resistance (also in cases No 2 4 and 5) We diagnosed the capillary vessel disorders of several family members as vascular hemophilia and angiohemophilia A (von Willebrand Jurgens syndrome pseudohemophilia) Both the Rumpel Leede test and the Goethlin test were strongly positive in the adult family members (No 2 4 5 6) whereas the suction test in the midaxillary line (Bigot and Schaerfe apparatus) was positive with two adults only with a vacuum of 100 200 and 300 mm Hg The Rumpel Leede test and the Goethlin test were however negative in four children up to a maximum

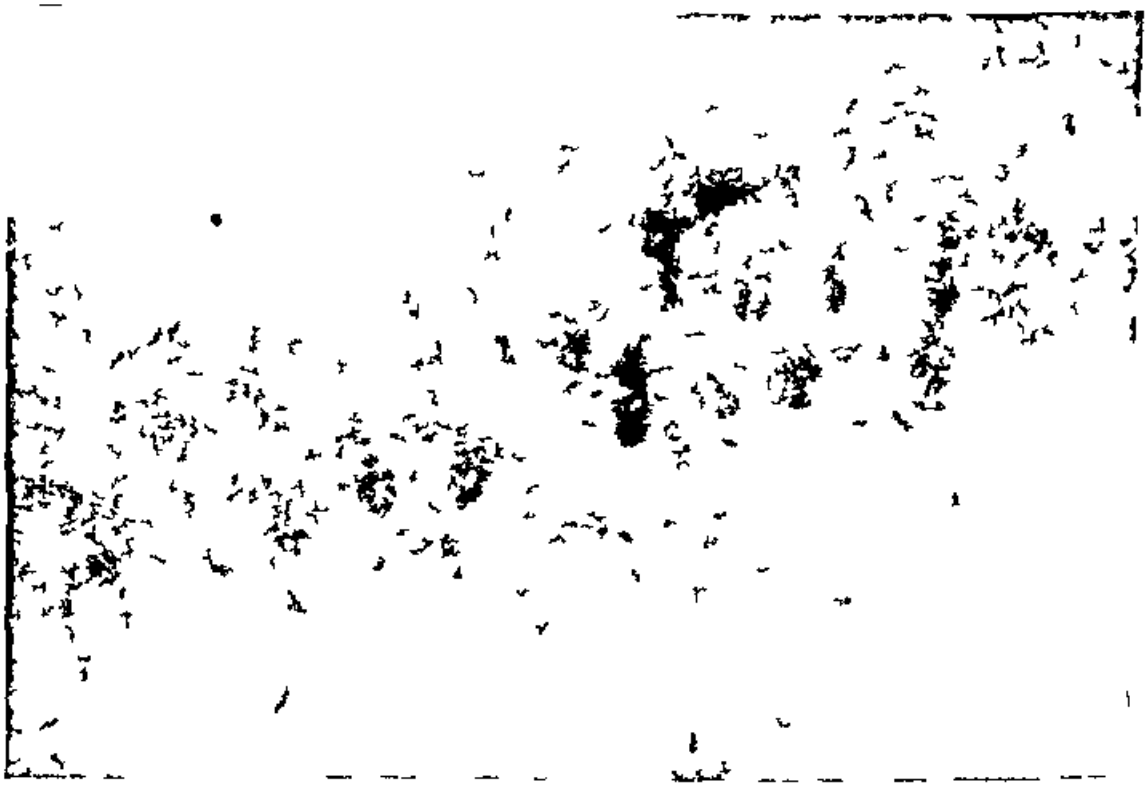


FIG 4 Carpet Beater Capillaries in the Nail Bed
Patient No 6 Family G (Vascular Hemophilia A)

age of 9 years (No 8 9 10 11) and the suction test was occasionally positive with only one child Age must be taken into account when the capillary resistance is considered Children from families whose adults show a decreased hereditary capillary resistance may be found to have a normal capillary fragility

The agglomerogram and agglutinogram of the platelets in the capillary blood smear must be compared with identically prepared capillary blood smears of normal blood In judging this test we consider that an appreciable disorder of platelet agglomeration or agglutination is present only if per 100 counted separate platelets less than 17 are arranged in groups of two less than six in groups of three less than three in groups of four and none of them in groups of five It is not sufficient if one group is below the

norm The physiological range of fluctuation does not permit one to designate as pathological all agglomerograms or agglutinograms which are below the values mentioned by Rovatti Several blood smears should be counted and median values from various auricular punctures be utilized since in venous blood there is a much greater dispersal of platelets in the agglomerogram and conditions considered normal in venous blood would be pathological *in capillary blood Thus blood obtained by puncturing peripheral* vessels may show different conditions of agglomeration (probably a manifestation of viscous metamorphosis in its initial stages)

Vascular Hemophilia AB (angiohemophilia AB v Willebrand Jurgens syndrome pseudohemophilia) Female patient v d V 11 years old had a constitutional hemorrhagic diathesis with bleeding for several days after both hordeolum incision and tooth extraction gingival bleeding and easy bruisability The mother and family on the mother s side were clinically healthy History of father and family on father s side was unknown There were excessively prolonged BT (> 30 min) (Duke and Marx Ressel) normal platelet status (208 800 phase contrast microscope method) platelet factor 3 normal (Biggs test and Gross Schwick) platelet metamorphosis on zapon lac foil normal platelet agglomeration in the capillary blood smear normal and prolonged CT of venous blood 19½ min (Lee White) TEG values of r 21 min k_1 8 min ma 128 pathological serum prothrombin normal PT index 86 Factors V and VII 100% prothrombin Stuart factor complex 80% (Schultze Rieben) decreased Factor VIII (Biggs) and decreased serum thrombokinase factor activity Factor VIII 8% *Factor IX 21% (Geiger and co workers) angioscopic examination* showed short loop type in the nail bed without discernible morphological anomalies Rumpel Leede strongly positive RR 95/65 and retraction upper limit of normal (Fonio method)

A tentative diagnosis of angiohemophilia AB and vascular hemophilia AB (von Willebrand Jurgens syndrome pseudohemophilia) was made in view of the marked vascular hyperfragility without signs of any other disease As long as the vascular condition cannot be ascertained as hereditary nothing but a presumptive diagnosis can be made

***Plasmothrombopathy** (v Willebrand Jurgens syndrome) a) Family E* (four members examined) has a dominant hereditary hemorrhagic diathesis of varying expressivity with Factor IX deficiencies ranging between 39 and 50% moderately prolonged BT normal capillary picture (nail bed) and normal capillary resistance the female main bleeder had subarachnoidal bleedings and purpura decreased platelet factor 3 activity (repeatedly investigated) combined with normal platelet counts and normal platelet agglomeration

b) Family W has a dominant epistaxis diathesis (three persons two of them examined) with slight Factor VIII and Factor IX deficiencies (mother 17% Factor VIII 44% Factor IX son 14% Factor VIII 22% Factor IX)

inconstantly prolonged BT and normal nail bed capillaries The female main bleeder has a decreased factor 3 activity

Dominant Mild Hemophilia A with Prolonged BT (with incomplete expressivity) A large family Lei Ha has a dominant hereditary hemorrhagic diathesis (22) (61 members including 17 bleeders seven of which have been more thoroughly examined) with prolonged BT normal platelet factors and normal functional status (sporadically showing thrombopenia) decreased combined Factor VIII and IX content of plasma (2 23% Factor VIII and 4 68% Factor IX) normal capillary nail bed picture and normal capillary resistance It is of interest that this family includes individuals

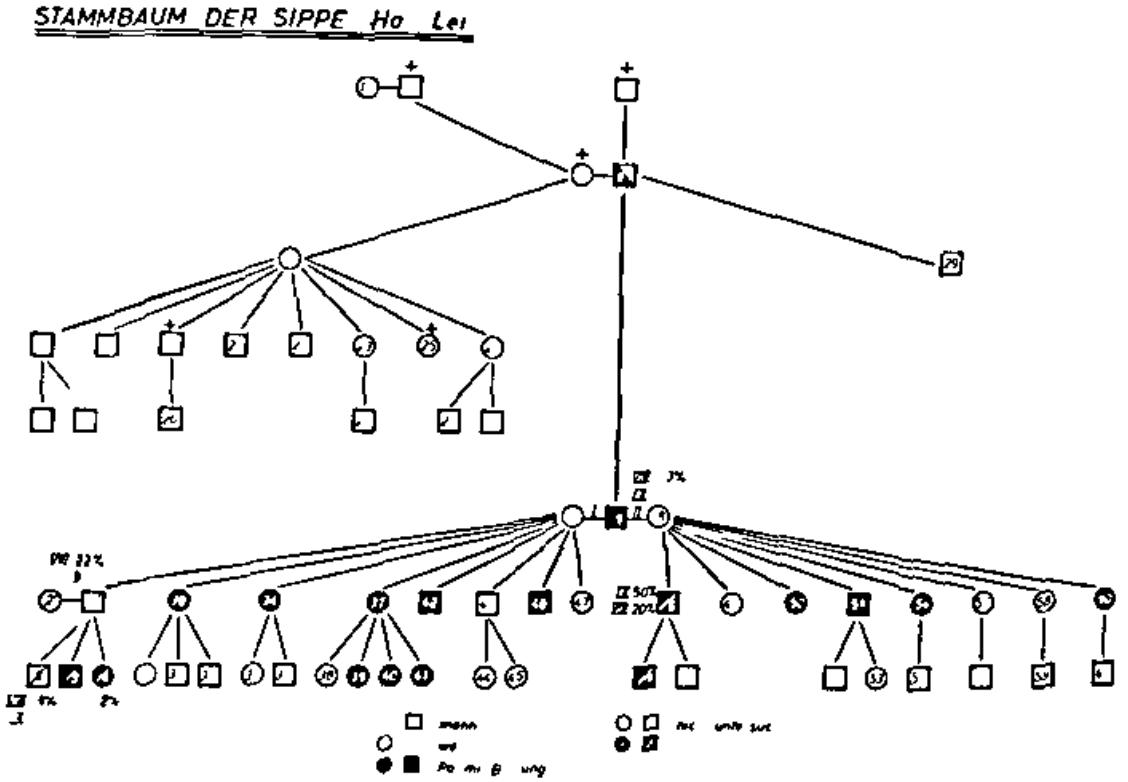


Fig 5 Pedigree of Family Ha Lei

with a consistent normal BT and merely a decrease of Factor VII only For the time being we describe the clinical picture as dominant mild hemophilia A with prolonged BT (with incomplete expressivity) (Fig 5)

Among the patients studied 16 members of 10 families showed pathological thrombocyte function and factor tests Seven members of five families were examined for platelet factor 3 (modified TGT Deutsch Johnson Seegers test) and were found to be pathological Two of these families showed a decreased platelet factor 3 activity with concomitant thrombokinase factor disorders of the plasma

Dominant Hereditary Hemophilia B with prolonged BT and disturbed platelet metamorphosis in citrate blood A single female patient H from a family with hereditary bleeding anomalies (one sister with epistaxis)

showed a moderately prolonged BT (11 minutes) Factor IX deficiency (9%) not related to liver disease a normal capillary status (nail bed capillary forms and Rumpel Leede) and absence of platelet anomalies as shown by platelet adhesion to glass agglomeration in the capillary blood smear me in the TEG retraction (Fonio) and platelet factor 3 (Gross Schwick) The case might be diagnosed as dominant hereditary hemophilia B (or Factor IX deficiency) with prolonged BT but disturbed platelet metamorphosis in citrate blood Whether or not the observed platelet disorder allows us to assign the case to the von Willebrand Jurgens syndrome cannot yet be determined since this disorder could also be a primary plasmogenic disorder The case illustrates the difficulties of classification

Cohn Positive Pseudohemophilia A A single female patient A H is from a family with hemorrhagic diathesis (menorrhagia epistaxis and bleeding after dental extraction in mother grandmother and great grandmother) There was a moderately prolonged BT (8 min) Factor VIII deficiency occasionally normal TGT normal nail bed capillary picture and normal platelets Her BT was normalized for some hours after infusion of one ampule of Cohn s fraction I We named the case Cohn positive hemophilia A with prolonged bleeding time or Cohn positive pseudohemophilia A

Purely Vascular Pseudohemophilia Two single cases (hereditary genesis undetermined) both had prolonged BT but no morphological capillary anomaly in the nail bed with normal coagulation factors and normal platelet status One of the patients who had previously suffered a retinal bleeding died of a subarachnoidal hemorrhage without having responded to an i v infusion of one ampule of Cohn s fraction I

Summary

The author has attempted to point out the problems of differentiating the pseudohemophilias and to classify the hereditary pseudohemophilias on the basis of methods available at present The question whether or not all hemorrhagic anomalies included under the term hereditary pseudo hemophilias are determined by *one* hereditary disorder is raised Hemostatic investigations of an increasing number of families are needed These investigations should include the blood vessels platelets and coagulation factors and should exclude latent liver disease

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which had to be incised At that time no unusual bleeding was observed In 1958 an operation of the osteomyelitis scar was done because of pain Following the operation heavy bleeding occurred in the wound area which was finally stopped after several blood transfusions and administration of vitamin K

In 1950 an infiltration appeared in the upper lobe of the left lung Because of her known bleeding tendency the patient was treated conservatively in a sanitorium In 1952 cavitation was observed and the sputum became positive A therapeutic pneumothorax was induced During one of the first refillings severe bleeding occurred and 7 units of stored blood were given The pneumothorax treatment was stopped and the hematothorax gradually became organized The cavity in the left apex persisted and the sputum remained positive In 1954 a severe hemoptysis lead to admission of the patient to the Second Medical Clinic She was found to have a fibro ulcerous tuberculosis in the left apex with several large cavities and with positive sputum Coagulation studies revealed the findings given below Her bleeding tendency made active therapy impossible She had to be hospitalized for nearly a year and during this time she received intensive chemotherapy cortisone and occasional blood transfusions (without recurrent hemorrhage) During that year roentgenograms indicated gradual improvement with regression of the recent lesions However the sputum remained positive Only after her discharge did her general condition improve considerably The patient married during this time Under continuous chemotherapy she gained 6 kg the lung condition became stationary leaving a cherry sized cavity but the sputum remained positive In 1956 while the pulmonary condition was still unchanged hemoptysis recurred necessitating a blood transfusion

In 1957 the patient became pregnant for the first time A decision had to be made whether a delivery could be risked (considering her illness) She was already at the end of the fourth lunar month of pregnancy when she consulted a physician The interruption of the pregnancy at that time would have probably resulted in considerable danger to the patient This influenced the decision in favor of a normal completion of the pregnancy since pelvic examination was normal Pregnancy progressed uneventfully For the time of delivery blood donors stored blood and Antihemophilic Plasma(R) were made available The delivery occurred without operative intervention Before the beginning of the second stage the patient received *100 mg Dolantin(R) and 0 05 gm Phenergan(R) with 1 25 mg Lorfan(R)* in order to ease the spontaneous bearing down as much as possible leading to a slow and careful stretching of the perineum during the delivery of the infant Thus an episiotomy could be avoided The delivery was made easier by the fact that the head was smaller than normal the infant weighing only 2900 gm The hemorrhage during the separation of the placenta was kept at a minimum by i v injection of 1 ml of Methergin(R) Close inspec

16

Diagnosis and Obstetrical Management of a Patient with Factor VIII Deficiency (Constitutional Thrombopathia von Willebrand Jurgens) *

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A DEFICIENCY of Factor VIII as found in patients with classical hemophilia causes a severe hemorrhagic diathesis In recent years a marked decrease in Factor VIII has been observed in some women who have no family history of hemophilia (1 2 4 6 10 11 18) Because of the rarity of this occurrence it was of interest to follow such a patient through her two pregnancies and deliveries

Case History M I A 34 year old housewife Bleeding tendency already in early childhood The patient bruised easily had occasional severe epistaxes and bled for several days after tooth extractions In late childhood hemorrhages occurred into the knee joints

Menarche at the age of 14 the menstrual flow always heavier than normal duration 4 5 days However there never has been severe bleeding during menstruation

The patient does not have any siblings Her parents live in Argentina and for that reason could not be examined but are said to be in good health

At the age of 8 she had osteomyelitis in the anterior part of the left foot

* The investigations were carried out with the aid of the Blood Research Foundation Washington D C U S A

Table 1

Test	*Normal Values*	*Mother*		*First Child*	*Second Child*
		Before Pregnancy	*Last Week of Pregnancy*		
Clotting Time	5 10 min	8 25 min			
Bleeding Time	1 3 min	2 min			
Platelet Count	100 000 300 000	184 000			
One stage Prothrombin	70 100%	75 95%	1 0%	80%	65%
Prothrombin	70 100%	88 100%	150%	68%	40%
Factor V	70 100%	87 100%	130%	—	—
Factor VII	70 100%	68 100%	180%	55%	40%
Stuart Factor	70 100%	—	—	12%	44%
Thromboplastin Generation	60 100%				
P s Plasma + P s Serum		6 5%	—	25%	42%
P s Plasma + Normal Serum		8 5 15%	17%	45%	63%
Normal Plasma + P s Serum		80 100%	—	61%	87%
Prothrombin Consumption	more than 15 sec	5 11 sec	—	15 sec	96 sec
Residual Prothrombin	less than 10%	24 80%	—	1 %	less than 1%
Heparin Tolerance Test	4 min	more than 45 min	—	more than 10 min	5 min 40 sec
Retraction		normal	—	—	—
Platelet Factor 1	50 100%	100%	—	—	—
Platelet Factor 3	30 000 Plat/mm³	0 000	—	—	—
Platelet Factor 4		reduced	—	—	—

Lowest concentration of platelets which induces normal thromboplastin generation after homogenization

tion of the birth canal postpartum revealed two small lacerations of the mucosa each 1 cm long which were closed immediately with 2 catgut sutures The patient then received 300 ml of fresh blood and 2 units of Antihemophilic Plasma(R) Under medication with oxytocics she had an uneventful puerperium As the patient was not allowed to nurse the infant because of her open tuberculosis lactation was prevented by giving estrogens three doses 10 mg each She was discharged on the 5th day post partum On the 8th day when a scab became detached from one of the small mucosal lacerations heavy bleeding occurred from the vagina The patient was readmitted to the Clinic To stop the bleeding local hemostyptic measures and 2 transfusions of 500 ml of fresh blood as well as 28 units of Antihemophilic Plasma(R) were used

The general condition of the patient had improved during the pregnancy and the cavity in the lung had become smaller Postpartum under continuous chemotherapy with INH(R) and 20 gm streptomycin the sputum became negative for the first time

In 1958 the patient became pregnant again The course of this pregnancy was also unremarkable With the experience gained from the first delivery the second delivery was handled in the same conservative manner and there were no complications As this delivery occurred one month prematurely and the infant weighing only 2500 gm was considerably smaller than the first one there was much less stretching of the birth canal During the first 24 hours postpartum she received one unit of Antihemophilic Plasma(R) every six hours and in the following days one unit every 12 hours After one week the treatment was stopped and the patient was discharged Lochial secretion was normal In order to suppress lactation the patient received 10 mg of estrogens on the first two days after delivery Two weeks later vaginal bleeding started initially slight but becoming more severe in the following days in spite of ergot medication Assuming that it might be a hemorrhage of an anovulatory cycle the patient was given 20 mg of estrogen in oily solution every two days On the day following the second injection the bleeding ceased promptly An additional 20 mg of depot estrogen was given and no bleeding occurred during the next 10 days Then 250 mg of depot progesterone was administered followed 12 days later by bleeding similar to normal menstruation The patient has been menstruating regularly ever since The cavity had disappeared completely at the end of the pregnancy and the sputum was negative

Coagulation Analysis Methods coagulation time—Lee White bleeding time—Duke platelet count—Fonio one stage prothrombin time—Quick prothrombin Factors V and VII—Deutsch and Schaden Stuart factor—Bachmann *et al* thromboplastin generation test—Biggs and Douglas prothrombin consumption—Soulier heparin tolerance test—Marbet and Winterstein platelet factors—Deutsch Johnson and Seegers plasma

the patient s plasma was considerably diminished but it was normal using the patient s serum Since the prothrombin consumption test and the re calcification time were normalized on the addition of a small amount of normal plasma (Table 2) the presence of an inhibitor could be excluded

Table 2

MIXING EXPERIMENT WITH NORMAL PLASMA

Amount of Patient s Plasma	*Normal Plasma*	*Recalcification Time*	*Prothrombin Consumption Time*
0 6 ml	—	4 30	14
0 5 ml	0 1 ml	10	43
0 3 ml	0 3 ml	05	62
0 1 ml	0 5 ml	2 00	81
—	0 6 ml	1 45	80

The plasma of the patient did not normalize the recalcification time of a patient with hemophilia A These findings alone pointed in great probability to a deficiency of Factor VIII To investigate this assumption normal plasma was fractionated with ammonium sulfate and the effect of fractions precipitated at 33 40 and 50% saturation was tested The fraction precipitated at 33% saturation with ammonium sulfate normalized thrombo plastin generation almost completely while the two other fractions were inactive In the thrombelastogram the reaction time was prolonged to 17 min and the clot formation time to 10 min (Fig 2) For the results of the tests for other factors refer to Table 1

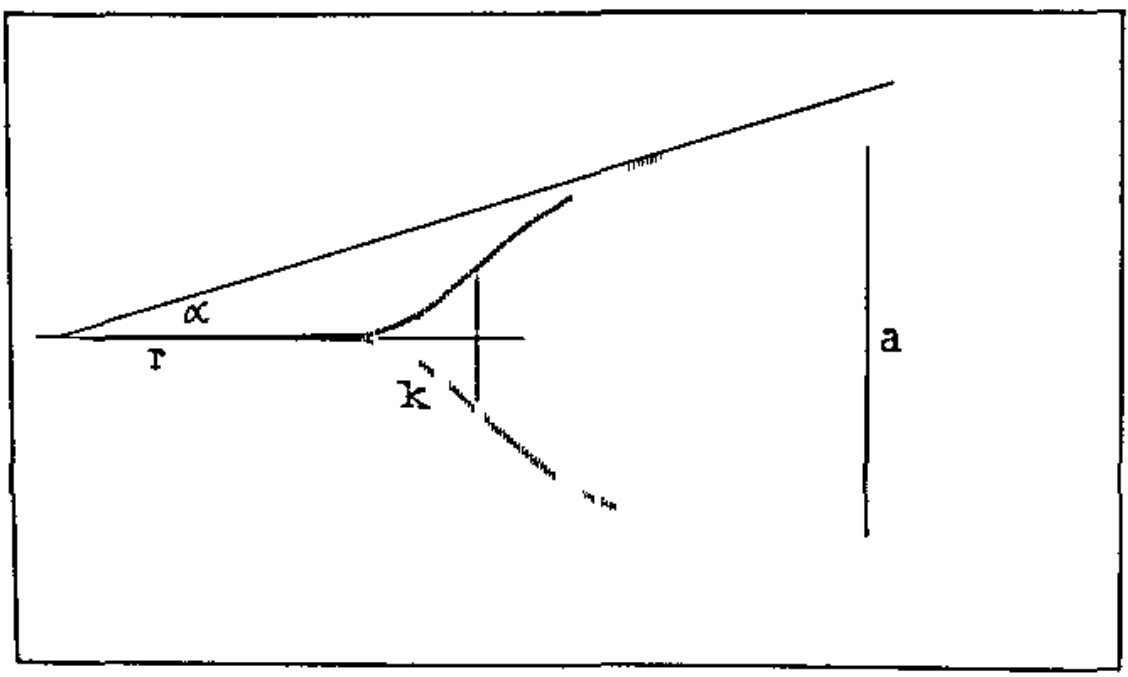


FIG 2 Thrombelastogram in the Blood of the Mother

mixing test—Deutsch thrombelastogram—Hartert as described in previous publications (7 8)

Results

1 Plasmatic Coagulation System During the 4 years we had observed the patient the coagulation disturbance had been investigated several times (Table 1) The coagulation time was always moderately prolonged (8 25 min) while the heparin tolerance was always greatly diminished Prothrombin consumption was markedly decreased the prothrombin content of the serum was increased Thromboplastin generation (Fig 1) using

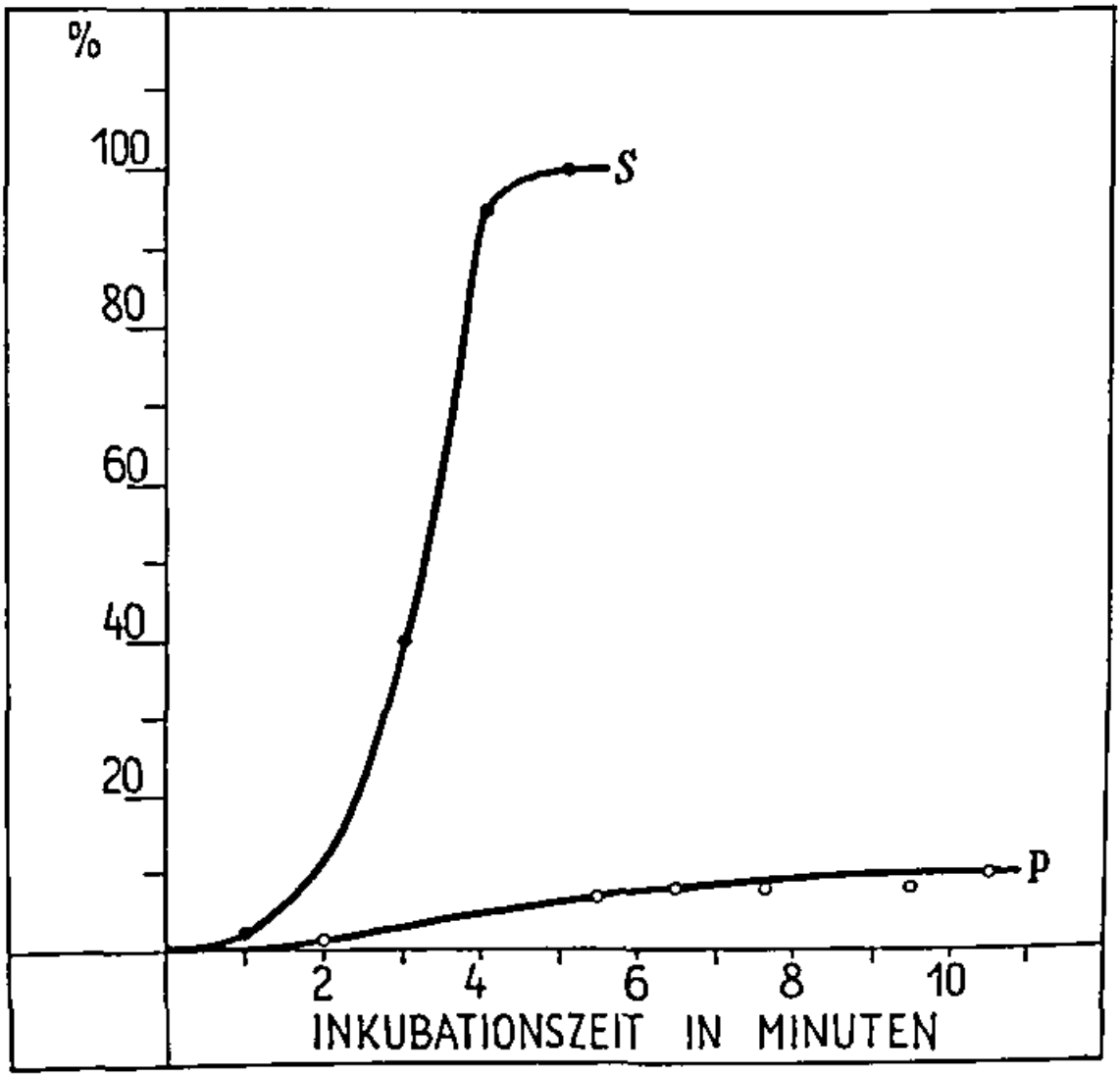


FIG 1 TGT in the Blood of the Mother
P Patient s $BaSO_4$ adsorbed plasma (1 5) + normal serum (1 10) + lipoid extract of brain (1 50)
S Patient s serum (1 10) + normal adsorbed plasma (1 5) + lipoid extract of brain (1 50)
P + S Patient s adsorbed plasma + patient s serum + lipoid extract of brain
Ordinate thromboplastin formed in % of normal

edly prolonged thromboplastin generation (Fig 4) These findings can be related to a deficiency in plasma and erum factors The disturbance in thromboplastin generation with the infant s plasma is slight but it cannot be explained by the age of the child Heparin tolerance was only slightly diminished and prothrombin consumption was normal

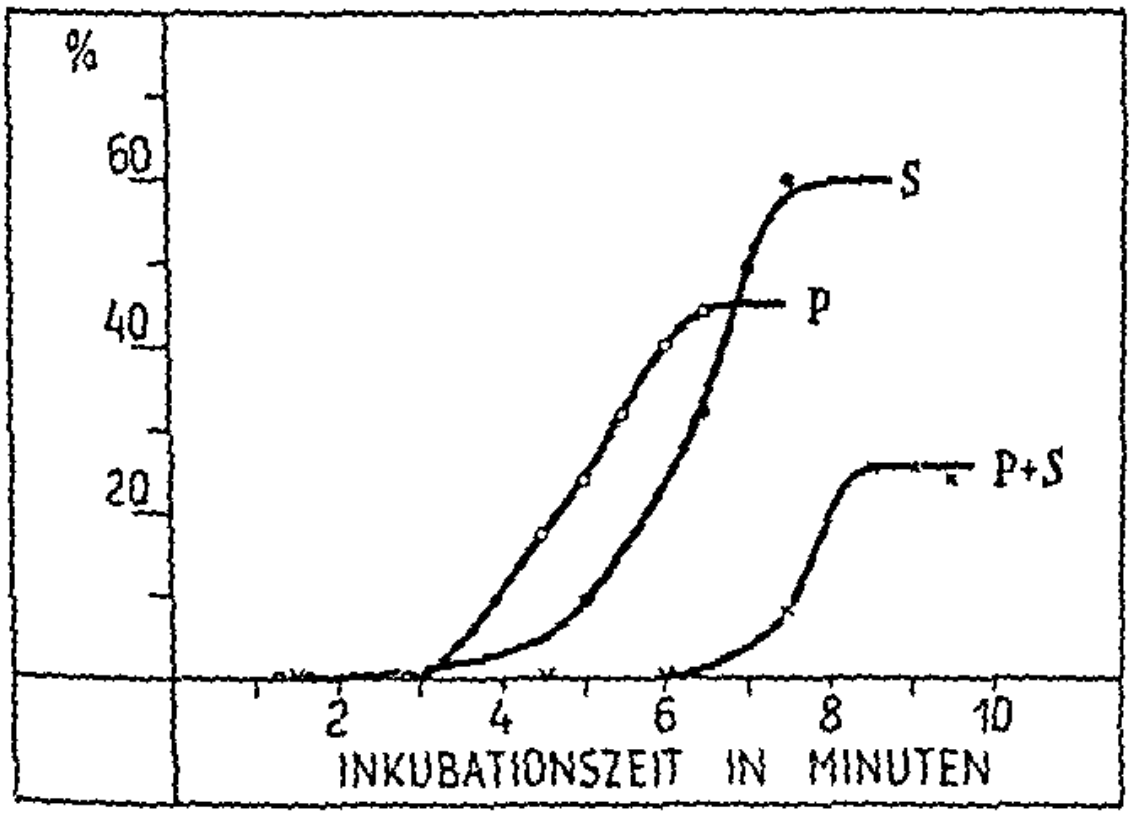


Fig 3 TGT in the Blood of the First Child Legend as in Fig 1

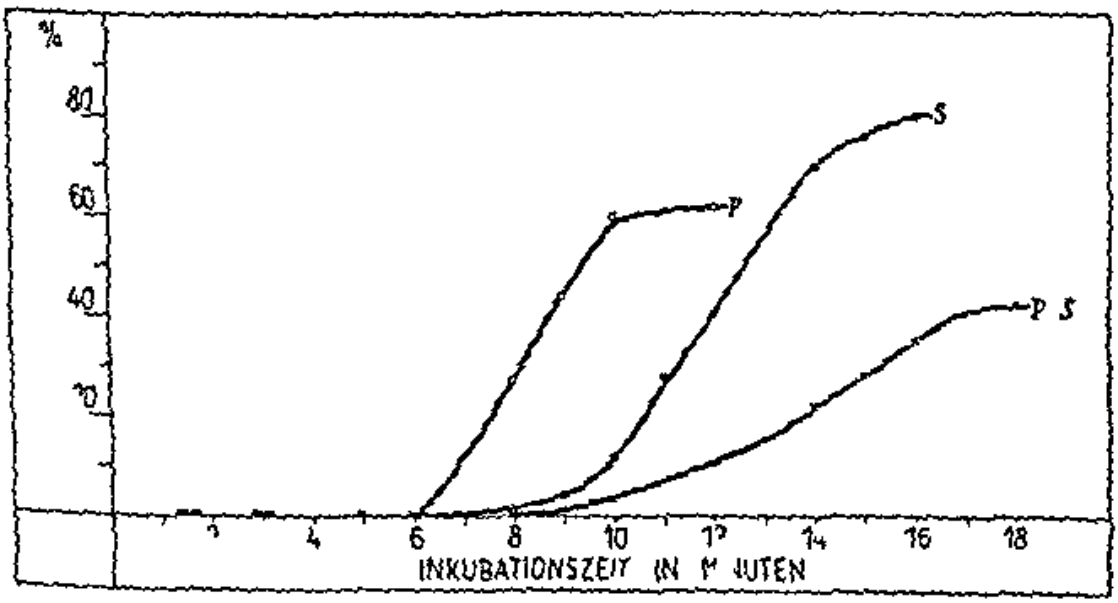


Fig 4 TGT in the Blood of the Second Child Legend as in Fig 1

2 Examination of Platelet Function Platelet count and platelet factors 1 and 3 were normal Thromboplastin generation was already optimal at 20 000 platelets/mm³ The antiheparin activity of the platelets however was distinctly decreased especially when the platelets had not been homogenized by freezing (Table 3) Electron microscopic examination by Dr Braunsteiner revealed that the platelets started to spread only after 30 min definitely a pathological finding The retraction and the maximal amplitude of 54 mm in the thrombelastogram were normal (Fig 2)

3 Examination of Vascular Function Bleeding time in all examinations was normal capillary resistance determined by the suction test was distinctly decreased Rumpel Leede test was usually positive

4 Behavior of Coagulation Factors During Pregnancy The coagulation factors were investigated one week before delivery (Table 1) It was found that prothrombin and Factors V and VII had as usual increased to values above 100% but that Factor VIII deficiency was not essentially affected

Table 3

PLATELET FACTOR 4

	Normal	*Patient*
Control without platelets and without heparin	16 sec	16 sec
Control without platelets and with heparin	45 55 sec	45 55 sec
Tests with 500 000 fresh platelets per mm³	20 25 sec	40 sec
Tests with 500 000 platelets per mm³ after homogenization	16 20 sec	31 35 sec

5 Behavior of Coagulation Factors in the Children Girl born on Nov 3 1957 The examination was made on the third day of life The results are shown in Table 1 A moderate decrease in prothrombin and Factor VII was found as well as a marked decrease in Stuart factor The thromboplastin generation using the infant s serum was pathological (Fig 3) These findings are explained by the physiological changes in coagulation during the first days of life (3) Apart from that the coagulation time after the addition of heparin and thromboplastin generation in the infant s plasma were distinctly decreased These findings cannot be explained by the physiological changes in coagulation during the first week of life (Fig 3) According to the parents the child has been healthy so far and no noticeable hemorrhages have been observed Therefore the parents could not be persuaded to bring the child for a follow up examination

M I M girl born on Jan 11 1959 The umbilical cord blood was examined (Table 1) A noticeable decrease in prothrombin Factor VII and Stuart factor was found as well as only slightly decreased but mark

time was normal although a prolongation is regarded as classical for this condition

The first child of the patient probably has inherited the disease from her mother We are not so certain about the second child In case this child also is a carrier the decrease in Factor VIII seems to be much less pronounced A definite diagnosis should be made later when the coagulation pattern of infancy is no longer present The variable manifestations of the disease in members of the same family present a characteristic difference between the illness described here and hemophilia

It seems important to us that during pregnancy the coagulation factors prothrombin Factor V and Factor VII increase in the same manner as in normal women (Table 1) but that no essential changes occur in the deficiency of Factor VIII

A normal delivery without traumatic tissue lacerations does not seem to present any special risk for such patients since hemostasis during separation of the placenta is effected predominantly by mechanical compression of the vessels by contraction of the uterine muscles Should operative intervention become necessary this would present considerable danger to the patient We avoided even a simple episiotomy in both deliveries and our patient did not lose any more blood than women with a normal coagulation mechanism The hemorrhage becomes a threat only during and after the puerperium Suitable therapy would probably have prevented the incident that occurred 8 days after the first delivery Therefore it is necessary to give such patients preventive treatment during and after the puerperium whether they bleed or not The treatment should consist of antihemophilic plasma or fresh blood to be given first at intervals of 4 6 hours and later at longer intervals as well as of application of oxytocics These should be given during delivery and continuously throughout the puerperium and should be gradually withdrawn after involution of the uterus decreases the danger of hemorrhage

The question raised in the beginning whether thrombopathia von Willebrand Jurgens constitutes an indication for interruption of pregnancy has to be answered negatively It is our opinion that a non physiologic intervention such as interruption of pregnancy is more dangerous for the patient than an otherwise uncomplicated delivery If the patient should consult a physician before pregnancy occurs she should be advised against it for two reasons 1) even a normal delivery represents a certain danger and 2) she is very likely to transmit the disease to her children In our special case there was still another reason not to interrupt the pregnancy the possibly favorable effect on the tuberculosis which could not be treated by pneumoperitoneum and pneumothorax because of danger of hemorrhage This favorable effect really occurred the cavity disappeared during the second pregnancy and could not be demonstrated any more even by tomogram The sputum has been negative for about two years

Discussion

A combined coagulation disturbance in this patient is the cause of an occasionally quite severe hemorrhagic diathesis Most important is the decrease in plasma Factor VIII which explains the prolongation of coagulation time decrease in prothrombin consumption and the results of the thromboplastin generation test The decrease in heparin tolerance is striking being greater than that ordinarily expected in classical hemophilia even with the same low Factor VIII activity and the same prolonged coagulation time Reduction of platelet factor 4 appears to be the basis of the very abnormal heparin tolerance test The changed morphological behavior of the platelets in electron microscopic examination should be mentioned as further evidence of a disturbance in platelet function On the other hand clot retraction other platelet factors as well as the firmness of the clot are normal (Fig 2) The decreased capillary resistance and increased capillary fragility have to be interpreted as signs of a disturbed vessel function The normal bleeding time repeatedly determined is extraordinary in this coagulation disturbance

The classification of this illness does not present any difficulties On the basis of family history and the combination of Factor VIII deficiency with disturbances in platelet and vascular systems classical hemophilia can be excluded For this condition Quattrin found many years ago the very pertinent and descriptive name plasmo teleangio thrombopathia (16 17) In 1933 von Willebrand and Jurgens investigated the first families with this condition in the Åland Islands They defined it as a constitutional thrombopathia and recognized then the combination of a platelet dysfunction and a vascular damage as the cause of the illness In recent years Jurgens re examined some of these patients and found that platelet factor 3 (12 14) and/or plasma Factor VIII (14) were decreased This shows that in the various members of the same family not all three systems have to be affected The degree of disturbance in different systems can be even more variable from family to family Thus Macfarlane (15) and Estren (9) described families that showed only vascular changes and used the name pseudohemophilia Schulman and Smith (18) used vascular hemophilia and Klesper and Achenbach (1 2) used angiohemophilia describing families with Factor VIII deficiency as well as vascular defects In our opinion we are dealing in all these conditions with only one disease that if fully developed affects all three systems—plasma (Factor VIII and/or IX) platelets and vessels However all combinations are possible with two or only one system being affected although we are not dealing with separate diseases in the incomplete syndromes for historical reasons we would like to keep the name constitutional hemorrhagic thrombopathia von Willebrand Jurgens and classify our patient s illness as such It is remarkable that despite involvement of all three systems in one patient the bleeding

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Summary

A 34 year-old patient with a hemorrhagic diathesis constitutional thrombopathia von Willebrand Jurgens affecting all three systems (plasma with Factor VIII deficiency platelet dysfunction and vascular damage) acquired open pulmonary tuberculosis 7 years ago Attempted induction of a pneumothorax resulted in hematothorax requiring 7 blood transfusions In the last few years the patient has had two pregnancies both with normal deliveries The older daughter has inherited the disease from her mother and most likely the younger daughter has too The best way to conduct a delivery and the risk of induced abortion are discussed The cavernous pulmonary tuberculosis was clinically healed probably favorably influenced by the two pregnancies

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Part 3

ACQUIRED "HEMOPHILIOID" STATES

17

New "Hemophilioid" Defects Some Clinico Laboratory and Experimental Abnormalities in Thromboplastin Generation *

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THE Tpl generation test has contributed enormously to our understanding of coagulation Many factors i e AHF PTC PTA Ac globulin Hageman factor Stuart Prower factor are necessary for blood Tpl formation Controversy still exists however regarding technical aspects of the test as well as the requirements for other components Data to be presented bearing directly on these points consist of abnormalities observed in Tpl generation consequent to (a) induction of the generalized Shwartzman phenomenon (b) the addition to blood of colloidal thorium oxide (Thorotrast) and (c) the influence of powdered glass and of other manipulative procedures on coagulating blood Although the direction of these investigations seems unrelated they are described in order to draw attention to several unexplored areas of the earliest phase of coagulation the elaboration of intrinsic thromboplastin Certain theoretical biopathological and practical aspects of the observations will be discussed

The Endotoxin Induced Generalized Shwartzman Reaction and Tpl Generation

Despite evidence suggesting hemostatic defects and hemorrhagic phenomena associated with the induced generalized Shwartzman phenomenon

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(1) no clear-cut definitions of some observed clotting abnormalities have hitherto been reported (2 3) That they exist however would seem likely since in this experimental condition fibrin is deposited in the small blood vessels associated with alterations in clotting time thrombocytopenia and fibrinogenopenia (2 3 7) Moreover the fibrin thrombi can be prevented by prior administration of anticoagulants or of the fibrinolytic activating a ent Streptokinase (4 5 6)

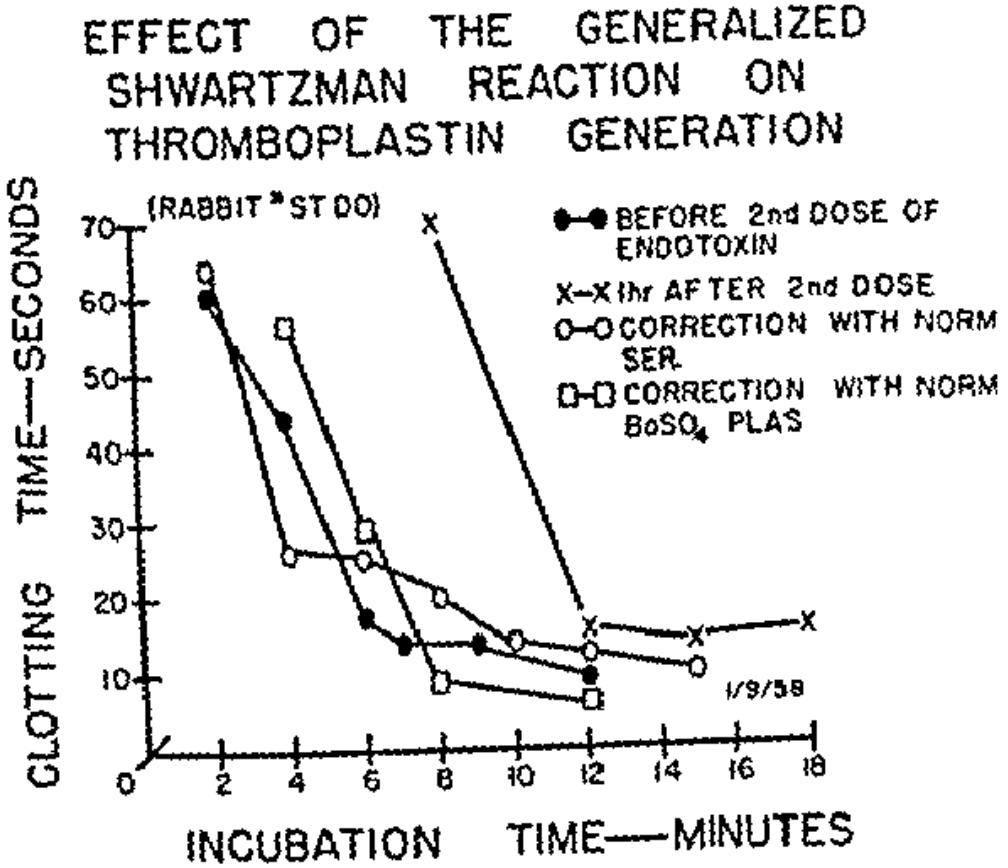


FIG 2 Corrective Effect in TGT of Normal Constituents on Shwartzman associated Tpl generation disturbance

In six of 11 rabbits subjected to the orthodox double dose Shwartzman inducing endotoxin (E coli) marked impairment of Tpl generation super vened (Figs 1 2 3) In addition to the six two other animals showed questionable abnormalities As seen in Fig 4 the defect became evident within one hour after the animals received the second dose of toxin and may become more severe within 3 hours In one surviving animal it was still evident at 48 hours The abnormality demonstrable in both plasma and serum could be substantially corrected by substitution in the TGT of the respective Shwartzman defective component (plasma or serum) with its normal or pre second endotoxin dose counterpart Although the precise

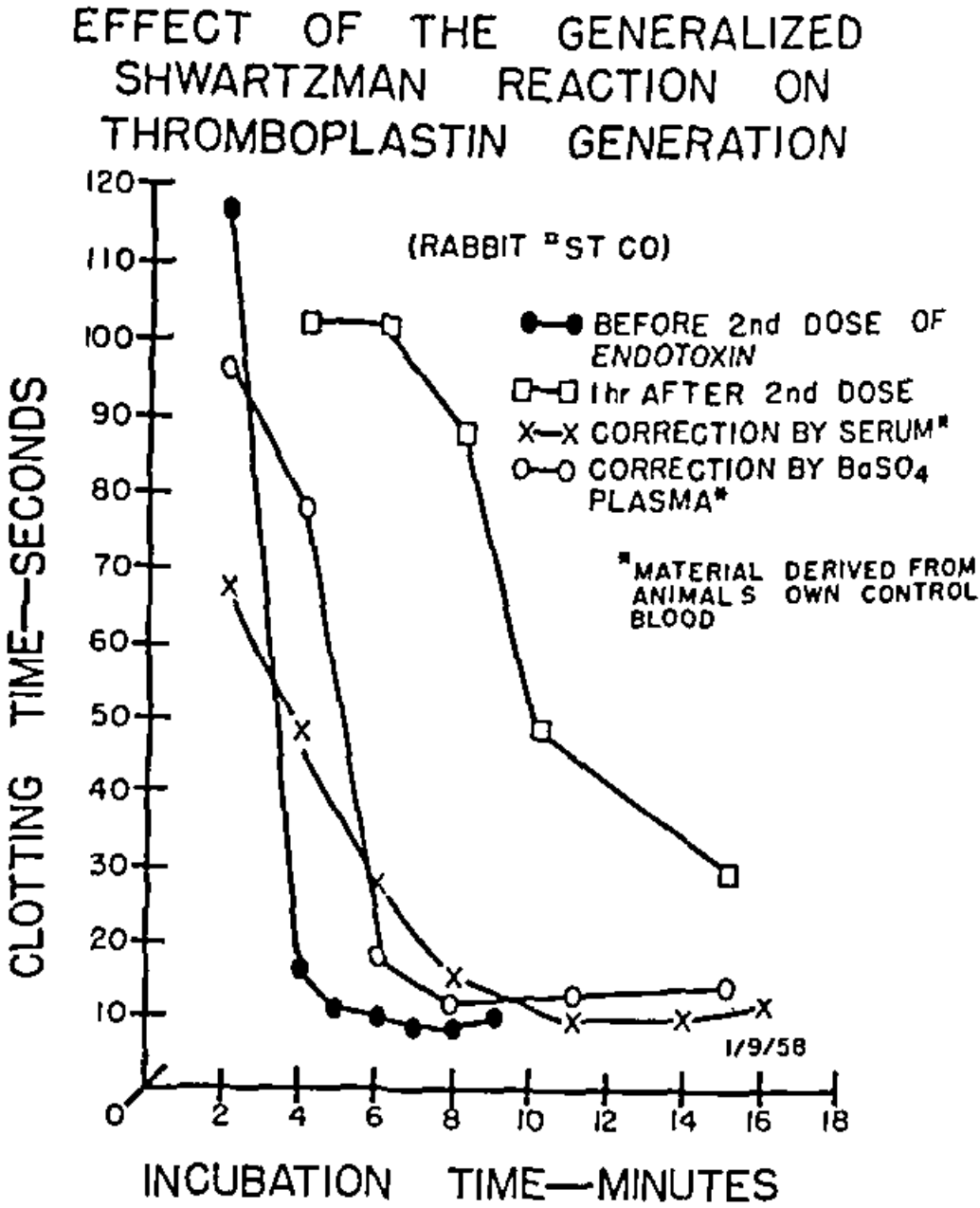


FIG 1 TGT on Hybrid Weanling Albino Rabbit 0 2 mg per kg of E coli endotoxin kindly provided by Dr F J Schweinburg of the Beth Israel Hospital were administered intravenously repeated 24 hours later Under the conditions of these experiments about 90% of animals studied in a separate control group exhibited the generalized Shwartzman reaction characterized by bilateral renal cortical necrosis and fibrin deposition in lungs liver spleen and kidney TGT according to method of Biggs and Douglas (18) with minor modification whereby Veronal Buffered Isotonic Saline (VBIS) was used as diluent of $BaSO_4$ adsorbed oxalated plasma and of serum Serum derived from normal blood 2 hr after coagulation Asolectin (Associated Concentrates Long Island N Y) was used in the Tpl generating mixture as source of phospholipid Same techniques employed in Figs 2 3 4

Table I

GENERALIZED SHWARTZMAN REACTION AND COAGULATION

Animal	*AO*		*BO*		*CO*		*DO*		*EO*	
	Bef	*Aft*	*Bef*	*Aft*	*Bef*	*Aft*	*Bef*	*Aft*	*Bef*	*Aft* *
CT (min)	9	11	9	1	8	10	5	5	8	14
Cl Retract	Good	Good	Good	Poor	Good	Poor	Good	Poor	Good	Poor
Plat (10^3/mm^3)	00	185	175	115	5	110	150	1 0	150	115
Proth Act (")	1 0	1 0	110	110	100	105	105	90	110	98
Res Ser Proth (") *	14	10	0	15	10	19	0	11	0	18
Fibrinogen (mg %)	530	550	650	350	650	380	5 0	430	500	480
Tpl Generat	Norm	Poor	Norm	Norm	Norm	Poor	Norm	Poor	Norm	Poor
Clin Course **	Surv till Sacrifice		Surv till Sacrifice		Died of Hemo-pericard		Died of Hemo-pericard		Surv till Sacrifice	

* 1 hr after 2nd dose of endotoxin

* Serum separated and allowed to stand 24 hrs at 37 C before assay

** 4 other animals not included died of cardiac puncture hemopericardium at

? 3 and 48 hours after nd dose of endotoxin Three showed poor Tpl generation

No excessive fibrinolysis was observed

clotting factor(s) affected is still unknown the fact that normal serum or $BaSO_4$ adsorbed plasma rectifies the defect indicates that if only one clotting factor is involved it is most likely PTA The TGT associated with endotoxin administration in the rabbit is remarkably similar to what is obtained in human PTA deficiency an example of which is shown in Fig 5

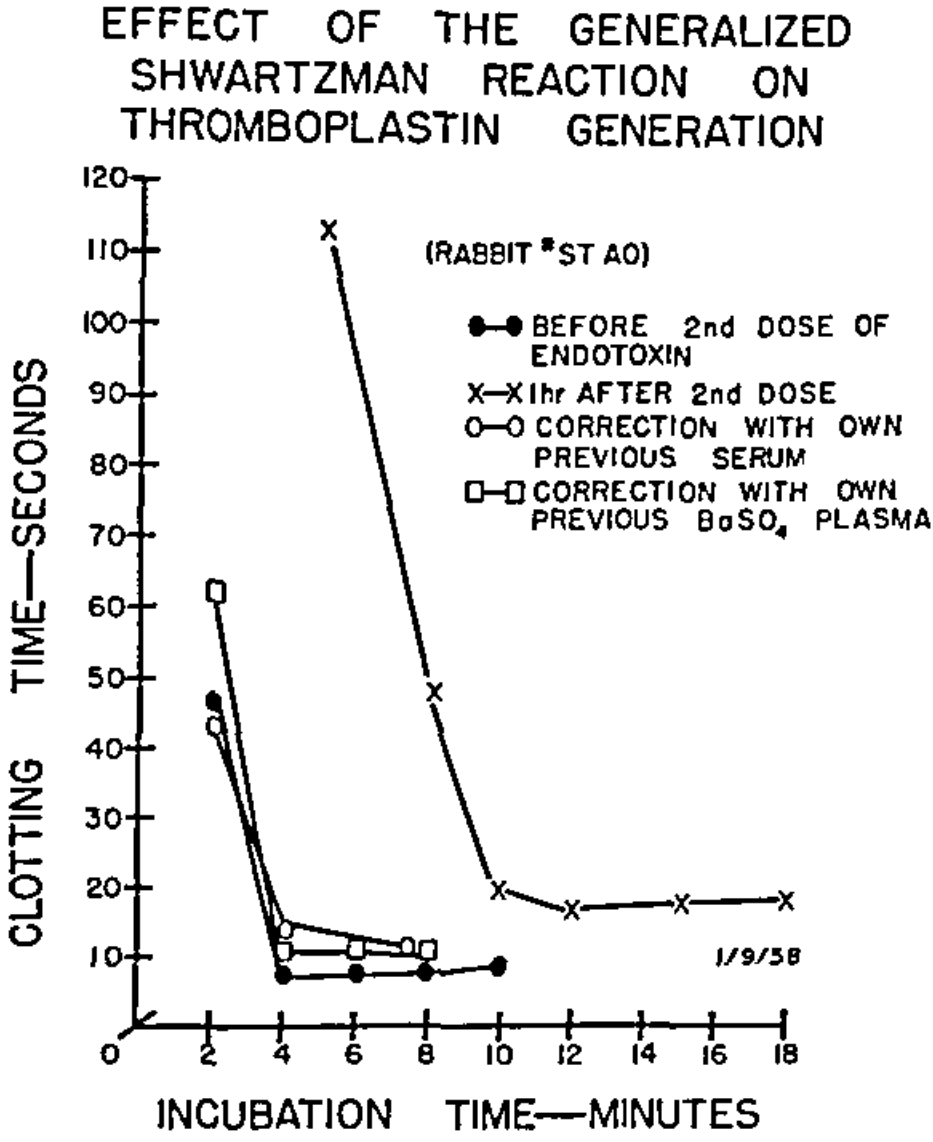


FIG 3 Corrected Effect in TGT of Normal Constituents on Shwartzman associated Tpl generation disturbance

The other known first phase factor common to both serum and adsorbed plasma Hageman factor is probably not the entity involved since the clotting time should have been distinctly elevated

Disturbances in other important coagulation parameters are noteworthy These include poor clot retraction some decrease in platelets and fibrinogen [in agreement with earlier observations (2)] and suggestive interference in prothrombin consumption (Table 1) That the overall hemostatic defect

bleeding phenomena in the gut and other viscera in traumatic and other forms of shock where endotoxin has been implicated (8) That the hemostatic disturbance in the rabbit has pertinence in man is indicated by recent reference to the close histopathologic resemblance between the Shwartzman phenomenon and what is observed in purpura fulminans (10 11)

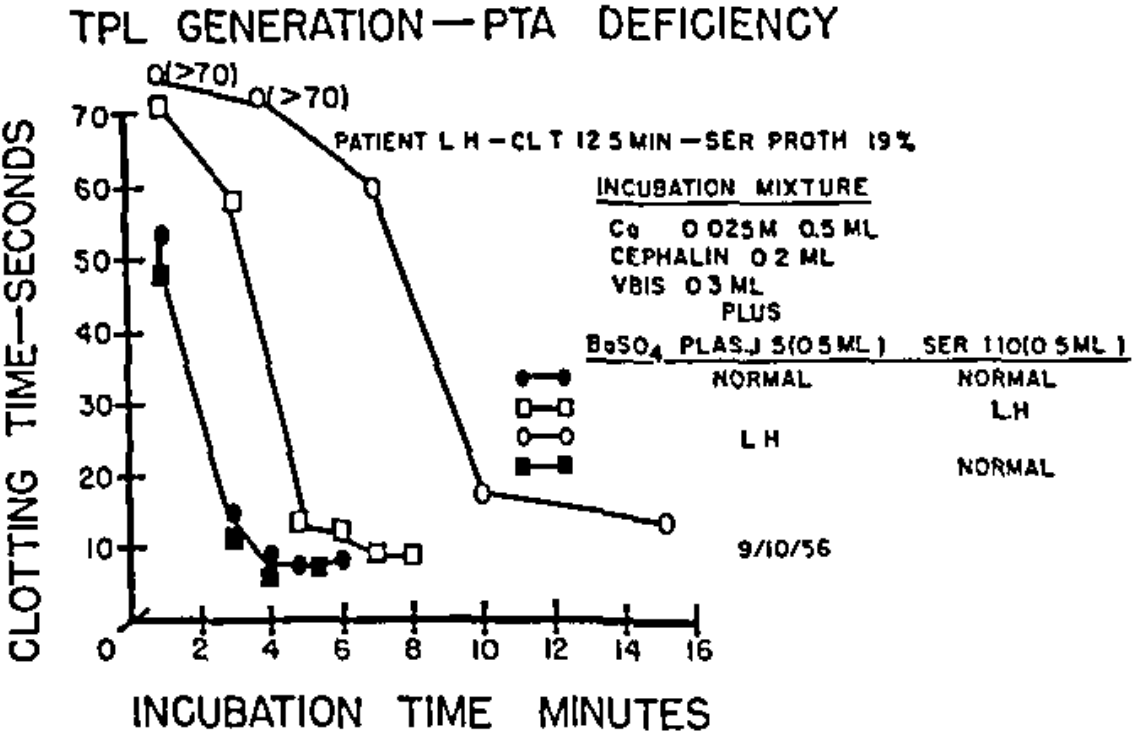


FIG 5 TGT on Patient with PTA Deficiency 67 yr Old Male Undue bleeding only following surgical procedures since age 17 brothers and sisters bleeders Except for occasional slightly elevated CT and diminished prothrombin consumption no other coagulation abnormalities were noted

The Effect of Thorium on Tpl Generation

The generalized Shwartzman phenomenon can be induced also by bacterial endotoxin followed by the infusion of colloidal thorium oxide (Thorotrast) (7 8) and hemorrhage has been observed following its use (8 9) even without prior administration of endotoxin (9) This led us to explore the coagulation mechanism following Thorotrast administration As shown in Fig 6 1 5 ml of Thorotrast * per kg of rabbit (one half that

More recent experiments to be reported in detail elsewhere (12) revealed that the same defects were obtained with thorium chloride (C P) the critical concentration being approximately $1 \times 10^{-}$ M which is about 1/50 that of the colloidal thorium oxide It may also be added that lanthanum chloride and neodymium nitrate induce the same defects in both plasma and serum This raises intriguing questions regarding the known anticoagulant action of the actinides (13 14) their similarity in this regard to thorium and their similar physico-chemical properties (15)

was serious is indicated by the fact that seven animals died of hemoperi cardium following cardiac puncture a sign of greatly impaired hemostasis

We thus have a new type of hemophiloid disorder experimentally in

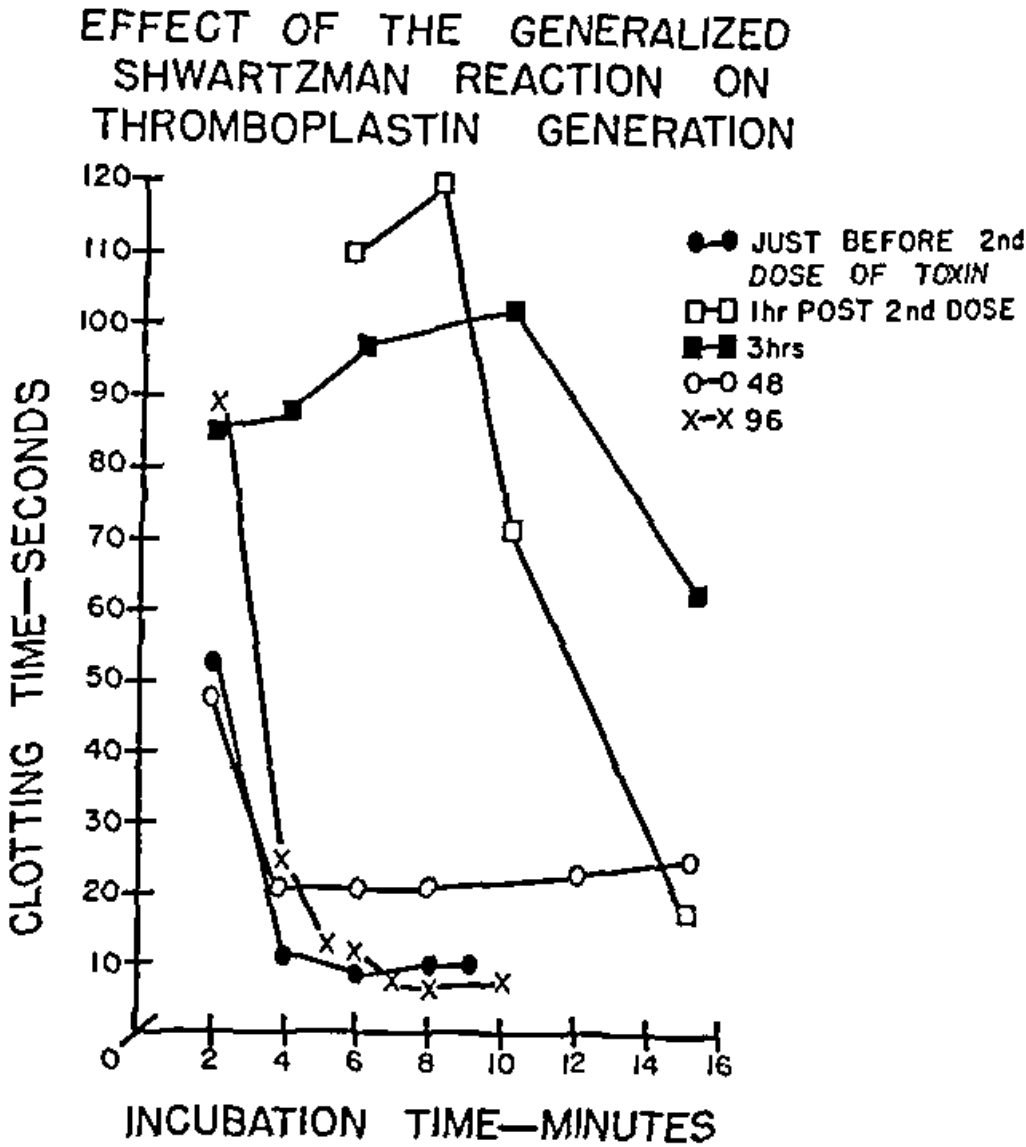


FIG 4 Relation Between Interval Following Second Dose of Endotoxin and Tpl generation defect Control curve obtained on same animal as *3 hr* curve *1 hr* curve on same animal as *48* hr curve *96* hr curve on entirely different animal Thus possible effects of excessive repeated bleedings per se were avoided Prior observations on other animals demonstrated that two comparable blood samplings via cardiac puncture do not affect the TGT

ducible by endotoxin associated with defective Tpl generation thrombocytopenia fibrinopenia poor clot retraction and perhaps disturbed prothrombin consumption manifested clinically by hemorrhagic phenomena Further study is urgently needed to explore how this relates to intravascular fibrin deposition and the Shwartzman phenomenon directly or to the

Not only did Thorotrast compromise Tpl generation but also as seen in Fig 9 when it was added to a normal mixture at the height of maximal generation there was rapid decay of the generated activity

Experiments were performed to define the factor(s) affected In *plasma* progressive destruction of Ac globulin to one third its prior level occurs within ½ 1 hr following the addition of thorium associated with slight elevation in PT while the prothrombin and proconvertin remain unchanged

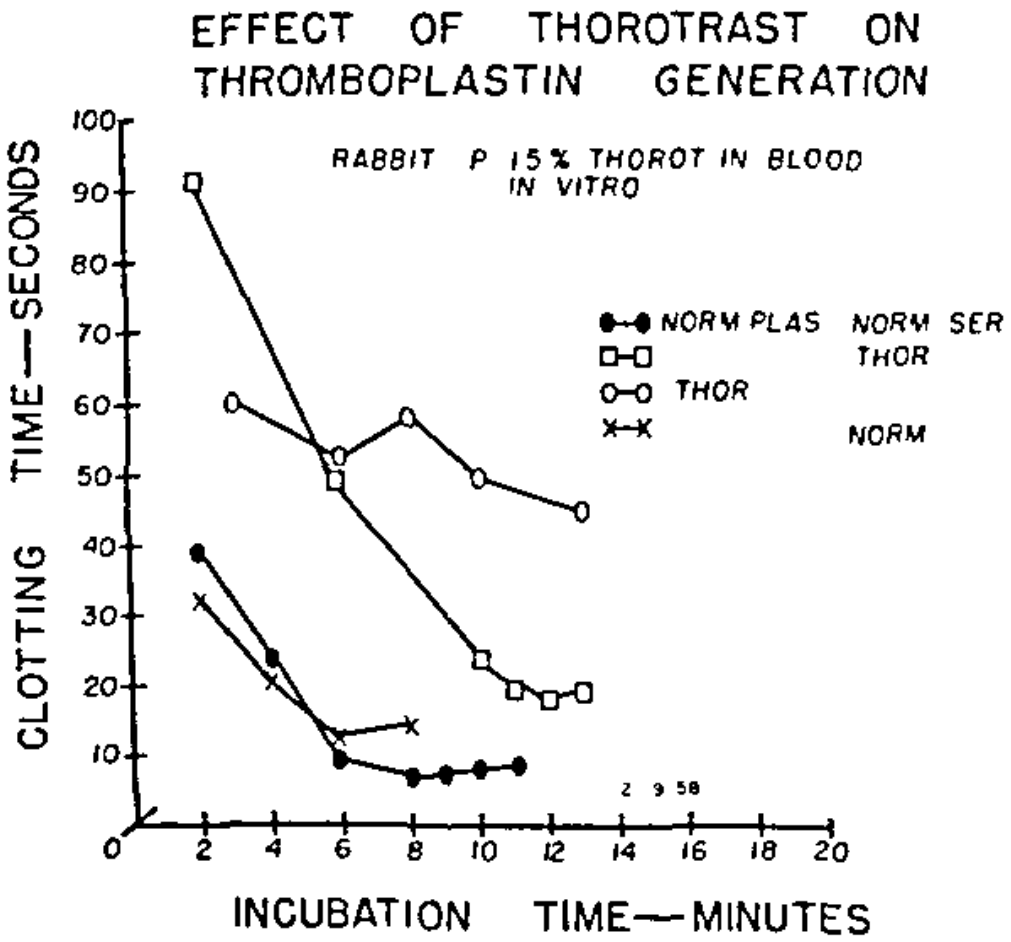


FIG 7 TGT on Blood of Same Animal as in Fig 6 obtained prior to Thorotrast infusion

AHF seemed not involved since substantial correction of the TGT fol lowed incorporation of fresh plasma from a classical hemophiliac with the *thorotrast treated plasma*

In serum similarly the untoward effect of the thorium was both immedi ate and progressive but the precise nature of the defect was more difficult to determine PTC could be readily excluded since PTC deficient serum and Thorotrast serum mutually corrected each other (Fig 10) The same was true with a combination of PTA deficient and Thorotrast -defective

generally used for the Shwartzman phenomenon) in and of itself promptly induced marked impairment in Tpl generation Not only was generation impaired but whatever Tpl activity did evolve rapidly disappeared in the TGT Associated with these abnormalities were an elevation in clotting time and a moderate drop in platelet count but no striking change in prothrombin time or proconvertin activity Much to our surprise the same

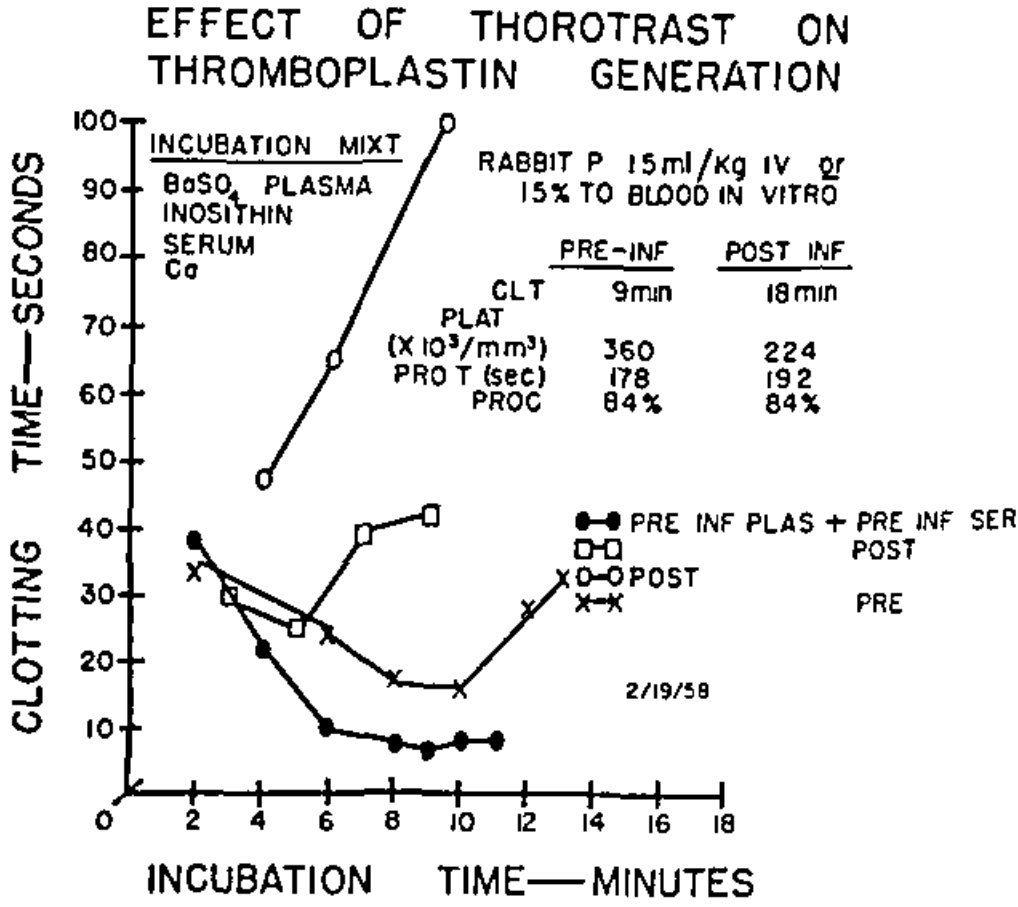


Fig 6 Tpl Generation Before and After Thorotrast Infusion to hybrid weanling albino rabbit Thorotrast added *in vitro* also (Fig 7) to a sample of preinfusion blood of same animal PT determined on whole oxalated plasma by Quick procedure (19) with human brain Tpl proconvertin by Owren method (20)

abnormality was produced by the addition of Thorotrast to blood of the same animal *in vitro* (Fig 7) both the plasma and serum were unable to support good Tpl generation As seen in Fig 8 the concentration of Thorotrast determined the magnitude of the serum defect the minimal critical level being approximately 1 7% Dextrin the other major component in the preparation was inert The same observations were obtained with human material

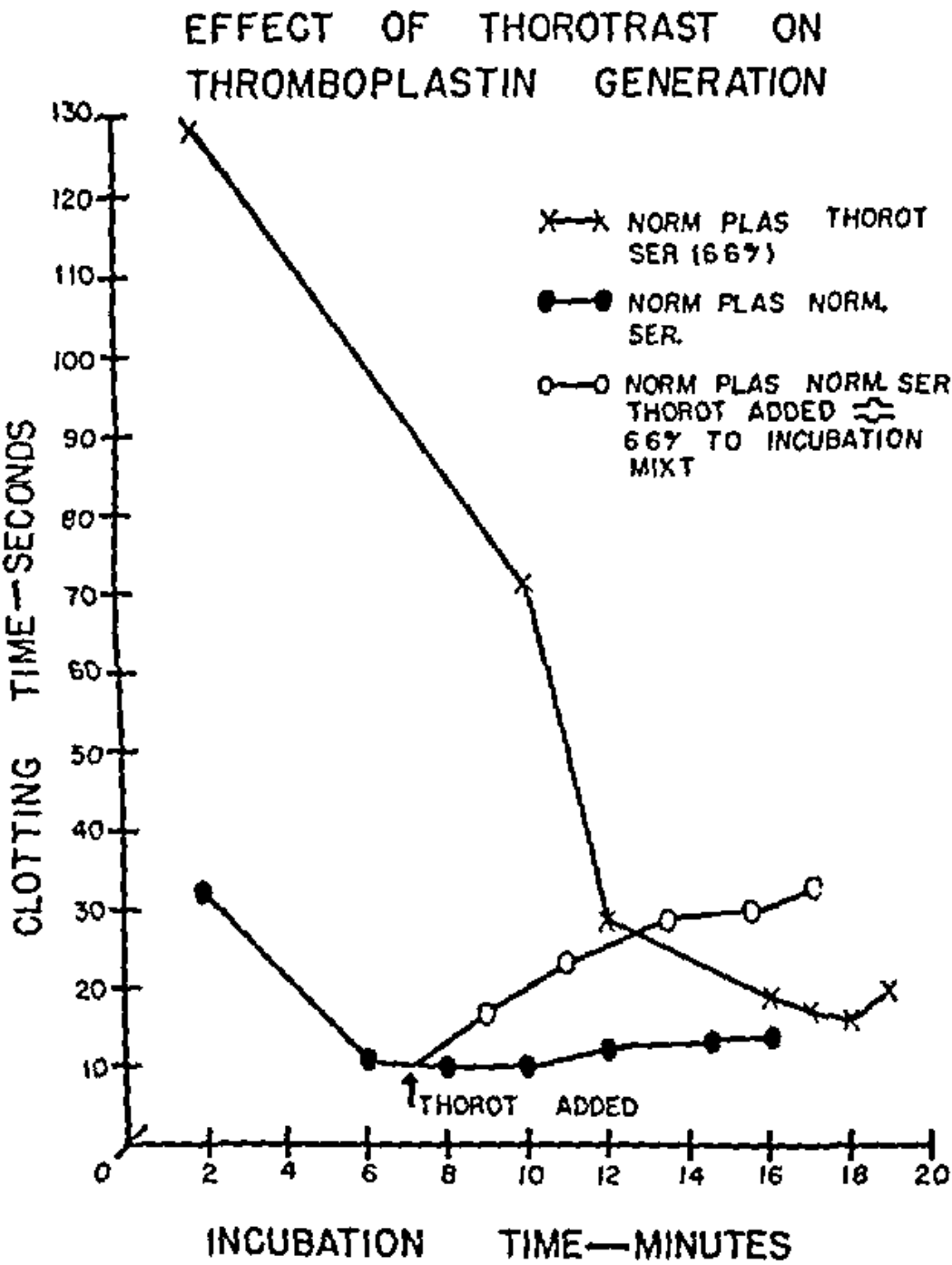


FIG 9 Destructive Effect of Thorotrast Added at maximal Tpl generation Human material

sera However the abnormality *resided in a component adsorbable by* $BaSO_4$ perhaps Stuart Prower * factor

One additional point is noteworthy it appears that Thorotrast does not act by potentiation of an inhibitor

The obscure mechanism by which Thorotrast adversely influences the first phase of coagulation is intriguing in view of the clotting abnormalities known to be associated with dysproteinemia and other conditions involving

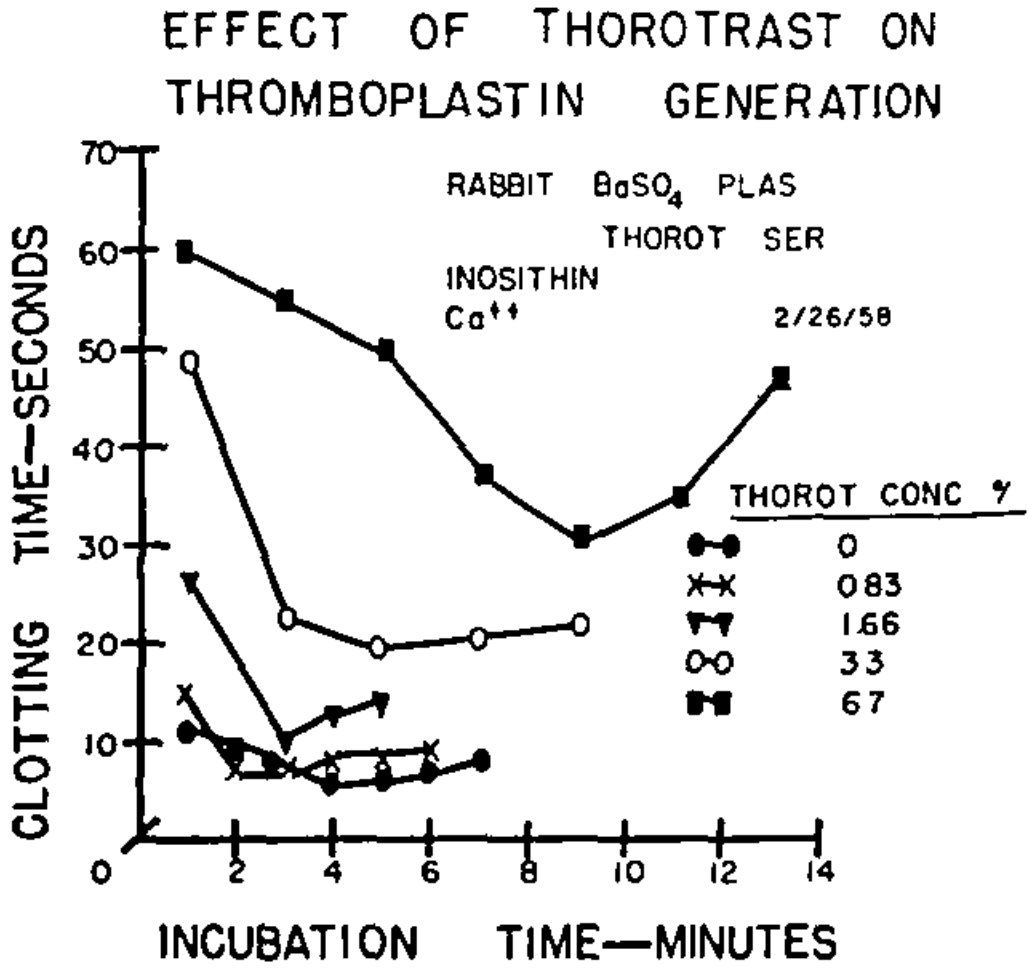


FIG 8 Quantitative Relationship Between Thorotrast concentration and Tpl generating potential of serum

electrical charge and surface phenomena It is striking that the untoward effect of the agent is both immediate and progressive suggesting that the *specific* clotting components involved are not only blocked but also ren dered labile Also significant in this regard is the destructive effect on formed Tpl

* More recent observations exclude Stuart factor Stuart deficient sera obtained from 2 patients with this abnormality (one the original Stuart) corrected 'Thoro trast defective serum Moreover normal serum aged for 2 3 weeks at 0-4 C was not corrective in contrast to *fresh serum or serum aged for 2 days This* suggests that Factor X is the serum entity involved (16)

as 0 1 mgm glass per ml of freshly shed blood yielded a serum which was abnormal in its ability to support Tpl generation No adverse effect was seen when the glass was added to serum after it was separated from the clot or to a generating mixture at the height of generation Obviously the manipulative procedure involving the glass somehow yielded a serum devoid of certain clotting factor(s) important for thromboplastic activity as it evolves in the TGT Tpl generation is compromised irrespective of whether platelets cephalin Inosithin or Asolectin are used in the generat

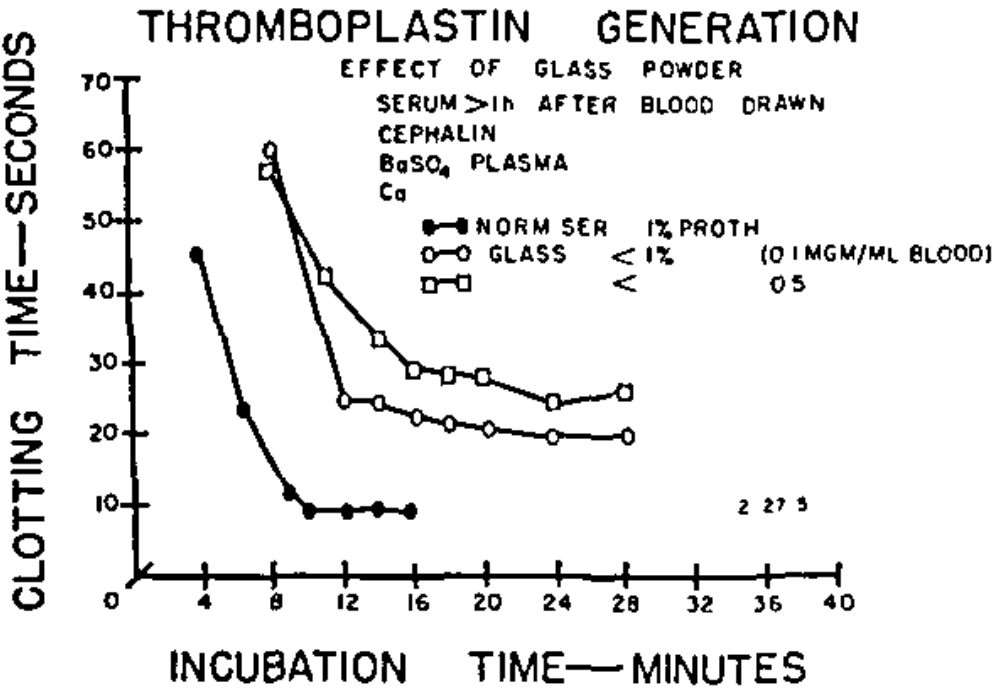


Fig 11 Effect of Powdered Glass on Tpl Generation Normal Venous blood obtained with ordinary glass syringe and needle aliquot oxalated to obtain $BaSO_4$ adsorbed normal plasma another aliquot allowed to clot in tubes containing powdered glass in amounts indicated These were stirred during clotting allowed to stand for over an hour and the sera separated Residual serum prothrombin was determined by Owren procedure

ing mixture The only other differences discernible in the glass -derived sera were significantly increased Factor VII (proconvertin-convertin) ac tivity less residual prothrombin and frequent hemolysis

In elucidating the glass incurred defect at least 3 variables had to be considered 1) mechanical manipulation associated with the glass addi tion such as stirring 2) the resultant hemolysis frequently observed and 3) the possibility that the difference between normal and glass serum was directly due to elimination of residual serum prothrombin Many observations an example of which is seen in Fig 11 seemed to exclude all three of these While it is true that blood clotted in the presence of powdered glass generally yields a serum containing less prothrombin one

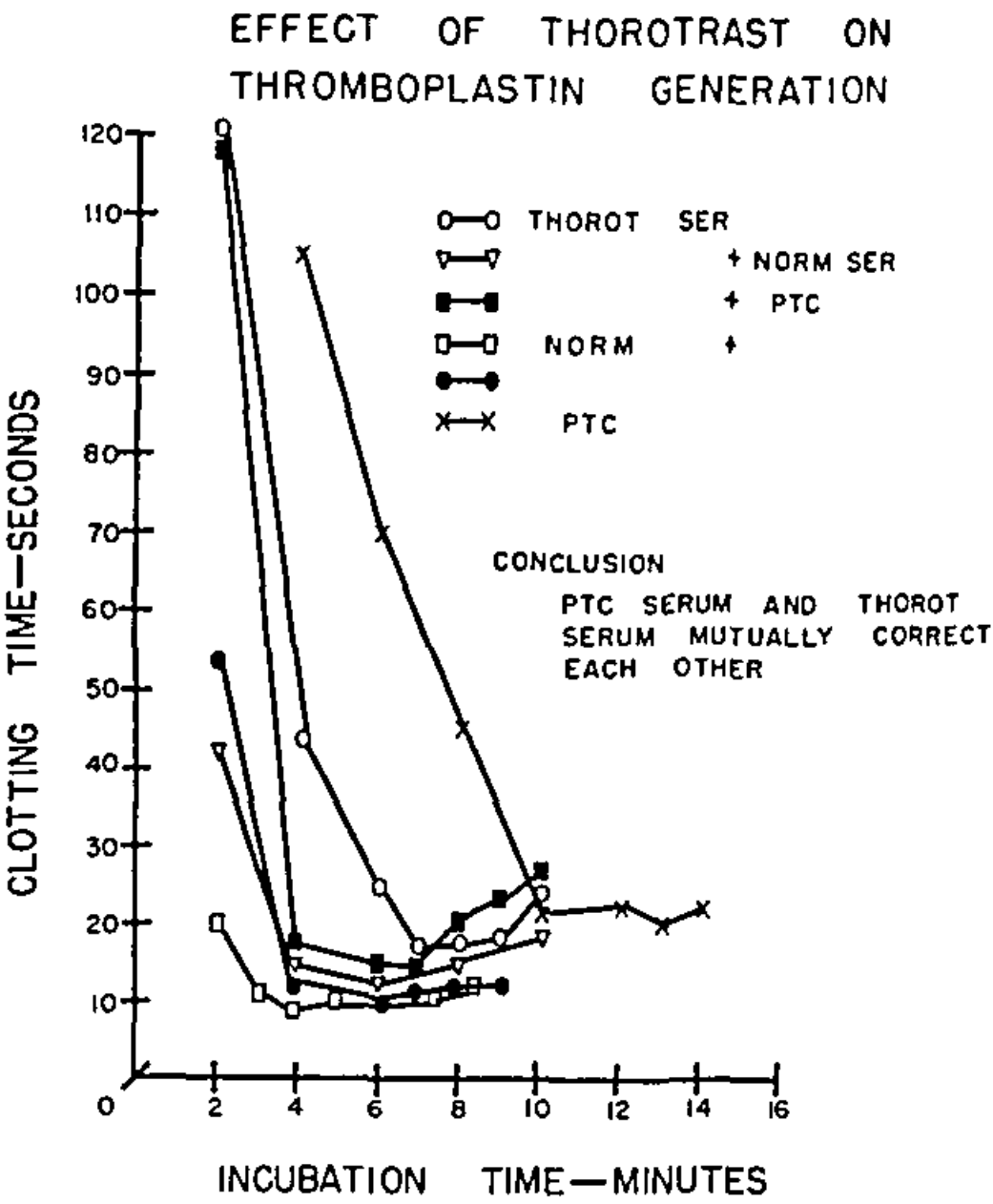


FIG 10 Corrective Effect of PTC Sera on Thorotrast Induced Serum defect Incubation mixture contained Inosithin normal human $BaSO_4$ adsorbed oxalated plasma and various sera (normal Thorotrast treated PTC deficient and combinations thereof)

Effect of Glass and Other Manipulative Procedures

In our experience the TGT has occasionally proven erratic Attention has been drawn by many investigators to possible artefactual errors introduced by small amounts of residual serum prothrombin To reduce this to a minimum by pushing coagulation to a maximum blood was clotted in the presence of either powdered glass (Pyrex MacAlister Bicknell Co Cambridge) or tissue thromboplastin In Fig 11 it will be noted that as little

ing effect of glass has long been known most recently attributed by Ratnoff and Rosenblum (17) to release of Hageman factor from a binding inhibitor we hardly expected to find a *defect* Fig 14 shows that the abnormality in glass serum is not referable to PTC since PTC deficient serum corrects

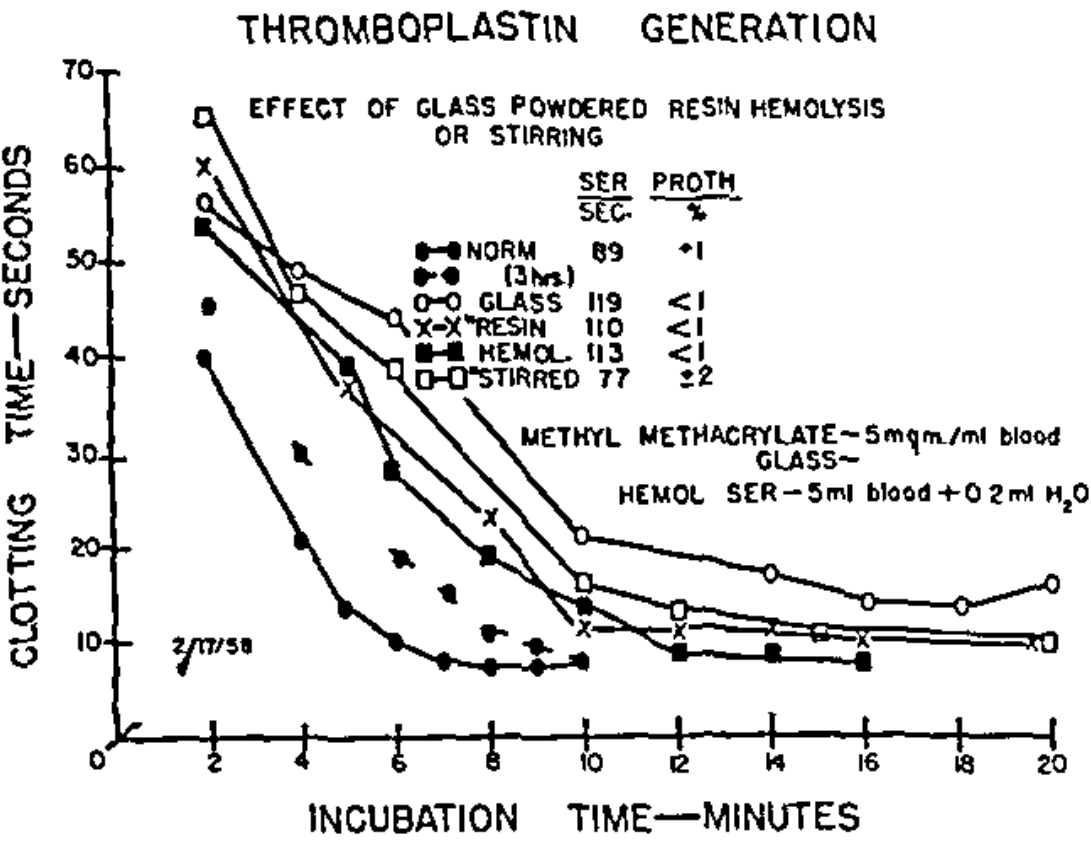


FIG 13 Effect of Various Manipulative Procedures on Serum Tpl Generating potential Freshly shed normal blood was divided into several aliquots which were then subjected to powdered glass to powdered resin (methyl methacrylate) to H O to induce substantial hemolysis and to stirring with glass rod during clotting process Respective sera tested in TGT and assayed for prothrombin by Owren procedure It should be noted that glass serum and stirred serum showed slight hemolysis the serum from the H O blood combination substantial hemolysis and the resin serum no hemolysis The broken line represents the normal control repeated 3 hrs after all the other tests had been performed

glass serum Moreover the glass serum does not seem to be truly corrected by normal serum (Figs 14 15) in mixing the two the glass serum appears to act as a simple diluent of the normal serum which is finally present in only one half the original amount Moreover a combination of glass serum with $BaSO_4$ adsorbed normal serum is even less active than the glass serum alone (Fig 15) Similarly admixture of 'Thoro-

may obtain comparable sera in this regard with or without glass (Fig 12) From many such observations it is concluded that more complete removal of prothrombin is not the answer This and the effect of other variables is shown perhaps more strikingly in Fig 13 It is noteworthy that of all the manipulations *glass powder non glass resin powder stirring hemolysis*

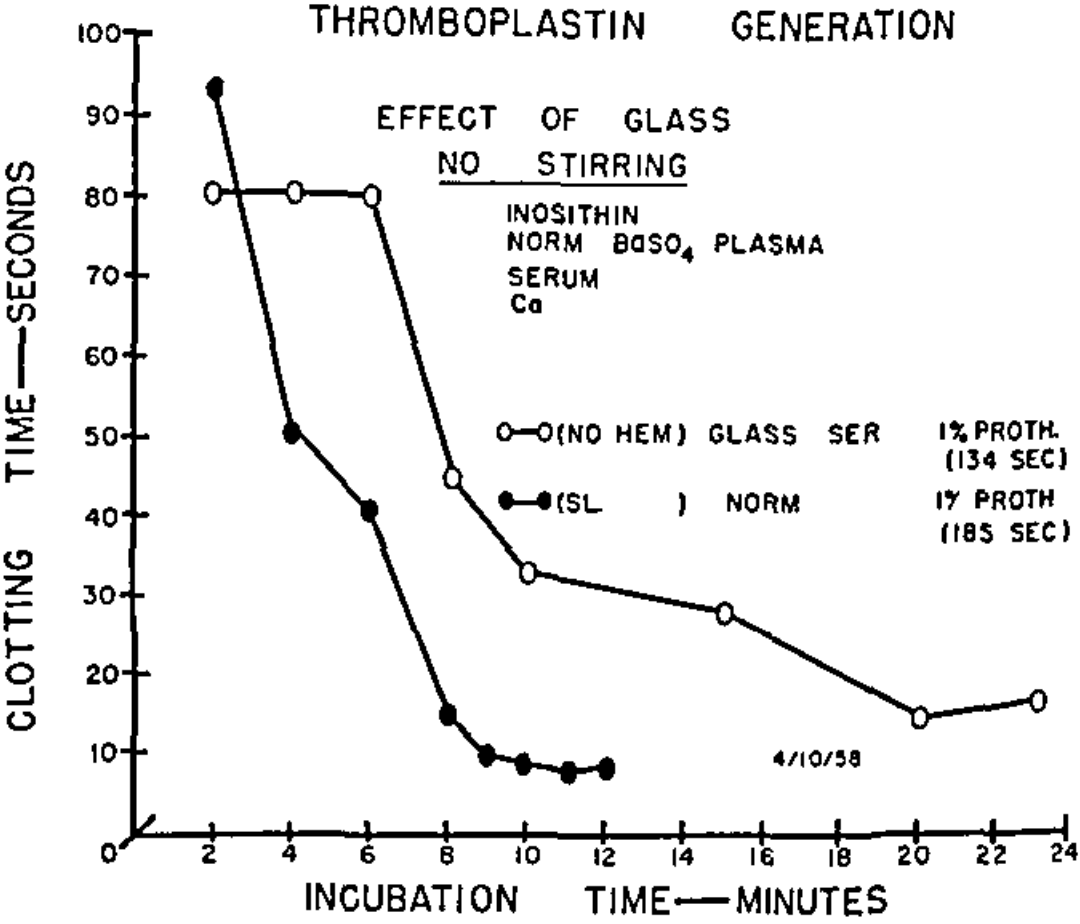


FIG 12 Effect of Powdered Glass on Tpl Generation In This Experiment the mixture of blood and powdered glass (5 mgm/ml) and the untreated blood was not stirred but the tubes were inverted until clotted The prothrombin times (Owren) and residual serum prothrombin activities are given It is to be noted the glass derived serum showed *no hemolysis* and *more prothrombin* compared with its non glass counterpart yet its Tpl generating potential was poorer

the worst serum was obtained with glass although the other manipulations had similar untoward effects again unrelated to residual traces of prothrombin Application of any of these manipulations to the serum *after* it was separated from the clotted blood had no effect Moreover collecting the blood in anticoagulant in the presence of powdered glass had no untoward effect on the TGT supporting potential of the plasma

What then is the nature of the glass defect? Whereas the clot promot

trast serum with glass serum also makes it worse (Fig 16) Apparently the glass induced defect involves the same serum component required for Tpl generation which is blocked or otherwise inactivated by thorium and which is adsorbable by $BaSO_4$

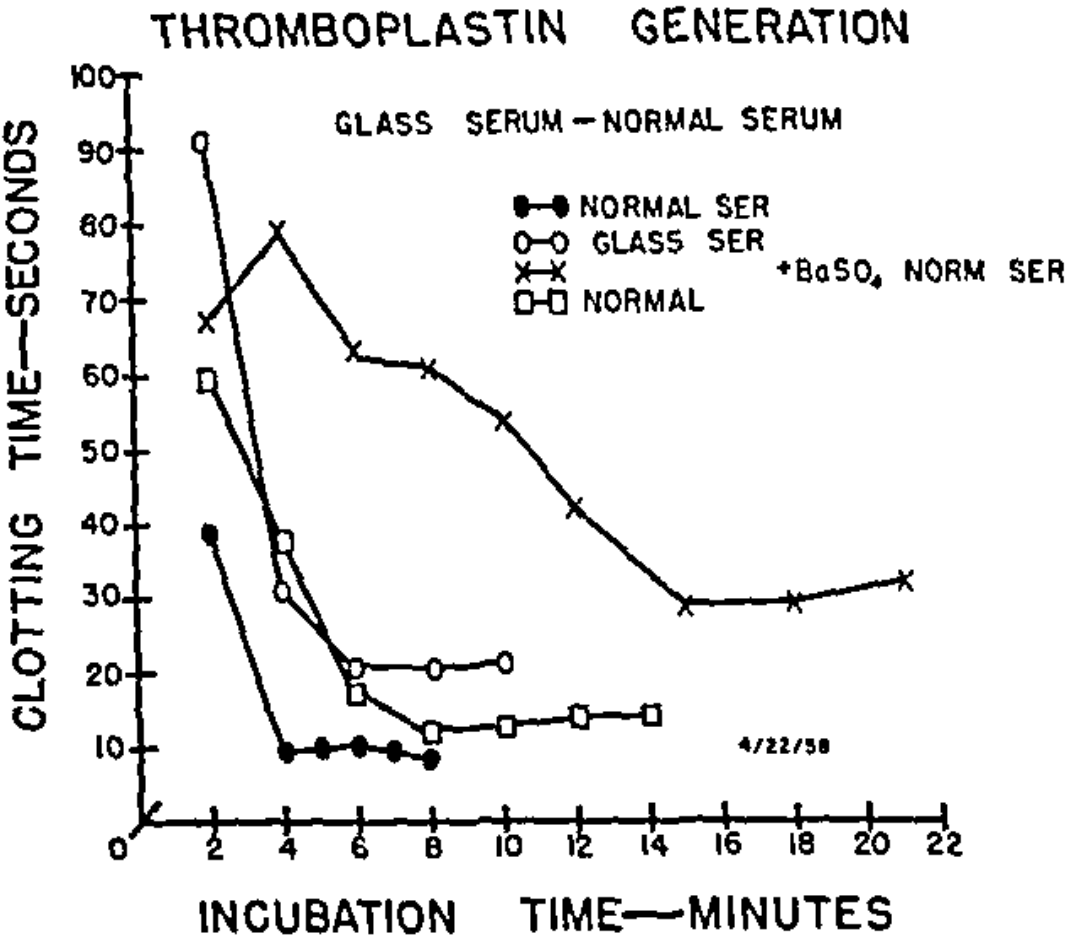


FIG 15 TGT with Various Combinations of Normal and Glass Sera The normal serum was slightly hemolyzed contained ± 1% prothrombin (PT 118 sec) Glass serum obtained by inversion mixing of blood with glass without stirring showed no hemolysis contained less than 1% prothrombin (PT 185 sec) The mixture of Ca++ Inositin $BaSO_4$ plasma and $BaSO_4$ serum alone gave a lowest clotting time of 116 sec

It is unlikely that the glass acts simply by adsorption since the addition of glass to citrated blood or to serum after clotting has run its course has no effect It must be present in actively clotting freshly shed blood Experiments are underway to correlate the defect with the nature and size of the glass particles used In any event we have here too an experimentally inducible abnormality in a factor required for the first phase of coagulation It is postulated that the effect of glass is to induce more complete consumption of an essential clotting component not prothrombin which is

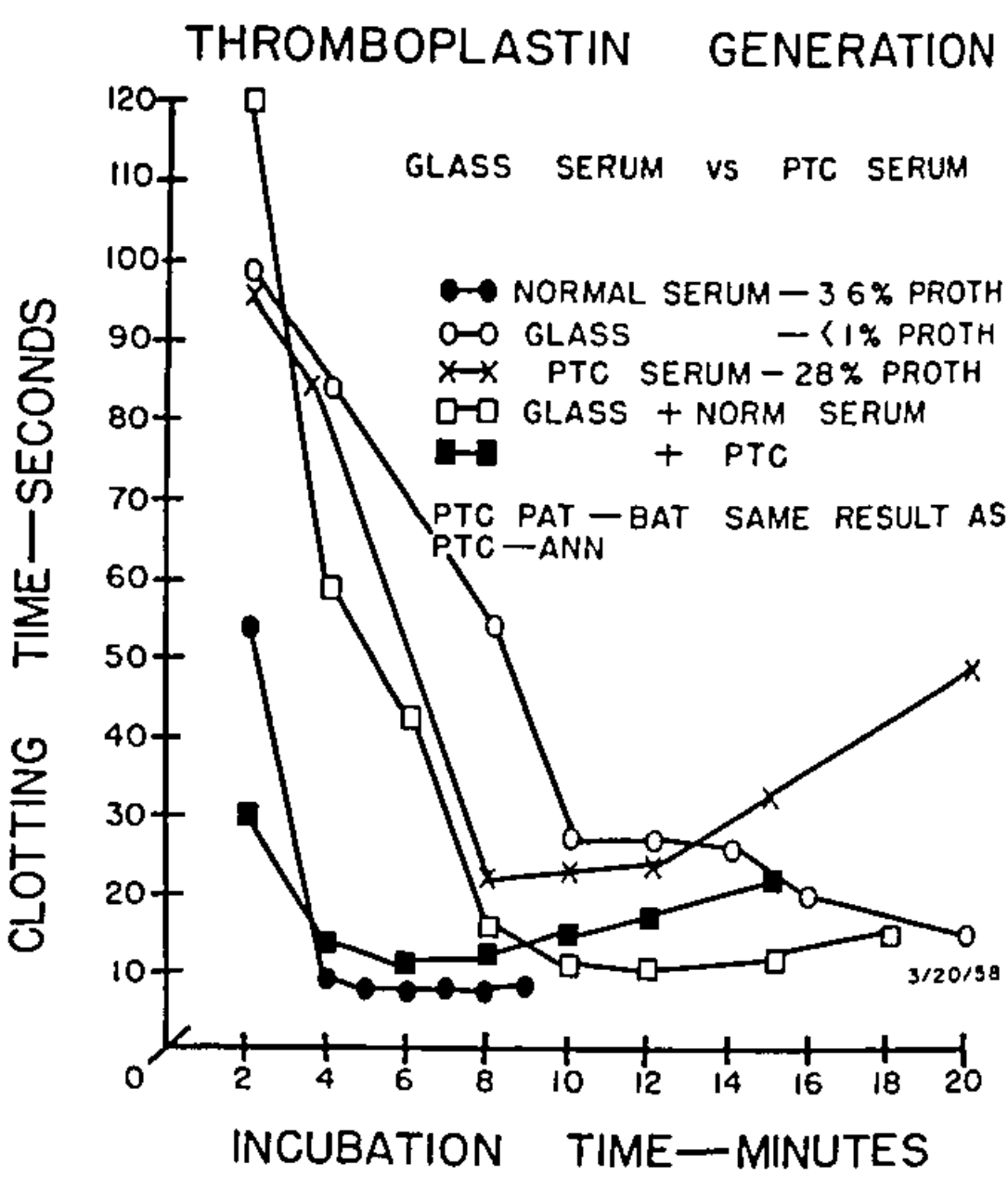


FIG 14 Corrective Effect of PTC Deficient Serum on Tpl Generation obtained with glass defective serum Incubation mixtures contained Inosithin (Assoc Concentrates L I N Y) normal $BaSO_4$ adsorbed plasma Ca^{++} and sera indicated Glass serum was derived from normal blood clotted with 5 mgm glass per ml It showed some hemol ysis Serum combinations were made by mixing in 1 1 vol propor tion and then diluting 1 vol of the mixture to 10 with the VBIS in the usual manner Not shown are observations obtained on serum derived from an aliquot of the same blood subjected only to stirring It con tained 1 2% residual prothrombin The curve was practically super imposable on that obtained with the glass serum normal serum com bination It should be noted that in this combination the concentration of normal serum is only one half that in the test with normal serum alone The greater Tpl generating potential of the combination of glass and PTC deficient sera is probably referable to the large re sidual prothrombin in the latter

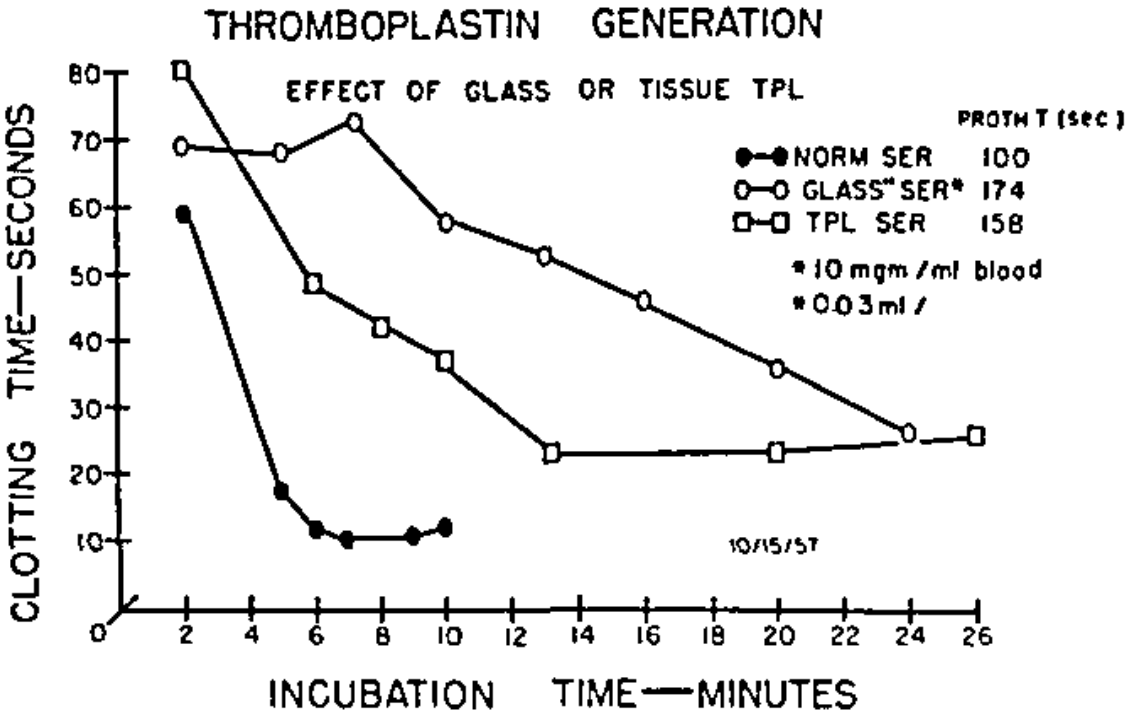


FIG 17 Comparable Untoward Effects on Tpl Generating Potential of sera derived from blood clotted in the presence of tissue Tpl extract (human brain) or powdered glass Cephalin (Associated Concentrates L I) used in TGT incubation mixture Addition of glass or tissue Tpl to normal serum *after* separation from the clotted blood does not alter its Tpl generating potential

Summary

A new group of hemophilioid defects is described comprising 1) the endotoxin induced generalized Shwartzman reaction which is associated with defective Tpl generation and a serious hemostatic defect Although the specific coagulation factor(s) involved awaits elucidation the evidence suggests that it is PTA Other parameters adversely affected are platelet number clot retraction and fibrinogen This constellation constitutes a new type of hemophilioid disorder which perhaps reflects triggering of intravascular coagulation by the double dose administered toxin

2) Thorium *in vivo* and *in vitro* impairs Tpl generation in plasma by rapid and progressive Ac globulin destruction and in serum by progressive blocking or destruction of a component distinct from most known serum factors The evidence suggests that it is Factor X Besides explaining the bleeding of Thorotrast treated animals the observations raise provocative questions regarding the highly specific effect of this and certain physicochemically related elements acting as colloid or in ionic form

3) Various manipulative procedures notably exposure of coagulating blood to powdered glass mechanical agitation and tissue Tpl yield sera

expended to a variable degree during coagulation under the usual circum stances

The manipulative procedures namely glass and stirring which produce so to speak poor sera are known to accelerate clotting presumably push ing the reaction as rapidly and as far as possible Since tissue Tpl will do the same it was therefore of interest to study the effect of tissue Tpl added to freshly shed clotting blood Fig 17 typical of many experiments shows

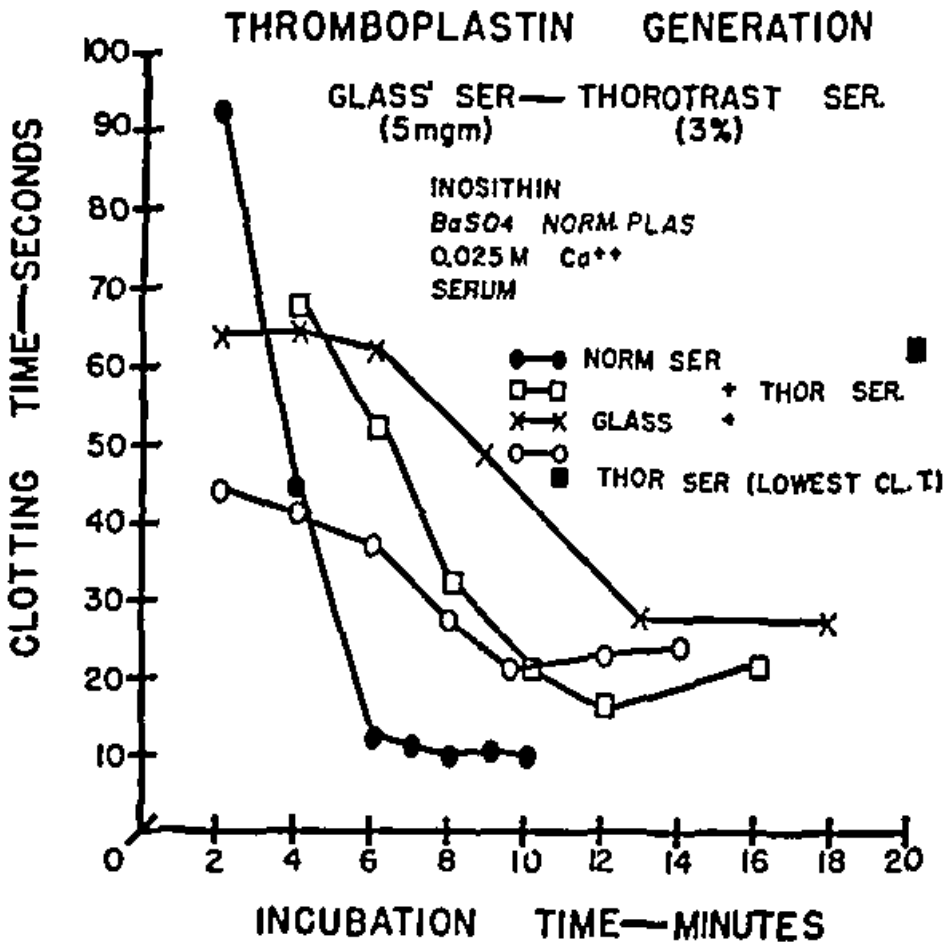


FIG 16 TGT with Human Sera Treated with Thorotrast or derived from blood clotted in the presence of powdered glass

that the serum thus obtained is similarly poor in the TGT The implications of this pointing up the importance of meticulous venipuncture which avoids contamination of blood with tissue juice is obvious

More important perhaps is the question whether the normal Tpl genera tion curve obtained with serum derived in the orthodox manner is really normal or artefactually too steep reflecting incomplete and variable inter mediary degrees of coagulation It is possible that the true normal curve is that obtained when the serum used in the test is derived from blood pushed to complete coagulation

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defective in their ability to support so called normal Tpl generation An essential component seems lacking apparently identical with that affected by thorium The effects seem related to the extent to which coagulation is rendered complete Aside from their general important biopathologic significance the observations focus attention on several technical aspects of the TGT and help explain some of its variations even in experienced hands

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Materials and Methods

Blood was obtained from the anticubital vein by venipuncture The blood was mixed either with 1 34% sodium oxalate in a ratio of 9 parts blood to 1 part oxalate solution or with 3 2% sodium citrate in a ratio of 8 1 Blood was centrifuged for 15 min at 3200 x *g* and *plasma* withdrawn by pipette Plasmas of the patient were tested on the day they were obtained Plasma from Mr Stuart was prepared as above and used either fresh after storage at −20 C or after reconstitution from the lyophilized state immediately before testing

Serum was prepared by placing 0 1 ml of human brain thromboplastin (2 5% suspension) in a conical centrifuge tube and adding 9 9 ml of blood This was mixed by inversion and allowed to clot The clotted blood was incubated 2 hours at 37°C and the serum expressed by centrifugation at 3200 x *g* The serum was considered satisfactory for use in the thromboplastin generation test (TGT) if it failed to clot in 10 minutes when CaCl brain thromboplastin and fibrinogen were added

Clotting time was determined by the method of Lee and White as described by Jaques (5) *Prothrombin time* was determined by the method of Quick (6) using human brain thromboplastin *Partial thromboplastin time* was determined by the method of Langdell and associates (7) as described by Rodman and others (8) *Stypven time* and *Stypven plus cephalin time* were determined as described by Hougie and others (1) The *thromboplastin generation tests* were performed by the method of Biggs and Douglas (9) as modified by Graham and Barrow (10) *Prothrombin concentration* was determined by the Iowa 2 stage method as described by Wagner and others (11)

$BaSO_4$ supernatant was prepared by adsorbing oxalated plasma with 100 mg $BaSO_4$ per ml at 5°C The $BaSO_4$ was sedimented at 3200 x *g* and the supernatant plasma decanted and stored at −20°C The $BaSO_4$ precipitate was resuspended in a volume of 3 2% sodium citrate equivalent to the original volume of plasma inverted several times and resedimented The second supernatant solution was dialyzed 18 20 hours against 032% sodium citrate in isotonic saline designated *$BaSO_4$ eluate* and stored at −20°C

Observations

Clinical History A 53 year-old white male farmer was admitted to the North Carolina Memorial Hospital in Chapel Hill on 18 December 1956 because of an unexplained generalized bleeding diathesis Prior to the present illness he had shown no evidence of a hemorrhagic tendency and there had been no bleeders in his family His leg had been cut rather

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Stuart Clotting Defect III
An Acquired Case with Complete Recovery

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Department of Pathology University of North Carolina Chapel Hill

STUART factor deficiency has been distinguished only recently from other deficiencies of the prothrombin complex (1 2 3) Initially it had been thought that SPCA proconvertin stable factor and Factor VII were synonyms for a single clotting factor It now appears that there are *at least* 2 factors (SPCA and Stuart factor) which have the following characteristics in common they are present in both plasma and serum are adsorbed by certain of the insoluble alkaline earth salts are reduced by administration of coumarin compounds and have a marked effect on the prothrombin time test (PT) of Quick

Since the identification of Stuart factor a number of patients with proconvertin deficiency in many parts of the world have been restudied Our information suggests that approximately half of the restudied patients have proved to have the Stuart defect and half SPCA deficiency (4) All of the patients with the Stuart defect who have been described in the literature appear to have been affected at birth and none have been improved appreciably by various drugs

This communication reports an example of apparently acquired Stuart factor deficiency accompanied by symptoms of hemorrhage which responded poorly to intensive Vitamin K and transfusion therapy but remitted completely over a period of about 1½ years

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1 Professor of Pathology
2 Research Assistant
3 Student Fellow in Pathology

Diagnostic Blood Clotting Studies The patient s plasma and serum were investigated in an effort to determine the cause of the prolonged PT Providentially Mr Stuart the prototype patient with the Stuart defect appeared in the laboratory for his semi annual blood donation the day after the patient was admitted and it was possible to compare fresh samples from the two men simultaneously

The data shown in Table 2 strongly suggest that the patient and Mr Stuart had identical defects for the following reasons (a) both men had long prothrombin and partial thromboplastin times corrected by normal control plasma (*indicating absence of inhibitors*) but not mutually corrective (lines 1 6) (b) plasma samples from the patient and Mr Stuart

Table 2

DIAGNOSTIC BLOOD CLOTTING STUDIES ON ADMISSION

	Type of Plasma					*Clotting Test in sec*			
Line No	*Control*	*Patient*	*Stuart*	*$BaSO_4$ Supernatant*	*$BaSO_4$ Eluate*	*Prothrombin Time*	*Partial Thplin Time*	*Stypven Time*	*Stypven and Cephalin Time*
1	1					14	70	19	8
2		1				65	09	67	21
3			1			86	343	54	19
4	05	05				71	88		
5	05		05			18	8		
6		05	05			59	231		
7	05			05		15	86		
8		05		05		54	214		
9			05	05		80	83		
10	05				05	—	1 7		
11		05			05	—	179		
1			05		05	—	11		

Fresh

were affected little if at all by the $BaSO_4$ supernatant (lines 7 9) indicating that the prothrombin time prolongations were not the result of significant deficits of Factor V (c) samples from both men neither of whom had a deficit of *prothrombin* (2 stage) were corrected by the citrate *eluate* from $BaSO_4$ adsorption of oxalated normal plasma (lines 10 12) (d) the clotting times of both the patient and Stuart were not corrected by Stypven (Russell s viper venom) or Stypven plus cephalin indicating that the abnormality did not result from deficiency of SPCA (right half of lines 1 3) (e) in all tests clotting times of plasma samples from both men were approximately the same

Table 3 contains data obtained on the two men with the TGT It will be noted by comparison of lines 1 2 and 4 that the patient had a serum defect but not a plasma defect The same pattern was obtained on Mr

severely at 32 years of age without excessive bleeding and all of his teeth had been extracted without difficulty at age 47

Four days before admission he had impregnated the earth in his tobacco seed bed with a volatile commercial fungicide commonly used in tobacco cultivation in Eastern North Carolina Four to five hours later he noted soreness of the metacarpo phalangeal joint of the left index finger Within several hours the entire left hand had become swollen This was followed in a few hours by pain in the right knee The next morning the right knee was swollen reddish purple and hot He also noted black stools and deep red urine at about this time He visited his local doctor who found evidence of generalized hemorrhage an hematocrit of 38% and a PT of 45 sec

On admission in Chapel Hill 4 days after first symptoms were noted he was found to have many large subcutaneous ecchymoses swelling of the left hand and hemarthrosis of the right knee His feces reacted strongly to benzidine and his urine contained many red cells There was no jaundice or enlargement of liver spleen or lymph nodes

Routine laboratory findings on admission are contained in Table 1 It will be observed that the only abnormal laboratory findings were the slightly prolonged clotting time (line 7) and the markedly prolonged PT (line 11) It should be noted that prothrombin concentration by the 2 stage method was normal 91% of control (line 12)

Table 1

LABORATORY RESULTS ON ADMISSION

Line No	*Test*	*Result*
1	Hemoglobin	12 grams
2	Hematocrit	34%
3	*White blood count*	*10 500/mm³*
4	Differential blood count (pmn/others)	79/21
5	Platelets (on smear)	Numerous clumped
6	Bleeding time (Duke)	2½ mins
7	Clotting time (Lee and White)	14 mins
8	Clot lysis	None in 16 hours
9	Tourniquet test (Rumpel Leede)	Negative
10	Clot retraction	Good in 1 hour
11	Prothrombin time (Quick)	71 secs (control = 14 2)
12	Prothrombin concentration (2 stage)	210 units—91%

Tests of liver function with the exception of BSP clearance were normal (a) the cephalin flocculation was 1 + at 24 and 48 hours (b) the alkaline phosphatase was 2 9 3 5 Bodansky units and the acid phosphatase 0 1 Bodansky units (c) free bilirubin ranged from 0 2 0 3 mg % and total bilirubin from 0 9 1 3 mg % (d) total protein was 6 6 gm % and the albumin/globulin ratio was 4 2/2 4=1 75 (e) blood urea nitrogen was 25 mg % and (f) bromsulfophthalein retention was 15 0% of the administered dose after a 45 min period on one occasion 23% on another

variation in the control PT during this period At the top are recorded the amounts and types of Vitamin K administered and at the bottom the amounts and types of transfusions

It would appear that there was little or no response to the Vitamin K either natural or synthetic The drop in PT on the second day which appears large on a PT scale is really slight if put in percentage terms A change of this extent might have resulted from the transfused blood and plasma which was roughly equivalent to ⅓ his plasma volume As the chart indicates the PT slowly decreased toward normal still being 29 6 sec when he was discharged 11 days after admission

Table 4 shows that the patient s prothrombin time was still slightly prolonged (17 sec) when he visited the clinic 44 days after admission The patient s plasma now appeared however to correct the clotting defect of the sample of Stuart s plasma obtained 44 days earlier and stored at −20 C

Table 4

CLOTTING STUDIES 44 DAYS AFTER ADMISSION

Line No	*Type of Plasma*			*Clotting Test sec*			
	Control	*Patient*	*Stuart*	*Prothrombin Time*	*Partial Thplin Time*	*Stypven Time*	*Stypven and Cephalin*
1	0 1			14 6	79	13 8	5 9
2		0 1		17 0	68	12 7	6 3
3			0 1	68	2..6	33	15 5
4	0 05	0 05		—	70	—	—
5	0 05		0 05	—	77	—	—
6		0 05	0 05	15 8	72	—	—

Fresh
Frozen and stored 43 days at − 0 C

Table 5

CLOTTING STUDIES 591 DAYS AFTER ADMISSION

Line No	*Type of Plasma*			*Clotting Test sec*			
	Control	*Patient*	*Stuart*	*Prothrombin Time*	*Partial Thplin Time*	*Stypven Time*	*Stypven and Cephalin*
1	0 1			1..?	79	15 1	7 0
2		0 1		12 0	58	15 2	5 6
3			0 1	99	>300	>100	41 3
4	0 05	0 05		12 2	57	—	—
5	0 05		0 05	14 7	91	—	—
6		0 05	0 05	15 1	79	—	—

Frozen and stored 24 hours at −.0 C
Lyophilized and reconstituted.

Stuart s plasma as shown in lines 1 3 and 5 This is the pattern expected with the Stuart defect (2)

All of the data obtained on the patient were thus consistent with the hypothesis that the PT prolongation resulted from specific deficiency of the Stuart factor without significant reduction in prothrombin SPCA or Factor V It is assumed of course that the hemorrhagic diathesis was also related to the deficiency of Stuart factor

Table 3

THROMBOPLASTIN GENERATION TESTS ON ADMISSION

Line No	Test Mixtures (all with cephalin) Plasma	Serum	Clotting time sec Incubation time min 1	2	3	4	5	6	7	8	9
1	Control	Control	27 4	14 4	(13 0)	13 2	14 4				
2	Control	Patient	>45	>45	>45	>45	>45	>45	62		
3	Control	Stuart	>45	>45	—	>45	>45	53	36	(32)	39
4	Patient	Control	32 4	19 0	14 8	(13 8)	15 0	15 6			
5	Stuart	Control	37 4	26 0	18 2	16 0	(15 4)	19 0			

Clinical Course of the Malady The patient s course for 591 days after admission is shown in Fig 1 The upper solid curve traces the secular change in PT and the lower broken curve indicates the narrow range of

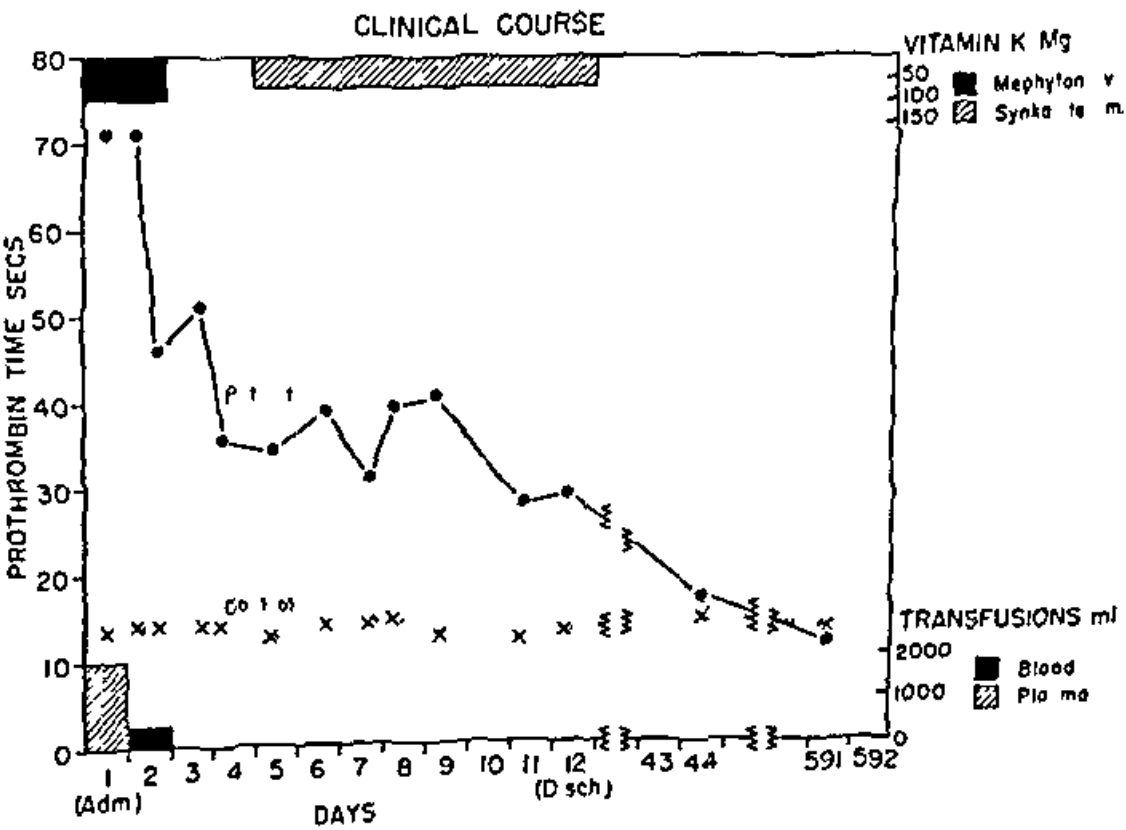


Fig 1

hydrocarbon methyl bromide mixed with a small quantity of a lacrimator chlorpicrin as a warning agent Methyl bromide is known to be a very active alkylating agent which might inactivate enzymic active sites in the liver It is widely used by tobacco farmers like the patient who happens to live in the region producing most of the cigarette tobacco for the United States It is of interest that no similar cases have been reported despite the wide use of this agent

We considered the possibility that the patient was heterozygous at the Stuart locus and perhaps more vulnerable to toxic agents than a normal homozygote would be The data of Table 5 seem to exclude this as a possibility because his clotting tests 591 days after admission were those of a normal person not a heterozygous one (2)

We made several unsuccessful attempts to produce the Stuart clotting defect in rats and dogs with the commercial fungicide Our failure was due in part at least to difficulties in handling methyl bromide It is a gas at 4 6 C odorless and very toxic The only information obtainable from the manufacturer s toxicity laboratory was with respect to findings in rats The substance has been shown to be absorbed through the skin (which it blisters severely) and toxic in low concentration The industrial pharmacologists had not been impressed by hemorrhage in the dead rats which had been autopsied (13)

Summary

1 A patient the first with a temporary (presumably acquired) and specific deficiency of the Stuart factor and a hemorrhagic diathesis is described
2 Recovery occurred slowly and was not greatly influenced by Vitamin K or transfusions
3 The only toxic agents suggested by history are methyl bromide and chlorpicrin
4 Attempts to reproduce the syndrome with methyl bromide and chlorpicrin in experimental animals have not been successful to date

Addendum

The BSP test was repeated on March 16 1959 763 days after the original symptoms began There was 26 residual BSP 45 minutes after intravenous administration

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Finally the patient was visited at his home by one of us (T R W) on 31 July 1958 591 days after the original admission The patient reported that recovery had apparently been complete and that there had been no re currence This is borne out by the clotting data in Table 5 which are com pletely normal (The patient emphasized that on returning from the hospital he had delegated the fumigation of seed beds to his eldest son)

Discussion

This patient appears to have had a temporary acquired clotting dis turbance characterized by a very long PT apparently due entirely to de ficiency of the Stuart factor This conclusion is based upon both negative and positive evidence Negatively there was no deficiency of prothrombin by the 2 stage method and the prolonged PT was corrected neither by $BaSO_4$ adsorbed normal plasma which is rich in Factor V (Ac G) nor by Stypven which is known to correct prolongations due to deficiency of Factor VII (SPCA) (8) Positively *the patient s plasma did not correct the clot* ting defect of the plasma of Mr Stuart and the two plasmas acted similarly in all tests

To our knowledge this is the first recorded example of a temporary de ficiency of the Stuart factor alone It emphasizes a conclusion drawn from the study of congenitally affected patients i e that the site of synthesis of the Stuart factor is highly specific and may be blocked without blocking the synthesis of the other parts of the so called prothrombin complex

There was no history of administration of coumarin drugs nor could a history of contact with rat poison (warfarin) be elicited Furthermore the normal prothrombin concentration (2 stage) and failure of the PT to respond to large intravenous injections of natural Vitamin K are unusual for coumarin overdosage A recent report of homicidally intended coumarin administration for example showed good response to Vitamin K (12)

The liver function tests indicate that generalized liver disease was not present although BSP excretion was moderately impaired It is of some interest however that a liver function test other than the level of Stuart factor was abnormal in our patient It is conceivable that the BSP ab normality accompanying the Stuart factor deficiency constitutes additional evidence for slight but rather specific liver enzymic inhibition by a toxic agent Theoretically an enzyme inhibitor might exist which inactivates only the active sites for BSP clearance and Stuart factor synthesis without affecting albumin synthesis glucuronidation of bilirubin or release of phosphatase It is interesting that Mr Stuart clears BSP normally (4% residual at 45 min) This suggests from another direction that two active sites were affected in our patient (See Addendum)

The history suggests that exposure to the fungicide caused the Stuart factor deficiency This agent proved to be a concentrated halogenated

19

Stuart Prower Factor in Newborn Infants *

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RECENT studies (1 5) have provided information on a clotting factor indispensable to the first and second phases of coagulation i e to the formation of blood thromboplastin and the production of thrombin from prothrombin The factor has been called Stuart Prower the names of two patients in whom the deficiency was recently defined (1 3) Bachmann *et al* reported diminished levels of this factor in heterozygous relatives of a patient with a congenital Stuart Prower deficiency (4)

Stuart Prower factor is present in normal plasma and serum is relatively heat and storage stable is adsorbed by Seitz filters $Al(OH)_3$ and $BaSO_4$ and is elutable from $BaSO_4$ after plasma adsorption A deficiency of the factor results in an abnormal thromboplastin generation test (TGT) with serum Quick prothrombin time (PT) Russell s viper venom CT and plasma recalcification time are prolonged and in measurements of proconvertin with reagents lacking adequate Stuart Prower factor a deficiency of the latter gives false low values for proconvertin (Factor VII stable factor cothromboplastin SPCA) (1 4)

It is the purpose of this paper to present studies on the Stuart Prower defect in healthy premature infants Other coagulation defects have long been recognized (6 7 15 17 19) The relationship of low Stuart Prower

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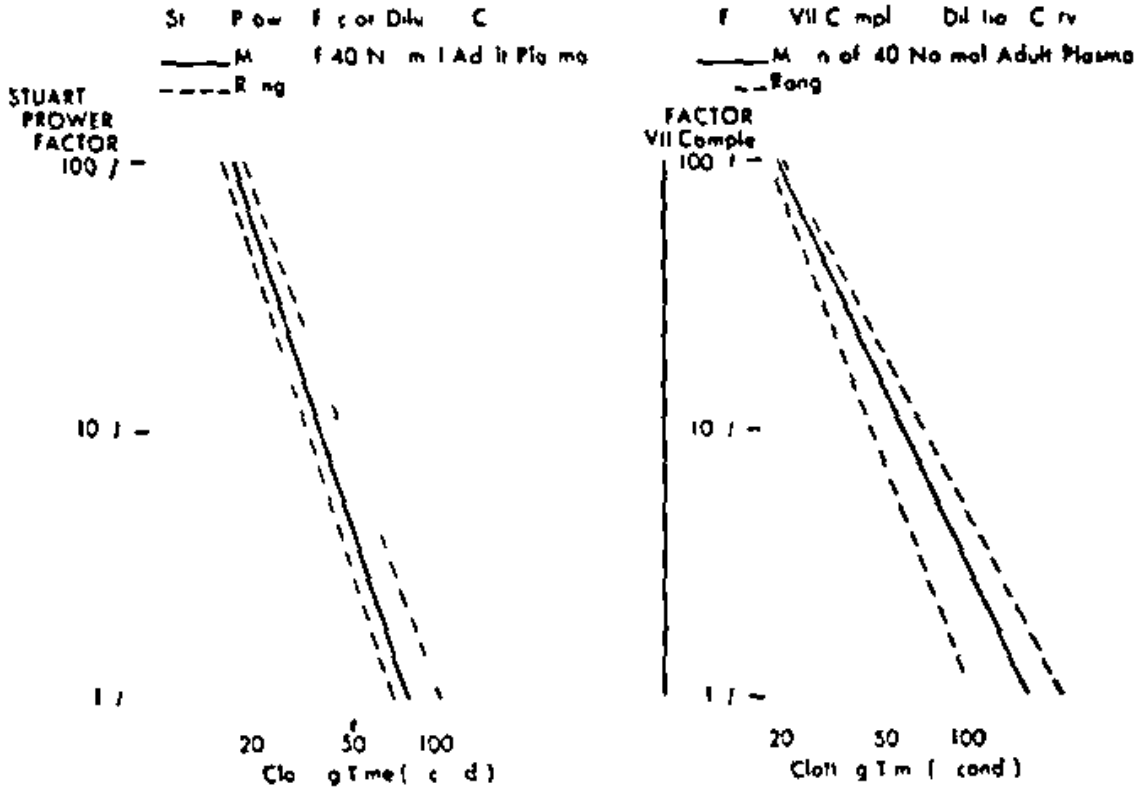


FIG 1

Table 1

COMPARISON OF VENOUS AND CAPILLARY PLASMA LEVELS OF STUART PROWER FACTOR AND FACTOR VII COMPLEX IN NORMAL ADULTS

A—Venous blood collected in uncoated glass tube
B—Venous blood collected in siliconized tube
C—Capillary blood collected in siliconized tube

Subject		*% Stuart Prower Factor*	*% Factor VII Complex*
1	A	100	100
	B	100	88
	C	100	90
2	A	80	96
	B	78	99
	C	78	97
3	A	100	100
	B	100	90
	C	104	100
4	A	78	90
	B	79	88
	C	84	100
5	A	115	100
	B	100	100
	C	115	100
6	A	96	88
	B	98	79
	C	90	78

Percentages referred to 100% of 5 other pooled normal plasmas. All plasmas stored in uncoated glass tubes

factor levels in the newborn infants to clinical manifestations of a hemorrhagic disorder or to a reversibility of such manifestations by vitamin K is still problematical None of the infants studied had symptoms of bleedin and no infant was excluded from the group because of such symptoms—none developed in the prematures seen during the four month period Some infants were necessarily dropped from the study for other reasons e g anoxia

Of the 24 premature infants investigated most were part of a large group in which an assessment of vitamin K prophylaxis was made (8)

Methods

Blood was obtained by heel prick in infants on the first third and sixth days of life The puncture was sufficiently deep to insure a free flow of the blood into a siliconized tube containing 3 8% sodium citrate (1 vol per 9 vols of blood) Many specimens were discarded because of small clot formation *Plasma* was obtained by centrifugation at 3000 rpm for 20 min and was stored at $-20°C$ in uncoated glass tubes until used (1 2 days after withdrawal of the blood) *Serum* from blood collected in uncoated glass tubes was incubated at 37°C for 3 hours and at 20°C for 24 hours before storage at −20°C *Veronal buffer* (Michaelis) pH 7 35 was used for all dilutions *Quick PT* Thrombokinase ACF (N V Amsterdamse Chininefabriek) was the source of thromboplastin *Specific prothrombin determination* the two stage procedure of Ware and Seegers was used (9)

TGT of Biggs and Douglas (10) was modified in the following way (a) brain extract of Bell and Alton (11) was substituted for platelets (b) adsorbed plasma was normal oxalated plasma (1 9 0 11 M sodium oxalate) shaken for 10 minutes at room temperature with washed $BaSO_4$ (12) 100 mg per ml of plasma (c) 0 3 ml of 1 5 dilution of adsorbed plasma 0 3 ml of 1 10 dilution of serum and 0 3 ml of 1 100 dilution of brain extract were incubated with 0 9 ml of M/40 CaCl (13) At intervals of 2 4 6 8 10 and 12 min 0 3 ml of the incubation mixture was added to 0 1 ml of citrate plasma substrate and CT recorded Normal plasmas and sera give CT of 10 11 sec after 4 to 6 min incubation Normal plasma and serum from the same person were used for all control tests in this study

Factor VII complex and Stuart Prower assay were done according to Bachmann *et al* (4 5) *Prothrombin content (two stage method)* of the Seitz (20 + 30%) filtered ox plasma was 25 30% Blank CT were 80 to 110 sec with Russell s viper venom (Stypven Burroughs Wellcome) cephalin reagent and 300 to 400 sec with brain thromboplastin

Fig 1 shows the range of dilution curves in 40 normal adults Because blood was obtained from the infants by heel prick measurements of Stuart

in only two of the infants and was normal in both Since in our experience and that of others (15 19) plasma Ac globulin is usually normal in the neonate repeated measurements were not done

Fig 2 is a graph of Stuart Prower levels in premature infants on the first third and sixth days of life with and without vitamin K_1 (Konakion F Hoffman La Roche & Co) intramuscularly as prophylaxis at the time of birth The mean values for Stuart Prower factor are slightly higher on the third day of life in infants who received vitamin K_1 than in those who did not

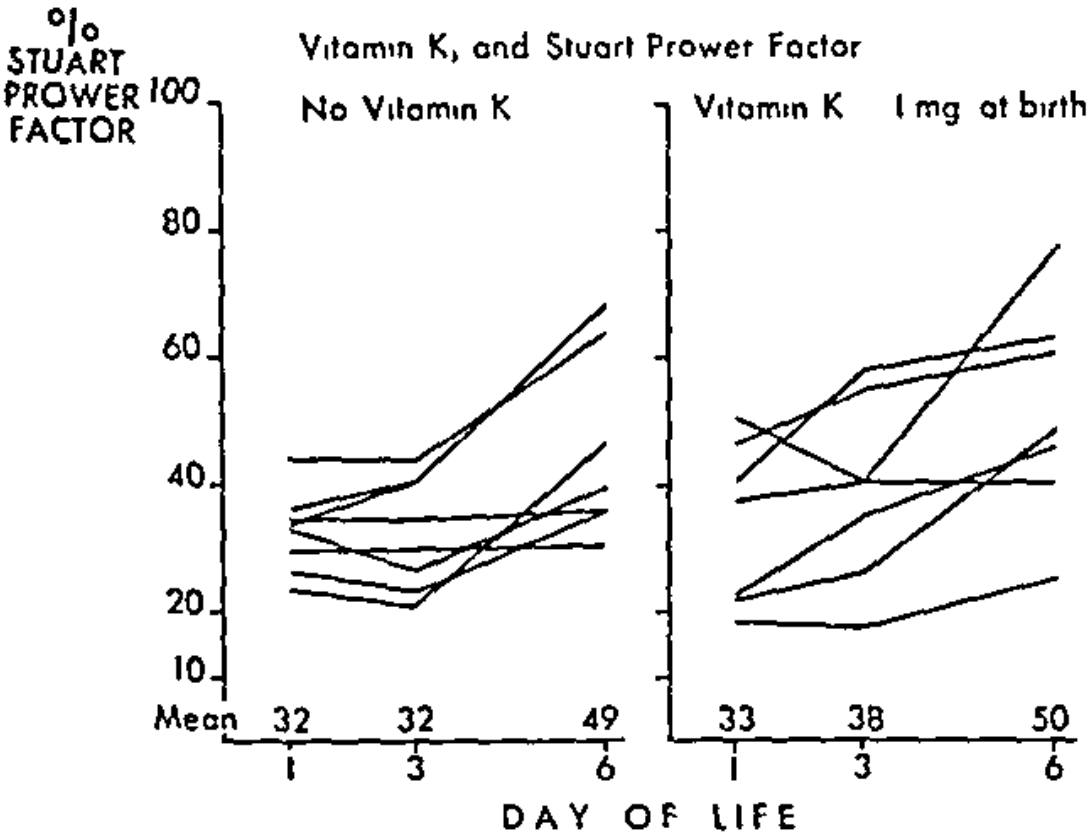


Fig 2

The results of the TGT using serum from 1 to 3-day old premature infants are shown in Table 3 No vitamin K was given to this group The defect in the serum of the infants was completely corrected by normal serum (diluted 1 10) PTC (Christmas factor Factor IX) deficient serum did not correct the abnormality as well as did serum from a patient with congenital Stuart Prower defect *

Kindly supplied by Caen J Laboratoire de l'Administration generale de l'Assistance Publique Paris and by Graham J B University of North Carolina Chapel Hill U S A

Prower factor and Factor VII complex in adult plasma obtained from (a) the antecubital vein and (b) a finger prick were done Venous blood was collected in both uncoated and in siliconized tubes Capillary blood was collected in siliconized tubes All plasmas were stored at −20°C in uncoated glass tubes and determinations were done in 1-2 days after the blood collections Table 1 shows that there are relatively insignificant differences in the three specimens and those that are apparent are not consistent for any one method of collection Though Factor VII (pro convertin) is influenced by glass contact (14) and Stuart Prower factor is not (5) storage of these plasmas in uncoated glass tubes tends to nullify such influences in these studies

Results

Table 2 shows the level of Stuart Prower factor Factor VII complex prothrombin and Quick time in eleven healthy premature infants in the first 12 hours of life Birth weight varied between 1780 and 2400 grams In general all coagulation factors measured were decreased In these infants

Table 2

PREMATURE INFANTS ON FIRST DAY OF LIFE

	Stuart Prower Factor *	*Factor VII Complex* *	*Prothrombin* * *2 stage*	*Quick Time* *
1	35	28	86	65
2	33	22	77	45
3	34	36	32	15
4	36	18	62	50
5	44	36	80	65
6	26	23	71	33
7	30	23	84	58
8	24	29	—	—
9	51	60	82	86
10	30	42	54	62
11	27	29	—	—

Figures in % of normal adult plasma values

the Quick time was more closely correlated with the level of prothrombin than with the value for Factor VII complex Since the latter levels which lie between true Factor VII and Stuart Prower factor levels (4) correlate well with Stuart Prower values it would be expected that Factor VII (pro convertin) is also low in these infants Specific Factor VII determinations with Factor VII deficient plasma were unfortunately not possible

Plasma Ac globulin (Factor V proaccelerin labile factor) was measured

in only two of the infants and was normal in both Since in our experience and that of others (15 19) plasma Ac globulin is usually normal in the neonate repeated measurements were not done

Fig 2 is a graph of Stuart Prower levels in premature infants on the first third and sixth days of life with and without vitamin K_1 (Konakion F Hoffman La Roche & Co) intramuscularly as prophylaxis at the time of birth The mean values for Stuart Prower factor are slightly higher on the third day of life in infants who received vitamin K_1 than in those who did not

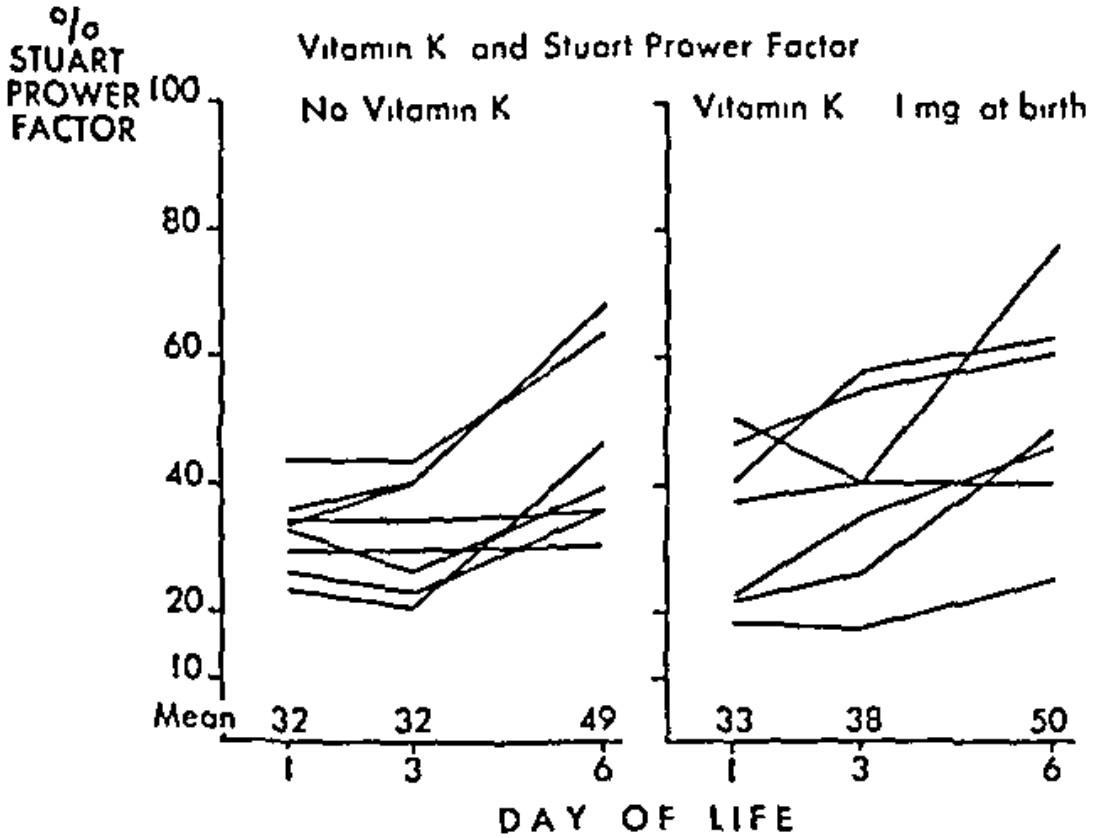


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Table 3

THROMBOPLASTIN GENERATION TEST IN 1 TO 3 DAY OLD PREMATURE INFANTS *

	Serum Used	*Incubation Time (min)*					
		2	4	6	8	10	12
		Minimum Clotting Time (sec)					
1	Normal adult			11			
2	PTC deficient						35
3	Stuart Prower deficient				25		
4	2 + 3 (1 1)			18			
5	Infant I			14			
6	+ 1 (1 1)		12				
7	+ 2 (1 1)				17		
8	+ 3 (1 1)			14			
9	Infant II				14		
10	+ 1 (1 1)			11			
11	+ 2 (1 1)				14		
12	+ 3 (1 1)		12½				
13	Infant III		17½				
14	+ 1 (1 1)					12	
15	+ 2 (1 1)			17½			
16	+ 3 (1 1)	14½					
17	Infant IV				25		
18	+ 1 (1 1)		15				
19	+ 2 (1 1)			20			
20	+ 3 (1 1)				16½		
21	Infant V						18
22	+ 1 (1 1)		15				
23	+ 2 (1 1)					21	
24	+ 3 (1 1)			16			
25	Infant VI		16½				
26	+ 1 (1 1)		12				
27	+ 2 (1 1)			18½			
28	+ 3 (1 1)		15				

* No vitamin K was given to these infants

The same normal adult $BaSO_4$ adsorbed plasma and platelet substitute was used in all tests

Conclusions

Stuart Prower levels were found to be low in premature newborn infants at birth with an increase toward normal by the sixth day of life whether or not vitamin K_1 was given to the infants at birth Conclusions concerning vitamin K_1 prophylaxis are not valid from data on this small number of infants However it appears suggestive that as with other coagulation factors (8 18 20 21) vitamin K may check the decline in levels during the first few days after birth

Other investigators have shown that PTC deficient serum does not completely correct the defect in thromboplastin generation noted with sera of newborn infants (7 22 23) It is probable that both PTC and Stuart Prower factor are responsible for delayed thromboplastin formation in young infants In the premature infants in this study however there was apparently a more critical lack of PTC for normal thromboplastin generation

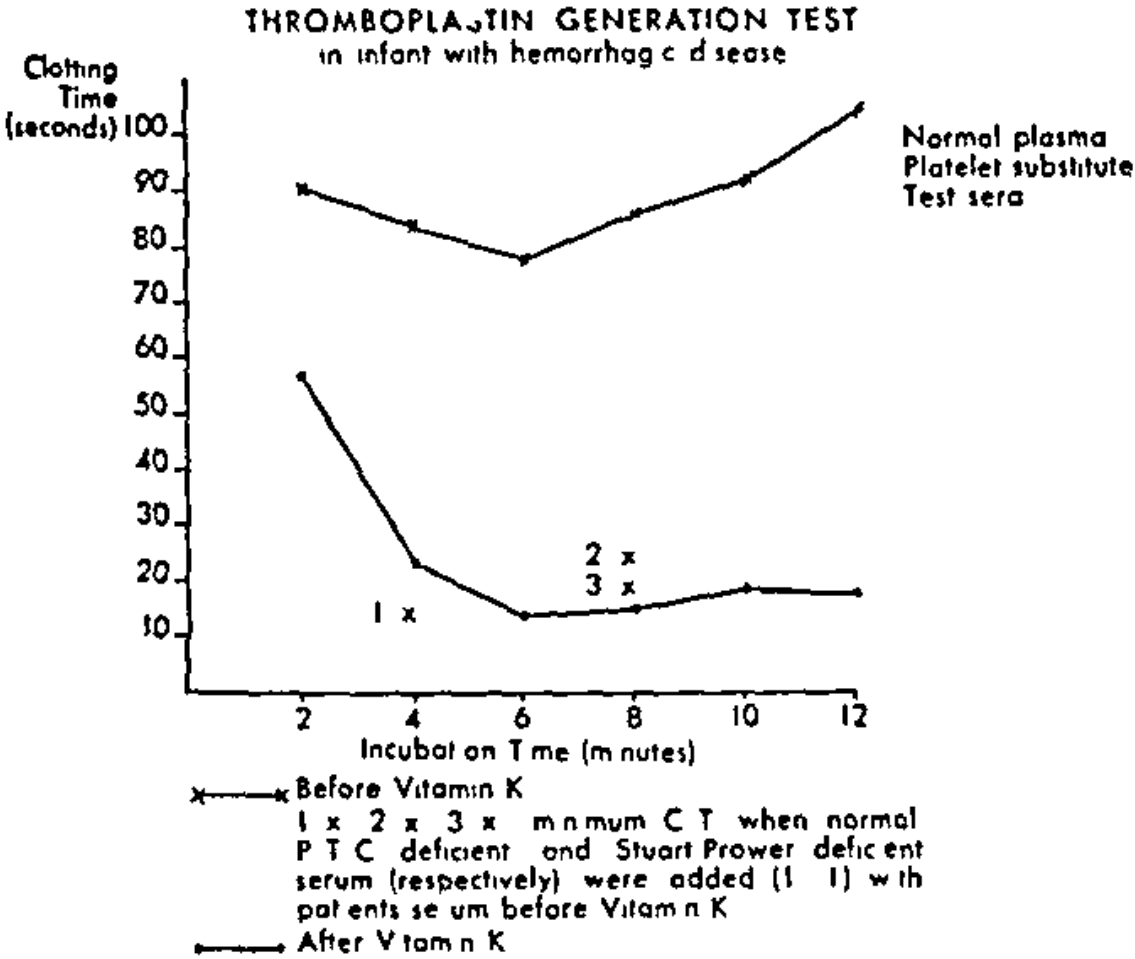


FIG 3

Coincidental to this investigation a newly born infant in another hospital was studied because of severe hemorrhagic disease The circumstances were similar to three other cases previously reported (24) The infant was born at term of a mother who was receiving phenobarbital daily for control of epilepsy A generalized bleeding tendency was noted on the fourth day of life and laboratory data revealed a Quick P T of + 30 sec (control 13 sec) prothrombin (two-stage) 48% Factor V 100% Factor VII complex 5% and Stuart Prower factor 7%

Levels of all coagulation factors approached normal one day after the administration of one mg of vitamin K_1 (Konakion) Fig 3 shows the TGT with this infant s serum before and after vitamin K_1 was given The

rapid return of coagulation factors to near normal levels is evidence that the multiple defect in this child was not due to liver immaturity at least not in the ability to respond to vitamin K It is possible that destruction and/or excretion of the medication may have been influential in the prompt recovery This too would militate against a marked inefficiency of hepatic function It is extremely difficult in the newborn to differentiate between the exogenous traumatic and endogenous influences affecting either the laboratory values for various clotting factors or hemorrhagic symptoms For this reason much of the evidence for the efficiency of vitamin K prophylaxis in the newborn infant seems equivocal The very prompt response of this infant and the previously reported ones (24) to one mg of vitamin K does however confirm the role of vitamin K in the therapy of a syndrome clinically indistinguishable in onset from the classical hemorrhagic disease of the newborn

Summary

1 Stuart Prower factor levels in the blood of 24 newborn premature infants were decreased when compared to normal adult values

2 Mean Stuart Prower factor levels were slightly higher on the third day of life in prematures who received one mg of vitamin K prophylactically than in those who did not

3 Serum deficient in Stuart Prower factor corrected the defect in the TGT of premature infants to a greater degree than did serum deficient in PTC

4 Stuart Prower factor levels in a case of hemorrhagic disease of the newborn were very low

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Part 4

HAGEMAN FACTOR AND SURFACE REACTIONS

20

Establishment of the Inheritance of Hageman Defect As an Autosomal Recessive Trait

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WE HAVE summarized in Table 1 the sparse information in the literature which might be helpful in unraveling the genetics of Hageman trait (1 8) The reader will undoubtedly infer correctly that only Ratnoff and Margolius (1 2 3) have deliberately attempted to obtain significant genetic data They succeeded however only in showing that Hageman trait is *probably* inherited by finding an additional affected sibling and evidence of inbreeding in one of the two kindreds studied The coefficient of inbreeding of the affected sisters was 1/32 This is not a particularly high degree of inbreeding and its significance is difficult to interpret in the absence of any knowledge of the frequency of the mutant gene

It is fair to say that while the Cleveland workers did not establish the mode of inheritance of Hageman trait with certainty their work emphasized that autosomal recessiveness was the leading possibility The family studies published by others (4 8) have emphasized further the likelihood of this mode of inheritance without contributing crucial information Only 2 parents and 5 children of affected persons have been tested in all studies prior to ours It should be noted however that not a single person with Hageman trait has been found in these seven tests

An adequate statistical test of the unifactorial genetic hypotheses requires

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Table 1

PERSONS WITH HAGEMAN TRAIT DESCRIBED IN LITERATURE

	AFFECTED PERSONS					TESTED RELATIVES OF AFFECTED PERSONS						
Authors	*No*	*Age Yrs*	*Sex*	*Ethnic Background*	*Whole Blood Clotting Time Min*	*Parents* Aff	Norm	*Children* Aff	Norm	*Other* Aff	Norm	*Total Tested*
Ratnoff and others (1 2 3)	1	37	Male	German	87	*	*	*	*	0	7	7
	2	46	Female	German	86 } *Sisters*	*	*	0	2	0	41	44
	3	50	Female	German	90 }			0	1			
Ramot and others (4)	4	60	Male	Lithuanian	60	*	*	0	2			2
Frick and Hagen (5)	5	32	Male	———	65	*	*	*	*	*	*	0
	6	35	Male	———	60							
Larrieu and others (6)	7	50	Female	French	17 21	*	*	*	*	*	*	0
Jim and others (7)	8	26	Female	German Irish French	60	0	2	*	*	*	*	2
Sjolin (8)	9 **	37	Male	Danish	—	*	*	*	*		*	0
Total						0	2	0	5	0	48	55

— = Categories in which no persons were examined
* = Not unequivocally established

information either on several large kindreds or on many small ones because affected persons other than index cases must be found (Index cases are dropped from statistical consideration to eliminate the bias of ascertainment) As mentioned earlier all but one of the pedigrees of Hageman trait published prior to ours have consisted of a single affected person and a statistical test has therefore not been possible

In this paper we shall describe our work on the inheritance of the Hageman trait in 4 families We shall show that the trait segregates as if it were a completely recessive autosomal characteristic a possibility which we have not been able to exclude statistically by an adequate test

Materials and Methods

Blood was obtained by venipuncture from the antecubital vein *Plasma* was prepared by placing whole blood in conical centrifuge tubes containing a buffered citrate anticoagulant in the ratio of 1 part anticoagulant to 8 parts blood The anticoagulant consisted of 3 2% trisodium citrate dissolved in 1 72% imidazole buffer at pH 7 25 The blood was centrifuged at 3200 x *g* for 15 minutes and the plasma withdrawn by pipette Plasma was tested sometimes immediately sometimes after storage at −20 C for periods up to 48 hours *Whole blood clotting time* was that of Lee and White as described by Jaques (9) *Standard partial thromboplastin time* was performed on the plasma of control and test subjects by the method described by Rodman and others (10) *Specific partial thromboplastin time* was performed on samples of control and test plasma after mixing with an equal part of plasma from a subject with a high grade Hageman defect This procedure was used throughout the study as the specific confirmatory test for Hageman trait Its specificity and utility in the diagnosis of Hageman trait have been pointed out previously (10) *Prothrombin time* was determined by the method of Quick (11) *Prothrombin utilization rate* was determined by the method described by Graham and others (12) *prothrombin concentration* being determined by the Iowa two stage method as described by Wagner and others (13)

Results

Description of the Kindreds Studied

"Ca Kindred The proband is a 17 year old white male of Anglo Saxon extraction referred because of a prolonged clotting time discovered by routine tests prior to tonsillectomy There was no history of excessive bleeding among his relatives He reported that the following accidents and operations were *not* accompanied by excessive bleeding tonsillectomy at age 6 tooth extraction at age 9 laceration of leg requiring 12 sutures at age 10 and appendectomy at age 15 His parents were examined tested

Table 2

CLOTTING DATA ON PERSONS WITH HAGEMAN TRAIT IN PRESENT STUDY

Probands

Kindred	Clotting Time Min	Bleeding Time Min	Prothrombin Time Sec (Proband/Control)	Standard Partial Thpln Time (PTT) Sec (Proband/Control)	Specific Partial Thpln Time (PTT) Sec (Proband/Control)	Prothrombin Utilized in one hour % (Proband/Control)
Ca	29	3½	17 7/12 5	308/72	453/123	——
I	40	3	14 1/13 6	324/76	215/107	——
M"	0	6½	13 7/13 6	147/86	164/102	63/94
Cu	35	—	13 9/10 5	1000/74	540/87	35/94
Additional Affected Persons Discovered Through Family Studies						
I" Kindred						
II 9	26 40	3½	16 2/13 0	435/70	222/98	——
II 16	17 37	3	14 4/12	457/70	242/112	——
M" Kindred						
II 5	35	2	16/12	204/57	195/73	——

and found unaffected The only sibling a brother died 36 hours after birth of a cause not known to the family Diagnostic clotting data on the pro band are shown in the top line of Table 2 The characteristic long clotting time long standard partial thromboplastin time and failure to correct the Hageman deficient plasma in the specific partial thromboplastin time test can be seen This patient also showed a slight but definitely prolonged prothrombin time a feature not generally reported in connection with Hageman trait but one which we have observed in 5 of the 7 cases recorded in Table 2 The immediate family of the proband are shown in the upper left part of Fig 1

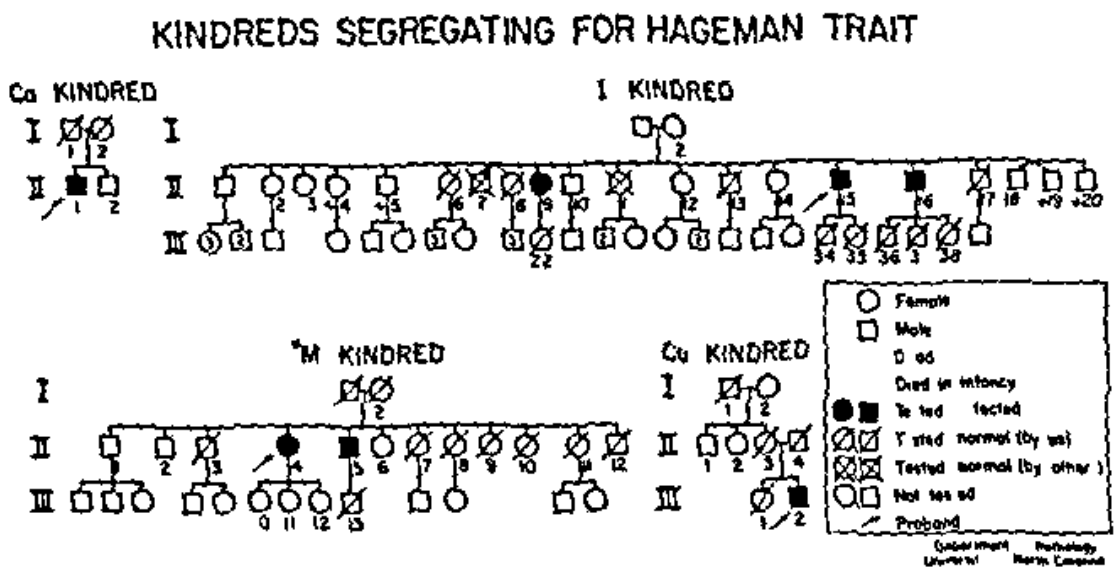


FIG 1

"I Kindred The sibship of the proband shown in Fig 1 consisted of 20 persons The proband II 15 is a 62 year-old white male of German extraction who was discovered to have a long clotting time prior to repair of a hernia He had had nosebleeds as a young boy and again intermittently for two weeks at age 47 He has had three operations recently without transfusions or bleeding sequelae i e cataract operations in 1957 and 1958 and hemorrhaphy in 1958 The patient has hypertension at present and had a stroke in 1955 at age 59 from which he has recovered almost completely Characteristic clotting data for the Hageman trait are shown in the second line of Table 2

The immediate relatives of the proband are shown in Fig 1 It will be noted that 9 of his 19 siblings are dead Six of the 10 survivors still reside in North Carolina and were tested by us Three of the four survivors living outside the state (New York Florida and Washington State) were con tacted and tests were carried out in laboratories in their own communities We discovered by these means two additional persons with Hageman trait a man (II 16) and a woman (II 9) The clotting data on these two persons are shown in the fifth and sixth lines of Table 2 To complete our studies all

six children of the three affected persons were also tested (The parents were dead) It will be observed on the pedigree chart that all children were found to be normal

"M Kindred The proband of this kindred is a 32 year old Negro female referred because of a prolonged clotting time discovered preparatory to tonsillectomy She has had no symptoms suggestive of a bleeding tendency and has been transfused only once receiving 7 units of blood following rupture of a tubal pregnancy Three children have been born to her without difficulty and she did not bleed excessively when four teeth were extracted

Diagnostic clotting data on Mrs M are shown in the third line of Table 2 The characteristic Hageman defect is clearly present but comparison of these data with those from the other probands suggests that she has the mildest defect in the group

The sibship of the proband shown in the lower left part of Fig 1 consists of 5 males and 7 females We were able to test 8 of the 11 siblings of the proband and 1 additional affected person (II 5) was discovered His clotting tests comprise the bottom line of Table 2 His only child the three children of the proband and the aged parents were tested and found to be normal

"Cu" Kindred The proband of this kindred is a 4 year old white male of Anglo Saxon extraction discovered to have a prolonged clotting time prior to tonsillectomy There was no family history of excessive bleeding The clotting tests performed by us on this boy are shown in the fourth line of Table 2 Typical data for Hageman trait can be observed It can be seen in Fig 1 that the proband has only one sibling a sister She and the parents were tested and found to be normal

Additional Affected Persons Three additional persons with the Hageman trait were discovered through the family studies described above As expected they were found in the larger sibships of the I and M kindreds Diagnostic coagulation data on these three persons are grouped together in the lower half of Table 2 Comparison shows that the clotting studies in the lower group differ in no respect from those of the probands although there is a tendency toward an intrafamilial correlation (cp affected persons in I kindred with those in M kindred)

Genetic Analysis of the Collated Material

The brothers and sisters of the probands of the 4 kindreds can be classified as shown in Table 3 a tabulation which shows the sex ratio to have been unity (16 16) It will be noted that the newly discovered cases included both males and females (The sex ratio among our seven cases of Hageman trait is 5 males 2 females) It should be noted (Fig 1) that 6 parents and 10 children of persons with Hageman trait were also tested and that not one showed Hageman trait These bits of information allow us

to eliminate three of the four simple unifactorial modes of Mendelian inheritance as follows Dominant inheritance either autosomal or sex linked can be excluded by noting that there was not a single affected parent or child of a person with Hageman trait in 16 instances studied The sex linked recessive mode of inheritance can be rejected because both males and females were affected and the fathers of the affected females were themselves normal

Table 3

COLLECTED DATA ON 4 SIBSHIPS SEGREGATING FOR THE HAGEMAN TRAIT PROBANDS EXCLUDED

Kindred	Males				Females				
	Affected	Normal	Unknown	Total	Affected	Normal	Unknown	Total	Total
Ca	0	0	1	1	0	0	0	0	1
I	1	4	5	10	1	3	5	9	19
M	1	2		3	0	5	1	6	11
Cu	0	0	0	0	0	1	0	1	1
Totals	2	6	8	16	1	9	6	16	3

These eliminations lead directly to a test of the fourth simple mode of inheritance autosomal recessiveness If the ratio affected normal among the siblings of the probands is formed by combining the sexes in Table 2 it will be observed to have been 3 affected 15 normal in a total of 18 tested With a total of 18 the expected numbers for an autosomal recessive (1 3 ratio) would have been 4 5 normal 13 5 affected The observed ratio is not therefore a significant deviation from the expected 1 3 ratio ($\chi = 6611$ P(1d f) 30 ¢) and the autosomal recessive mode of inheritance cannot be rejected The failure of rejection inclines us to believe that Hageman trait is indeed inherited in this simple fashion It would have been better had we studied a larger number of large sibships and also the segregation ratios among the offspring of all mating types Study of segregation ratios with the various mating types is not feasible at present because the trait is rare and as will become apparent later only one of the affected genotypes can be recognized with confidence in the laboratory Within these limitations we have shown that inheritance is probably unifactorial and in the manner of an autosomal recessive i e the affected persons are homozygous and their parents and children are heterozygous At any rate these will be our assumptions in the succeeding sections

Consanguinity

The relationships of the progenitors of the probands are shown in Fig 2 It will be observed immediately that the parents of the affected persons in the M kindred were second cousins The parents producing

affected children in the other three kindreds did not appear to be related although there was prior (and presumably irrelevant) inbreeding on both sides among the ancestors of the parents in the Ca kindred It is important also to note that no surname encountered in one family was ever encountered in another

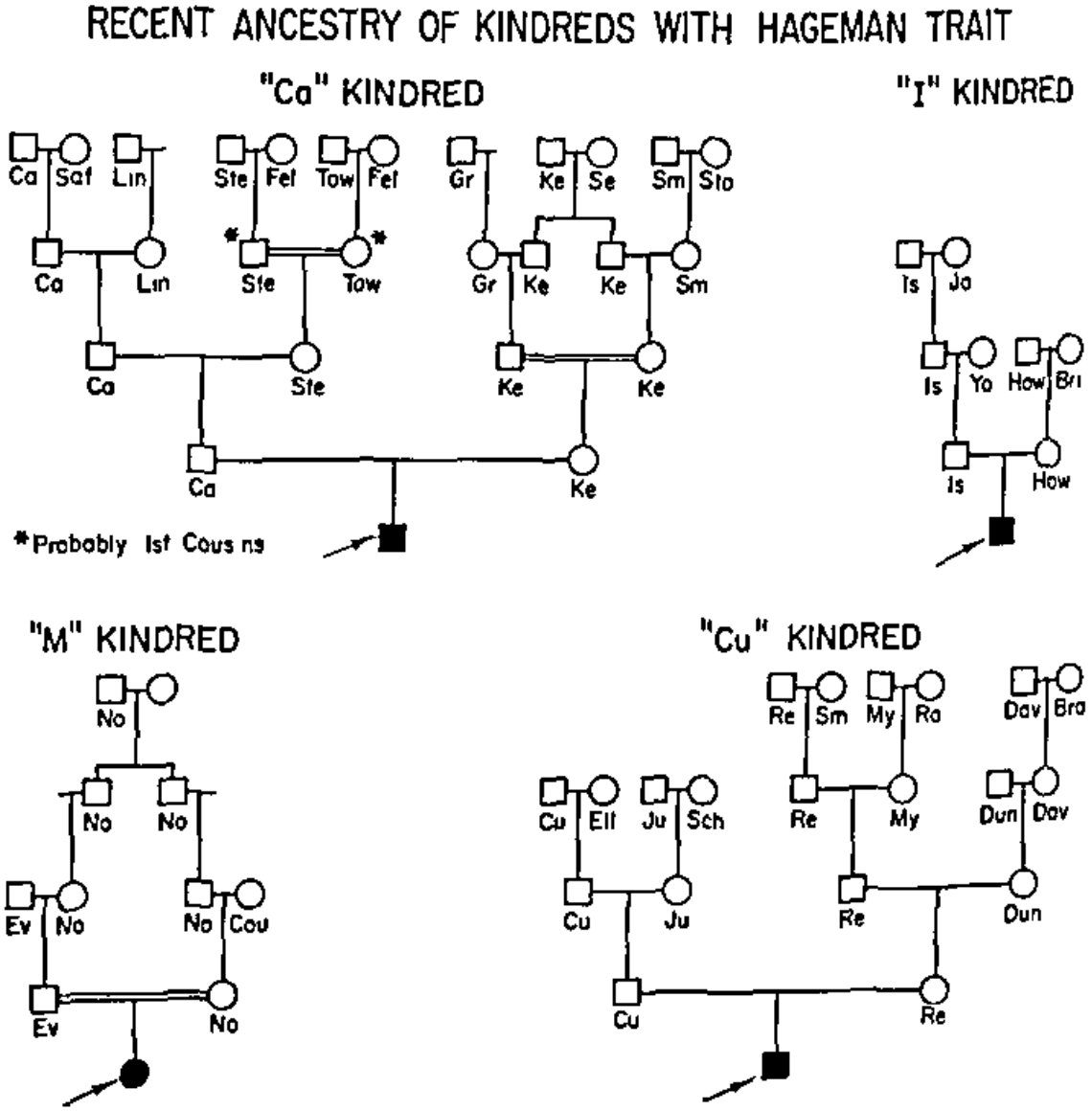


FIG 2

Effect of the Mutant Gene at the Hageman Locus on Clotting Tests

Group Effects in Homozygotes and Heterozygotes A comparison is made in Table 4 of the effects of homozygosity and heterozygosity at the Hageman locus on three clotting procedures *whole blood clotting time* standard PTT and specific PTT For the comparison we have averaged the values separately for 7 presumed homozygotes 15 presumed

heterozygotes (parents and children of affected persons) and 10 presumably normal laboratory workers of both sexes The values shown for the homozygotes were collected individually over a period of two months The plasmas of the 15 heterozygotes and the 10 normal controls were collected over a 4-day period and great care was taken to be certain that conditions of collection and storage were as similar as possible for all 25 These plasmas were quickly frozen after collection coded and stored at −20°C All standard partial thromboplastin times and specific tests were performed by one of us (K F McC) in triplicate during a single day The order in which the plasmas were tested was random and the same reagents were used throughout the tests

Table 4

GROUP MEANS FOR CLOTTING TESTS ON CONTROLS AND THE (PRESUMED) HOMOZYGOTES AND HETEROZYGOTES FOR HAGEMAN TRAIT

Group Tested	Blood Clotting Time (Min)	Standard Partial Thromboplastin Time (PTT) Sec		Specific Partial Thromboplastin Time (PTT) Sec	
	$\bar{x}$	$\bar{x}$	s e	$\bar{x}$	s e
7 Presumed Homozygotes	32	410 8	106 8	745 0	50 3
15 Presumed Heterozygotes	8	76 4	4 3	108 3	5 0
10 Presumed Normals	(6 10)	76 1	3 0	101 9	3 4

* Not established empirically Generally accepted range

t Tests of Significance
1) Standard PPT 15 heterozygotes v 10 controls $t = 05$ $p > 50$
2) "Specific PTT 15 heterozygotes v 10 controls $t = 9477$ $p > 30$

The top and middle lines of Table 4 show that homozygosity for Hageman trait has a very much greater effect on clotting tests than heterozygosity In all tests the clotting times of the homozygotes are significantly longer than those of the heterozygotes (or the normals) at the 1% point

In comparing the middle and lower lines of Table 4 however it becomes apparent that heterozygosity for Hageman trait (parents and children of affected persons) has little more effect than homozygosity for the normal allele (normal controls) There are only very slight and statistically insignificant differences between the group means These small differences are it should be noted in the direction which would be expected if heterozygosity had a slight effect one of prolonging clotting Also the effect is slightly greater in the specific than in the standard PTT

Individual Effects in Heterozygotes While the preceding test implies that a single mutant gene at the Hageman locus has little effect on a *group* of heterozygotes it should not be concluded necessarily that an occasional heterozygote does not show a significant effect Such individual effects were sought by the arrangement of the data with the standard partial thrombo

plastin time and the specific partial thromboplastin time test shown in Table 5 The 10 controls have been ranked in ascending order of standard partial thromboplastin times in the first column The 15 presumed heterozygotes have been similarly ranked with respect to the same procedure in the second column The third column contains clotting times of each normal control in the specific test for Hageman deficiency each number corresponding to the plasma with the same number in column 1

Table 5

ACTUAL CLOTTING VALUES ON THE 10 NORMALS AND 15 (PRESUMED) HETEROZYGOTES FOR HAGEMAN TRAIT

Standard Partial Thromboplastin Time Test				*Specific Partial Thromboplastin Time Test*			
10 Controls		*15 Heterozygotes*		*10 Controls*		*15 Heterozygotes*	
Subject No	*CT Secs*	*Subject No*	*CT Secs*	*Subject No*	*CT Secs*	*Subject No*	*CT Secs*
1	63 4	1	60 3	1	83 3	1	79 7
2	64 0	2	61 5	2	84 9	2	89.3
3	69 5	3	62 2	3	110 3	3	91 6
4	71 9	4	62 4	4	102 7	4	88 5
5	72 8	5	65 3	5	112 5	5	130 3
6	73 9	6	66 6	6	93 9	6	113 1
7	83 8	7	72 7	7	105 1	7	107 9
8	85 8	8	73 9	8	106 8	8	107 4
9	87 8	9	73 9	9	112 6	9	104 2
10	88 3	10	73 9	10	107 1	10	102 7
		11	78 1			11	90 7
		12	84 6			12	174 6
		13	85 3			13	124 5
		14	+107 0			14	+152 5 *
		15	118 2 *			15	118 1
$\bar{x} = 76\ 1$				$\bar{x} = 101\ 9$			
$\sigma = 9\ 6$				$\sigma = 10\ 8$			
$\bar{x} + 2\sigma = 95\ 3$				$\bar{x} + 2\sigma = 123\ 5$			

* Values for (presumed) heterozygotes which are more than 2 standard deviations greater than the mean for 10 controls

\+ (Presumed) heterozygote outside the normal range with both tests

The numbers of the plasmas in the fourth column refer to the same persons as the corresponding numbers in the second column It will be noted that the lower limits of the two populations are very similar with both tests Several of the heterozygotes however in both tests have longer clotting times than any of the controls

A discriminant can be devised which will help to decide whether any of the values of the heterozygotes are significantly prolonged The sum of the mean for the controls plus 2 standard deviations provides a point beyond which a heterozygote value may be considered abnormal with 95% confi

dence When such discriminants are prepared for the two tests (bottom of Table 5) it can be seen that only two presumed heterozygotes (Nos 14 and 15) fell outside this range with the standard partial thromboplastin time test and four (Nos 5 12 13 and 14) fell outside this range with the specific test Only one heterozygote Number 14 had significantly prolonged values in both tests She is incidentally a daughter of the pro band of the M kindred

Discussion

In adding a description of seven new patients with the Hageman trait to the nine previously reported we would emphasize the uniformity of the clotting disorder in our subjects The coagulation tests on our seven patients varied only slightly and most of the variety which we observed had nothing to do with coagulation Five of our patients were men two were women Three patients were of German extraction the ancestors of two emigrated from the British Isles and two were Negroes

We were able to establish significant consanguinity in only one of the four families There was consanguinity in another which was of a type not influencing the crucial mating Furthermore there is good reason to think that our four families were not related in any practical sense of the word

The 4 kindreds were ascertained in the same fashion All were discovered in hospitals where it is routine practice to perform whole blood clotting times before operation The precipitating operation was tonsillectomy in three instances herniorrhaphy in one All probands and the three additional affected persons gave histories remarkable for absence of hemorrhagic symptoms

The simplest and most sensitive test for detection of Hageman trait proved to be the partial thromboplastin time When performed on a mixture of test plasma and a plasma known to be deficient in the Hageman factor this clotting test was highly specific as well as sensitive a fact emphasized previously by Rodman and others (10)

Our study while not as large as might be desired is important because of the statistical test which it provides The study allows us to say with considerable confidence that Hageman trait is transmitted as a unifactorial genetic characteristic The mutant gene is probably carried on an autosomal chromosome and recessive The extent of recessiveness of the mutant is emphasized by the data showing that heterozygotes as a group are not different from a group of homozygous normals with respect to two relatively sensitive clotting tests Nevertheless it appears that an occasional hetero zygote may have clotting values falling outside the range of normal Spe cifically 2 of 15 heterozygotes had abnormally prolonged standard PTT tests 4 of 15 had abnormally prolonged specific PTT tests and one individual was abnormal in both tests If we define definite abnormality as

abnormality with both tests it would appear that about 7% of heterozygotes have clearly abnormal clotting tests Obviously clotting tests such as the ones we used are of no use in diagnosing heterozygosity for Hageman trait in individual cases

Finally our experiences throw no light on the real enigma of Hageman trait Current thinking in biochemical genetics would suggest that the Hageman factor has an as yet unknown function in normal physiology It is possible that heterozygotes are at some disadvantage with respect to this function and homozygotes at considerable disadvantage Whatever this function is however it apparently has nothing to do with hemostasis It is likely that the abnormal clotting tests only indicate the presence of the gene The sex ratio in our material is 1 1 and the I and M kindreds show no obvious reduction in reproductive capacity If the mutant has only slight deleterious effects or none at all it might be present at high frequency in a population The ease and certainty of diagnosis of homozygotes with the partial thromboplastin time suggests that a population survey is feasible The almost complete recessiveness shown by heterozygotes in this test implies however that a large population must be sampled because the gene frequency must be estimated from the square root of the frequency of homozygotes It is important to determine the frequency of this gene because of the insight this might give into the elective effects of this locus This is in fact the next task we intend to attempt with respect to the genetics of Hageman trait

Summary and Conclusions

1 Seven new patients with Hageman Trait are described These were found present in four unrelated families two of which showed consanguinity Affected men and women of both Western European and African extraction were observed

2 It is pointed out that three of the common forms of unifactorial Mendelian inheritance can be excluded by our results It is highly likely therefore that Hageman trait is inherited as an autosomal recessive characteristic

3 It is pointed out that presumed heterozygotes for the Hageman mutant do not show significant effects with certain sensitive clotting tests

4 Something less than 10% of presumed heterozygotes do however show prolongation of clotting which may be presumed to be due to slight reduction in Hageman factor

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21

Hageman Factor Activation and Related Processes *

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THE discovery that clot promoting activity in some fashion was associated with Hageman factor (1) has led to significant recent advances in studies of surface influences on blood clotting phenomena Shafrir and de Vries (2) and Margolis (3 4) found clot promoting activity could be developed by glass contact in plasma which had been adsorbed with $BaSO_4$ and then heated at 56°C for 10 15 min Subsequent investigations showed that Hageman deficient plasma failed to be activated by glass (1 5 7) or asbestos (8) contact Thus it seemed that Hageman factor (a) had a key role in surface activation and (b) was activated by surface contact Besides Hageman factor of the known clotting factors possibly PTA (9) could have been involved Studies on PTA deficient plasma (5 7 10) gave contradictory results regarding its participation in surface reactions

The changes in plasma that result from exposure to a variety of surfaces have been shown by standard (8) as well as special (4 6) clotting tests The partial thromboplastin time (PTT) test (11) was selected for the present studies because it is not sensitive to changes in platelet concentration Platelet rich platelet poor and platelet free plasmas were found to give similar PTT clotting times Therefore platelets could not have been responsible for changes in the test clotting times *Intact plasma* (4) for these studies was obtained in the following manner blood was drawn into

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a cold siliconized syringe containing one volume of 3 2% sodium citrate per 9 volumes of blood This was centrifuged at 3000 rpm for thirty minutes in a lusteroid tube and transferred by silicone coated pipettes into siliconized tubes To study the effect of surface contact asbestos or washed crushed pyrex glass (100 140 mesh) was used The details of asbestos activation are found elsewhere (8) Glass activation was accomplished by rotation of a 25 ml beaker containing 500 mg crushed glass per ml plasma for 5 min at 43 rpm The plasma was then filtered (to remove glass particles) into silicone-coated tubes and designated *contacted plasma* Samples were tested at different time intervals after contact

Most of our tests were performed in glass tubes which could be criticized as introducing additional surface contact However it was found (Table 1) by comparative tests on eight normal plasmas intact and contacted that once initial contact had been established there was little difference in test clotting times performed in either glass or siliconized tubes This confirms earlier experimental findings in our laboratory (12) Thus unless otherwise indicated the clotting tests were performed in glass tubes

Table 1

PLASMA PTT TIMES IN GLASS AND SILICONIZED TUBES BEFORE AND AFTER SURFACE CONTACT BY CRUSHED GLASS

	Clotting Test Surface	
Plasma Tested	*Glass*	*Silicone*
Intact	90 4	222 7
Contacted	66 8	70 4

Hageman Factor Activation The following summary of results in our experiments with asbestos activation of plasma (8) shows the intimate relationship of Hageman factor activation to surface phenomena (a) acceleration of clotting times in several different test systems occurs when normal plasma is exposed to surface contact (b) of the factor deficient plasmas and plasma fractions tested only Hageman deficient samples fail to show changes similar to (a) (c) in plasma mixtures contacted normal plasma shortens the clotting times of all deficient plasmas more than uncontacted normal contacted Hageman deficient plasma gives results which are not shorter than those obtained when intact Hageman deficient plasma is added (d) shortening of clotting times on the addition of increasing amounts of purified Hageman factor (6) showed no optimum (e) purified Hageman preparations not only correct the defect in Hageman deficient plasma they also simulate surface activation when added to normal or factor deficient substrates (f) comparable preparations from Hageman deficiencies are inactive and do not contain other clotting factors Differences from (e) must be attributed to the absence of Hageman factor Many similar con

clusions have been reported by investigators using other methods of surface activation (1 4 6)

Other Reactions In addition to the reactions summarized (A) inhib itory and (B) decay and exhaustion phenomena have been observed Such findings could not be ascribed to changes in the Hageman factor alone

(A) *Inhibitors* Ratnoff and Rosenblum (6) found that Hageman de ficient plasma impeded the action of glass on normal intact plasma They showed that the clot promoting effect of a mixture of normal and Hageman deficient intact plasmas was greater when the plasmas were contacted sep arately mixed and tested than when they were mixed contacted and then tested These results were confirmed in an experiment similar in design to theirs but which utilized the PTT rather than the recalcification time *in silicone* The controls and results are shown in Table 2 It can be noted

Table 2

SILICONE PTT RESULTS ON ADDITION OF NORMAL HAG PLASMAS AND MIXTURES OF EACH (INTACT AND CONTACTED) TO INTACT NORMAL SUBSTRATE PLASMA *

	Test Mixture	*PTT in seconds*
I	Normal Intact	260
II	Normal Contacted	67
III	Hag Intact	>1200
IV	Hag Contacted	>1200
V	Equal parts I and IV	257
VI	Equal parts I and II	94
VII	(Equal parts I and III) contacted **	157
VIII	Equal parts II and IV	117
IX	(Equal parts I and IV) contacted	106

0 05 ml test mixture + 0 05 ml intact normal substrate plasma
** Equal parts of each mixed then contacted and subsequently tested

(*cf* I V) that no essential change in clotting time resulted when contacted Hageman deficient * plasma was mixed with intact normal and tested However marked shortening was noted (*cf* I VI) when contacted normal was mixed with intact normal If the intact normal and Hageman deficient plasmas were mixed and then treated (VII) considerably less clot promot ing effect resulted (*cf* VI VII) The inhibition noted in VII was distinctly less when normal and Hageman deficient plasmas were contacted sepa rately before mixing and testing (VIII) The inhibition similarly was mini mal when normal intact plasma and contacted Hageman deficient were mixed then contacted and tested (IX)

The *inhibitory* phenomenon was studied in another way Again the PTT in silicone was used for testing however the tests were performed directly

* Plasma from patient L C proved to have Hageman trait (8)

on the plasma mixtures rather than on mixtures plus intact substrate plasma as in the previous experiment Normal plasma (100 Hageman factor concentration) was diluted with Hageman deficient plasma (assumed to contain 0% Hageman factor) to make mixtures containing 100 90 75 50 25 and 10% Hageman factor Three experiments were performed in the first which served as a control both intact normal and Hageman deficient plasmas were treated separately then mixed and tested (Fig 1 1) in the

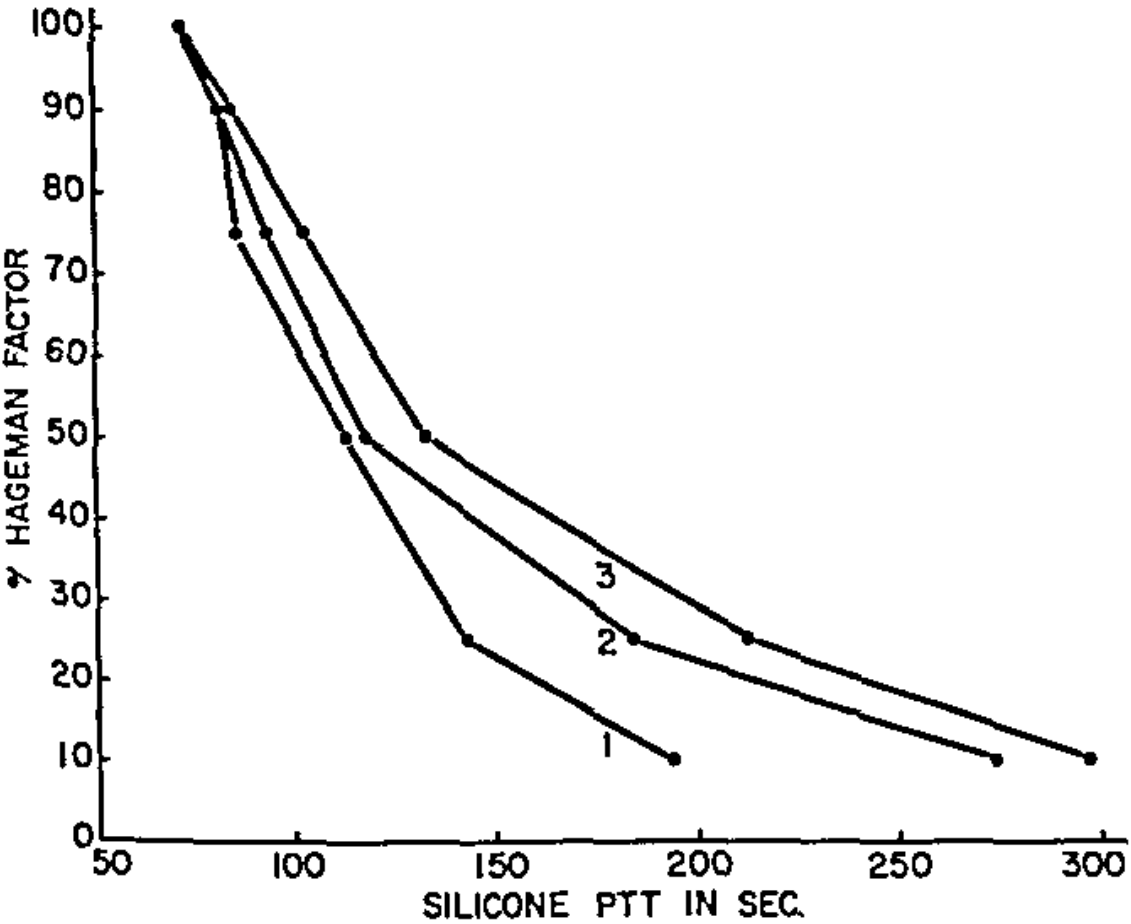


FIG 1 Enhancement of Inhibition of Normal Hageman factor activation by incubation prior to contact of various proportions normal and Hageman deficient plasmas

se ond and third the intact plasmas were mixed and incubated at 25 C for one and ten min respectively then glass contacted and tested (Fig 1 2 3) When compared with nonincubated samples the samples incubated for 10 min begin to show apparent inhibition at 50% Hageman factor concentration The one min samples in contrast do not show significant inhibition until the Hageman factor concentration is 25% Thus further evidence of an inhibitor in Hageman deficient plasma is shown It seemed apparent however that the inhibitor is a relatively weak one which is potentiated to some degree by incubation

(B) *Decay and Exhaustion* It was observed that contacted plasma removed from further surface contact underwent lengthening of clotting time on incubation This was described previously (4 6) Ratnoff and Rosenblum suggested that the destruction of clot promoting activity was enzymatic Margolis called the phenomenon decay and attributed it to an antagonist in whole plasma In later work, however Margolis (13 15) suggested that PTA *depletion was responsible for the clotting time prolongation* Since Hageman factor and PTA are said to survive heating at 56°C for 30 min (6 16) normal and Hageman deficient plasmas treated in this manner should contain respectively Hageman and PTA or only PTA Tests on heated plasma confirmed that AHF PTC AcG prothrombin proconvertin Stuart and fibrinogen were not present in measurable concentrations It was confirmed that Hageman factor survives this treatment

Table 3

PTT RESULTS ON ADDITION OF NORMAL AND HAG PLASMAS (INTACT AND HEATED) TO DECAYED PLASMA

	Test Plasma	*PTT in seconds*
I	Normal Intact—Immediate	92
II	Normal Intact—2 hrs incub	93
III	Normal Contacted—Immediate	67
IV	Normal Contacted—2 hrs incub *	137
V	Decayed + Saline	136
VI	Hag Intact + Normal Intact **	99
VII	Decayed + Normal Intact	84
VIII	+ Hag Intact	129
IX	+ Normal Heated ***	172
X	+ Hag Heated ***	250

* Decayed Plasma
Hag Intact PTT = 745
*** 56 C for 30 min

Normal plasma exposed to standard glass contact was removed from further contact and incubated in silicone at room temperature for two hours thus becoming decayed normal plasma Decayed plasma contained normal concentrations of prothrombin proconvertin AcG and Stuart by specific assays and corrected the abnormal PTT of AHF and PTC deficient plasmas Therefore no apparent change had occurred in these factors as a result of activation and decay of normal plasma The results of normal and Hageman deficient plasma (both intact and heated) addition to decayed normal are shown in Table 3 Normal intact plasma (I) corrected the defect in both the Hageman-deficient (VI) and decayed (VII) samples However the addition of intact Hageman deficient plasma (VIII) was little better than saline (V) since neither showed significant correction of the abnormal PTT of the decayed sample (IV) Serious doubts that decay is due to PTA depletion are apparent when it is noted that heated

Hageman deficient plasma (X) also fails to correct decayed plasma as well as normal heated (IX) On the basis of these data it would seem logical that following surface activation of plasma decay is the result of Hageman factor inactivation These results are in essential agreement with those of Ratnoff and Rosenblum (6) who further suggest that the mechanism is one of enzymatic destruction

Exhaustion was produced by repetition of the activation decay sequence several times (4) or by prolonged (8 24 hours) contact with the activation surface In addition to depletion of Hageman factor other effects were diminution of prothrombin and fibrinogen (17) as well as the labile factors (AcG AHF) At present there is no suitable simple explanation for the complex changes associated with exhaustion of normal plasma

Discussion

Some of the complex reactions that follow exposure of plasma to surface contact can be explained by currently available data It seems well established that the initial response to surface is dependent upon the Hageman factor since in its absence clot promoting activity is not developed (1 5 8)

It is reported (6) that inhibition of activation results when uncontacted normal and Hageman deficient plasmas are incubated together prior to surface contact In contrast to our earlier work (8) which did not employ incubation tests the present study confirms and extends this Since inhibition is noted on incubation and is potentiated thereby additional consideration is needed The action of the inhibitor is relatively weak and is overwhelmed readily by increased Hageman factor concentration (8) This inhibitor has been demonstrated in Hageman deficient plasma and it has been shown that inhibition of normal plasma does not result until the concentration of Hageman deficient plasma is present in excess relative to normal plasma (Fig 1)

Most of the above inhibitor is removed by surface contact (Table 2 IX) thus another explanation must be presented for the decay phenomenon This may be due to some agent that progressively inactivates Hageman factor (6) or to depletion of another clotting factor With the possible exception of PTA thought to have a secondary role (10 13 15) other clotting factors do not participate in the surface reactions described A partial defect in development of clot promoting activity in PTA deficient plasma has been reported (5) but others (6 7) fail to confirm this Some studies (13 15) suggested that PTA depletion was responsible for decay Data obtained by indirect experiment (Table 3) fail to confirm PTA depletion but indicate that progressive inactivation of the Hageman factor could be responsible Since the crucial experiment of correction of decayed plasma

by PTA deficient plasma is unreported it is not confirmed that PTA is involved in decay

On the basis of the results shown it seems possible to postulate that two inactivating mechanisms are related to the Hageman factor One tends to oppose the activation of Hageman factor and is removed by contact The other does not appear to be affected by contact but progressively inactivates or destroys the active Hageman factor produced These findings are in essential accord with those of Ratnoff and Rosenblum (6)

At the present time the relationship of these surface reactions to the clotting mechanism is obscure The inhibitors are incubation dependent and weak Surface contact removes the very inhibitor which tends to oppose the activation of Hageman factor Progressive inactivation of the Hageman factor is slow Knowledge concerning the chemical nature quantitation and possible relationships to other clotting inhibitors such as antithrombin is fragmentary Until such time that more data is available and the inhibitors better characterized it seems preferable to consider Hageman factor activation as the result of surface contact and inhibitory phenomena as related processes The demonstration that such are related to *in vivo* fluidity or clotting of blood must await convincing experimental evidence

Summary

Significant recent advances have been made in studies of the surface activation of plasma These have been shown to include Hageman factor *activation and two inhibitory mechanisms Other clotting factors* do not appear to participate in the described phenomena A connection between the observed reactions and the blood clotting mechanism remains to be demonstrated by future investigations

Addendum

Since the preparation of this manuscript the work of Waaler (18) has been reported He found that Hageman and PTA deficient plasmas only slightly corrected exhausted plasma In addition a defect in formation of activation product was apparent in both plasmas Thus it would seem that contrary to our data PTA may be implicated in surface contact activation of plasma Since the conditions of Waaler s studies differ from those presented here perhaps it is possible to infer that *in the decay* of activated plasma Hageman diminution occurs early and is followed by PTA disappearance later Thus sequential reactions would seem to occur It remains for studies with materials from PTA deficient patients to resolve the current dilemma

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by PTA deficient plasma is unreported it is not confirmed that PTA is involved in decay

On the basis of the results shown it seems possible to postulate that two inactivating mechanisms are related to the Hageman factor One tends to oppose the activation of Hageman factor and is removed by contact The other does not appear to be affected by contact but progressively inactivates or destroys the active Hageman factor produced These findings are in essential accord with those of Ratnoff and Rosenblum (6)

At the present time the relationship of these surface reactions to the clotting mechanism is obscure The inhibitors are incubation dependent and weak Surface contact removes the very inhibitor which tends to oppose the activation of Hageman factor Progressive inactivation of the Hageman factor is slow Knowledge concerning the chemical nature quantitation and possible relationships to other clotting inhibitors such as antithrombin is fragmentary Until such time that more data is available and the inhibitors better characterized it seems preferable to consider Hageman factor activation as the result of surface contact and inhibitory phenomena as related processes The demonstration that such are related to *in vivo* fluidity or clotting of blood must await convincing experimental evidence

Summary

Significant recent advances have been made in studies of the surface activation of plasma These have been shown to include Hageman factor *activation and two inhibitory mechanisms Other clotting factors* do not appear to participate in the described phenomena A connection between the observed reactions and the blood clotting mechanism remains to be demonstrated by future investigations

Addendum

Since the preparation of this manuscript the work of Waaler (18) has been reported He found that Hageman and PTA deficient plasmas only slightly corrected exhausted plasma In addition a defect in formation of activation product was apparent in both plasmas Thus it would seem that contrary to our data PTA may be implicated in surface contact activation of plasma Since the conditions of Waaler s studies differ from those presented here perhaps it is possible to infer that in the decay of activated plasma Hageman diminution occurs early and is followed by PTA disappearance later Thus sequential reactions would seem to occur It remains for studies with materials from PTA deficient patients to resolve the current dilemma

Methods

The technique of continuous flow of electrophoretic fractionation (9 10) and most of the blood coagulation methods (11) have been previously described Glass factor was assayed by adding 0 05 ml material to 0 2 ml normal silicone plasma in silicone treated 12 x 75 mm tubes and recalcify ing with 0 2 ml of 0 025 M CaCl Controls for tests on whole plasma contained 0 05 ml silicone normal plasma while those for fractions con tained 0 05 ml barbital buffer (pH 8 6) All fractions were obtained and studied in this buffer Concentration of glass factor was semi-quantitated by comparison with the ability of various dilutions of normal glass treated plasma to reduce the recalcification time and improve the prothrombin consumption Glass treatment of plasma was carried out by exposing the plasma to pyrex glass wool in a glass tube for 20 min at room temperature The continuous flow of electrophoretic procedure involved exposure of the plasma to glass and paper surfaces and no increased activation of fractions was obtained on additional glass treatment

Results

Glass Factor in Whole Plasma Platelet poor plasmas from 3 patients with deficiencies of AHF 3 of PTC 1 of proaccelerin 1 of Stuart factor and 1 of Factor VII * showed normal glass factor content as did $BaSO_4$ treated normal plasma Plasmas from two patients suffering from Hageman factor deficiency showed complete absence of glass factor Plasma from PTA (plasma thromboplastin antecedent) deficient patient † when fresh showed markedly reduced glass factor activity and also a similar reduction in Hageman factor After frozen storage these abnormalities disappeared

Glass Factor in Electrophoretic Fractions Normal Plasma Glass factor was assayed in 20 experiments on normal plasma In every case the greatest activity was obtained in the γ globulin area Fig 1 illustrates a typical experiment Paper strip electrophoresis of each tube fraction showed γ globulin in tubes 1 13 β in 14 15 α in 16 $\alpha + \alpha_1$ in 17 19 α_1 and albumin in 20 26 Glass factor activity was predominately in the γ area traces extending into the α areas Distribution of Hageman factor activity coincided almost exactly with the distribution of glass factor Substrate for assay of PTA (fresh PTA deficient plasma) was available for only a few experiments In two experiments on normal plasma (not illustrated) the ability of fractions to restore the defect in PTA plasma was found mainly in the γ area with some extension into the β and α areas

* Frozen plasma obtained through the kindness of Dr Henry Kupfer

† Blood was obtained from this patient through the kindness of Dr Robert Rosenthal

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"Glass Factor" Activity in Plasma Fractions Obtained By Continuous Flow Electrophoresis *

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THE clot accelerating effect of glass or other wettable surface has been widely studied and variously attributed to one of two mechanisms removal of an inhibitor by adsorption to glass (or other foreign surface) (1) or activation of a coagulation factor by contact with this surface Most of the evidence favors the latter theory but the exact mechanism is still unclear It has been attributed to changes in platelets (2) anti hemophilic factor (AHF) (3) plasma thromboplastin component (PTC Christmas factor) (4) proconvertin (5) and Hageman factor (6 7 8)

Employing the technique of continuous flow electrophoresis it was possible to separate plasma into 32 fractions in which the protein content was distributed according to the familiar γ β α α_1 and albumin pattern These fractions were studied for their content of the various coagulation factors Glass factor was the term used to indicate an activity present in a particular fraction or in whole plasma which when added to normal blood or plasma prepared and clotted in silicone treated containers shortened the coagulation or recalcification time and improved prothrombin consumption

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* This investigation was supported in part by a research grant (H 2254) from the Division of Research Grants of the National Institutes of Health United States Public Health Service Figures 1 2 and 3 are reprinted from *J Clin Invest* by permission of the Editor

Hemophilia A and B Figs 2 and 3 illustrate experiments on AHF and PTC-deficient plasmas The hemophilia A plasma showed complete absence of AHF in all fractions while that from the PTC deficient patient showed a lack of PTC in all fractions Distribution and concentration of glass factor

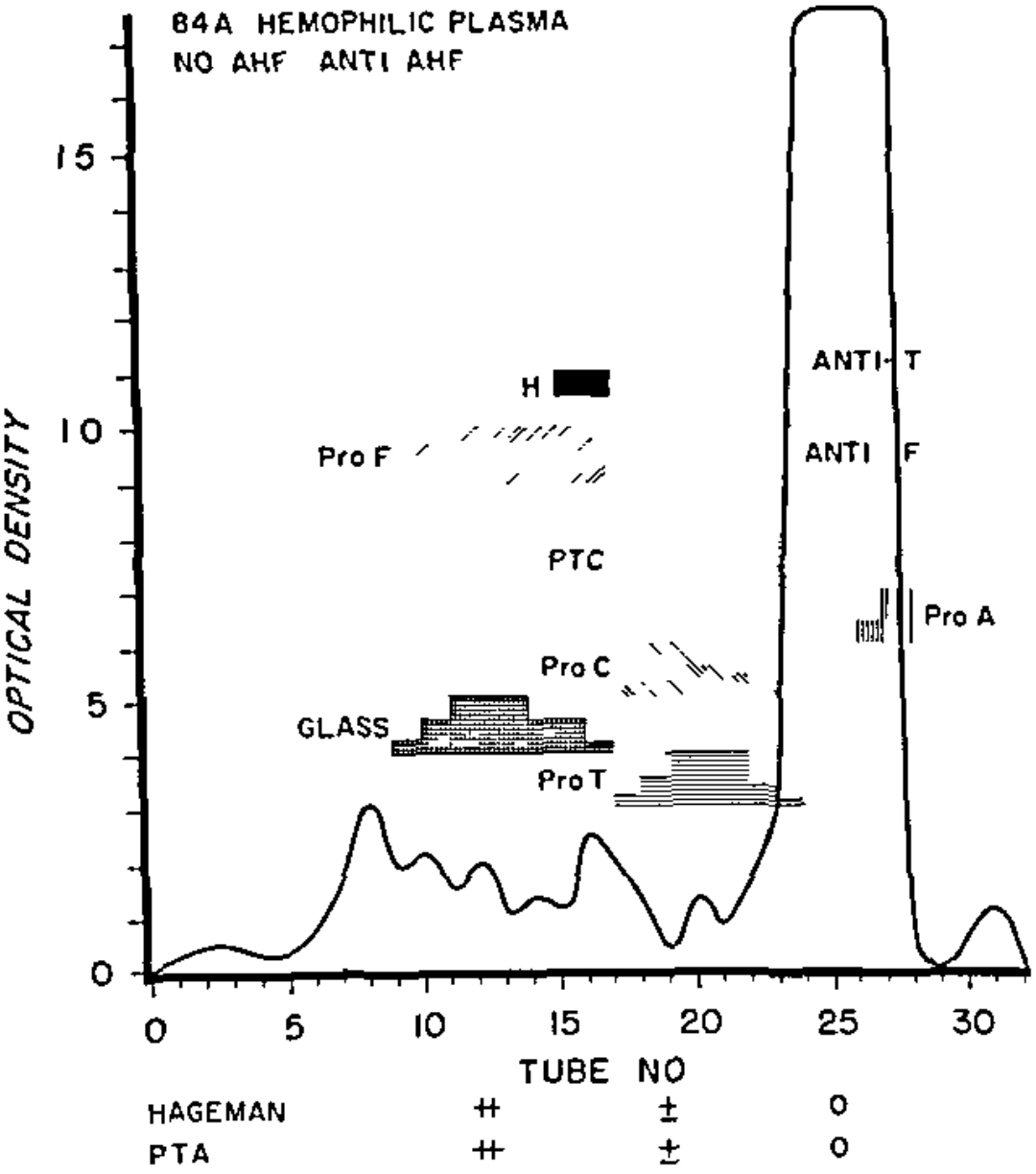


FIG 2 Distribution of Coagulation Factors after Continuous Flow electrophoresis of hemophilic plasma

and other coagulating factors appeared normal Hageman factor and PTA were present in the γ area as noted at the bottom of the figures

Hageman Factor Deficiency Continuous flow electrophoretic fractionation has been carried out on plasmas from two patients deficient in Hageman factor Fig 4 illustrates one experiment No Hageman factor or glass factor was found in any of the fractions In addition none of the fractions

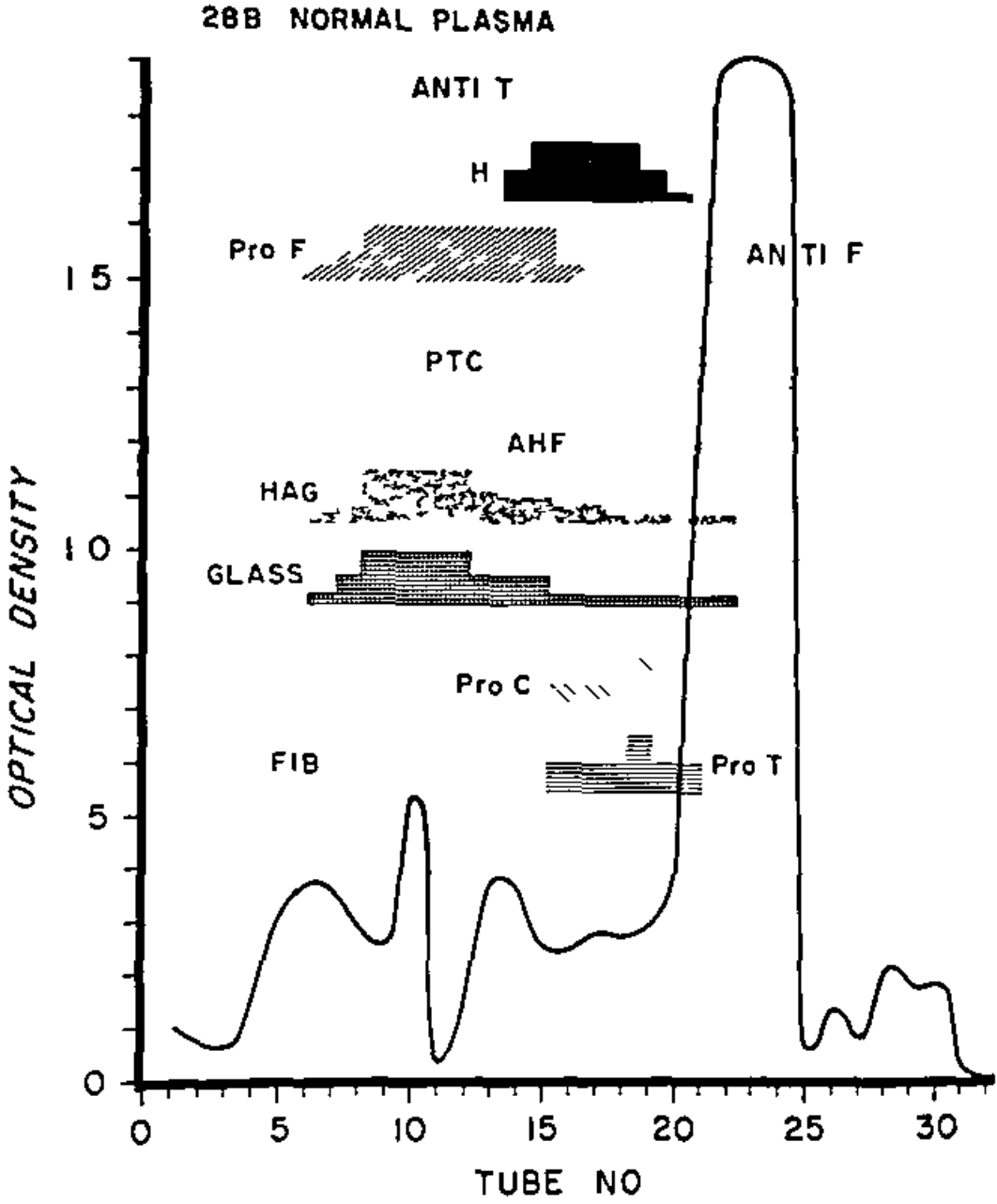


FIG 1 Distribution of Coagulation Factors after Continuous Flow electrophoresis of normal plasma The protein concentration of each tube (plotted as optical density) is related to the tube number on the abscissa as a continuous curve

Coagulation factors assayed FIB (fibrinogen) GLASS (glass factor) HAG (Hageman factor) ProF (profibrinolysin) ProT (prothrombin) ProC (proconvertin Owren method) AHF (antihemophilic factor) PTC (plasma thromboplastin component) Anti F (antifibrinolysin) H (*heparin cofactor*) Anti T (*antithrombin*) ProA (proaccelerin) Anti B (isoagglutinin)

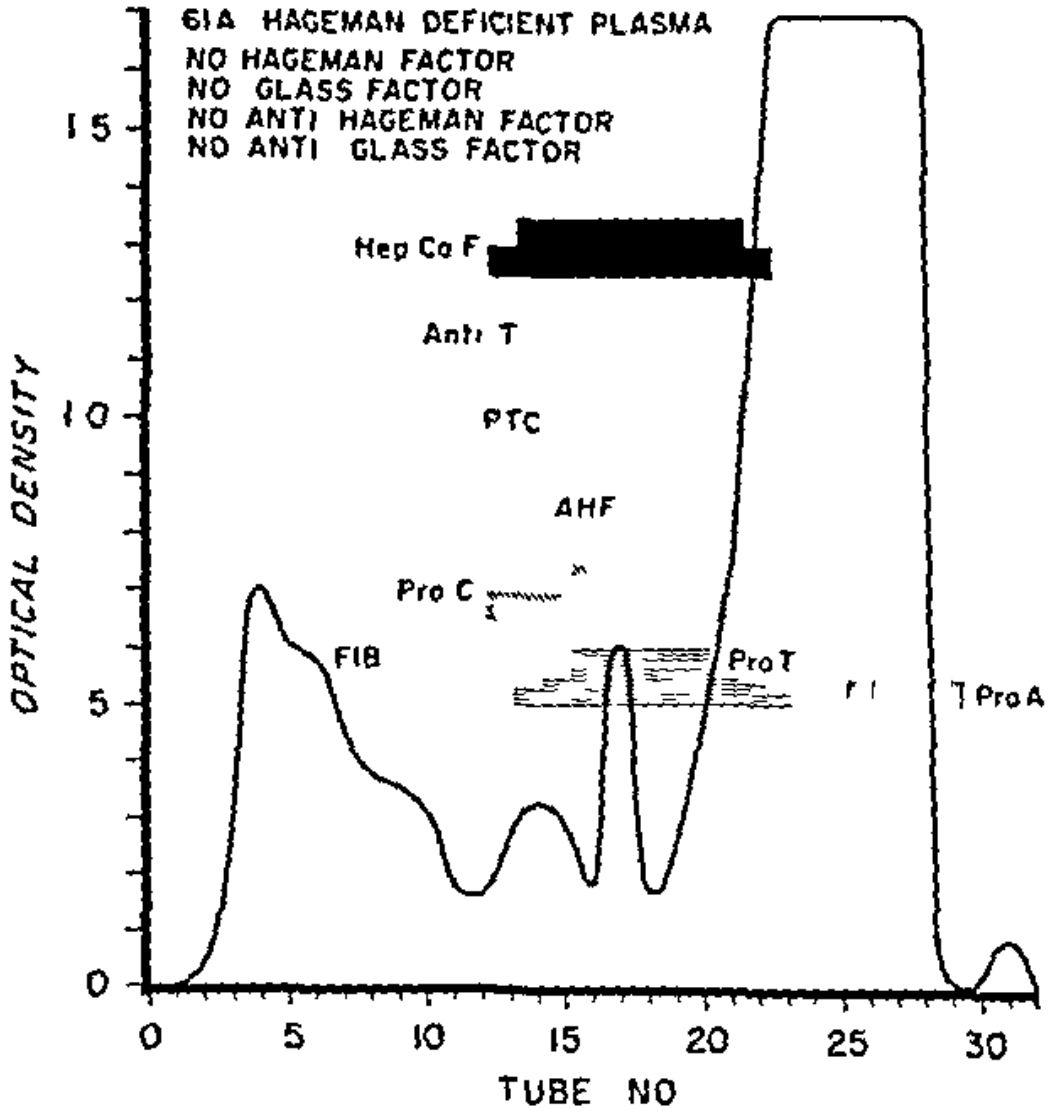


FIG 4 Distribution of Coagulation Factors after Continuous Flow electrophoresis of Hageman factor deficient plasma

Discussion

These studies confirm the previous findings of Shafrir and de Vries (6) that plasma "glass factor activity is independent of the content of AHF PTC proconvertin proaccelerin or platelets These authors and others (7 8) have suggested that glass factor and Hageman factor may be similar or identical

These studies pre ented in this paper suggest that glass factor is closely related to both Hageman factor and to PTA The three activities appear in the γ globulin fractions of normal hemophilic and PTC-deficient plasmas Hageman trait plasma is devoid of glass factor activity both in the whole plasma and the fractions Fresh PTA-deficient plasma and its fractions are partially deficient in both glass factor and Hageman factor activity Frozen

was able to inhibit a fraction from normal plasma which contained high concentration of glass and Hageman factors Distribution and concentration of other coagulation factors appeared normal

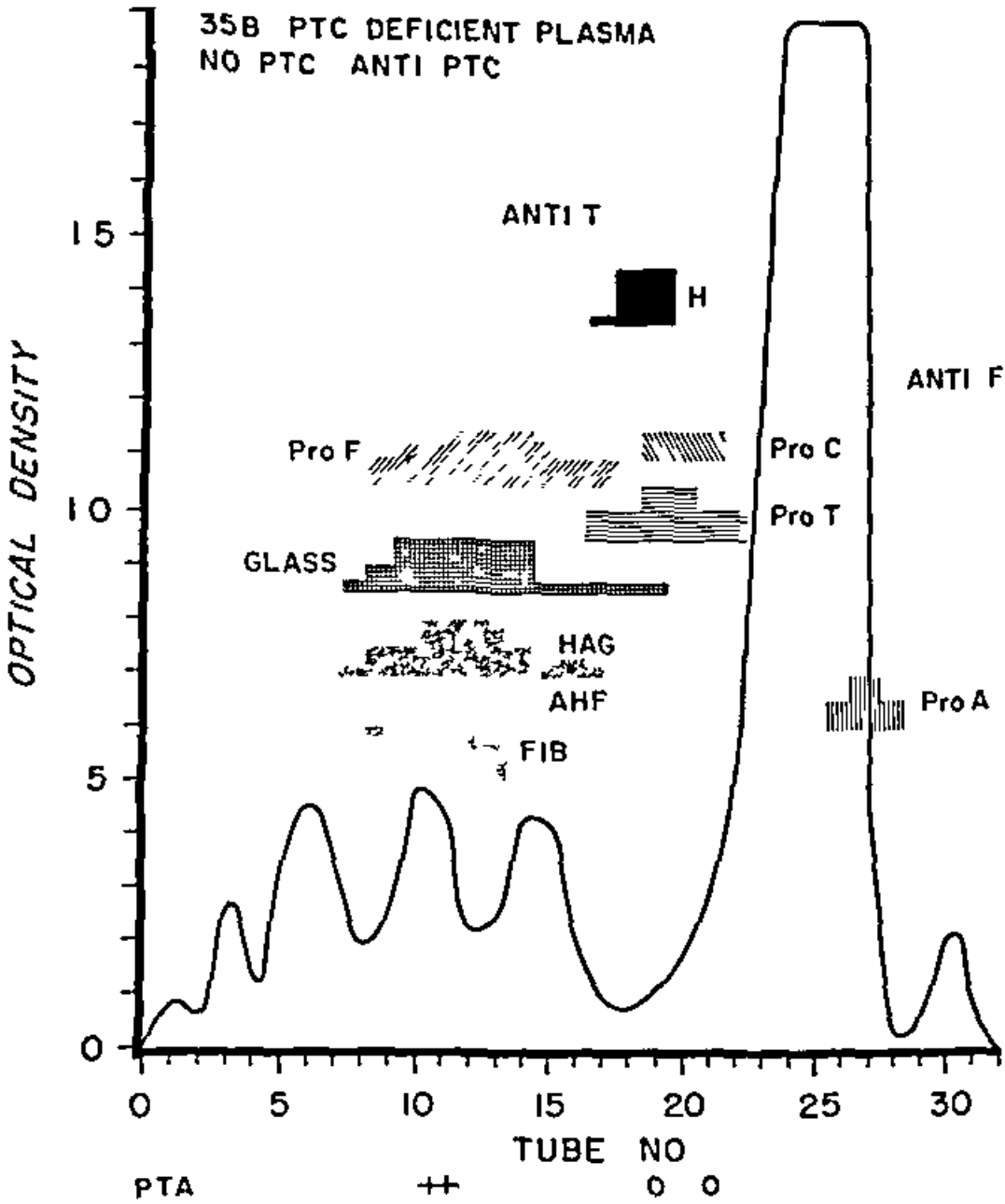


FIG 3 Distribution of Coagulation Factors after Continuous Flow electrophoresis of PTC deficient plasma

PTA Deficiency Fig 5 illustrates an experiment on PTA deficient plasma Only traces of glass factor and Hageman factor could be found in the fractions These traces appeared in the γ area In the albumin area there were 3 fractions which showed the ability to inhibit partially normal glass factor activity Distribution and concentration of other factors appeared normal

in the albumin area Inhibition of normal glass factor could not be demonstrated in the fractions prepared from Hageman-deficient plasma or from normal plasma

Summary

1 An activity called glass factor capable of shortening the recalcified clotting time and improving the prothrombin consumption of normal plasma prepared and clotted in silicone treated containers was found in glass treated plasmas from patients with deficiencies of AHF PTC proaccelerin Stuart factor and Factor VII Hageman deficient plasma was devoid and PTA-deficient low in this activity

2 On continuous flow fractionation glass factor was identified primarily in the γ globulin fractions of normal hemophilic and PTC-deficient plasmas It was absent in the fractions from Hageman factor deficient plasma and low in those from PTA-deficient plasma In addition a few fractions in the albumin area of the PTA plasma showed the ability to inhibit normal glass factor activity

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storage of PTA plasma appeared to correct these deficiencies This may account for the apparent discrepancy with the findings of Ratnoff and Margolius (8) who state that PTA deficient plasma is as effective as normal plasma in shortening the recalcified clotting time of Hageman deficient plasma These authors also noted that Hageman deficient plasma was only equivocally effective in correcting the clotting abnormality in blood deficient in PTA

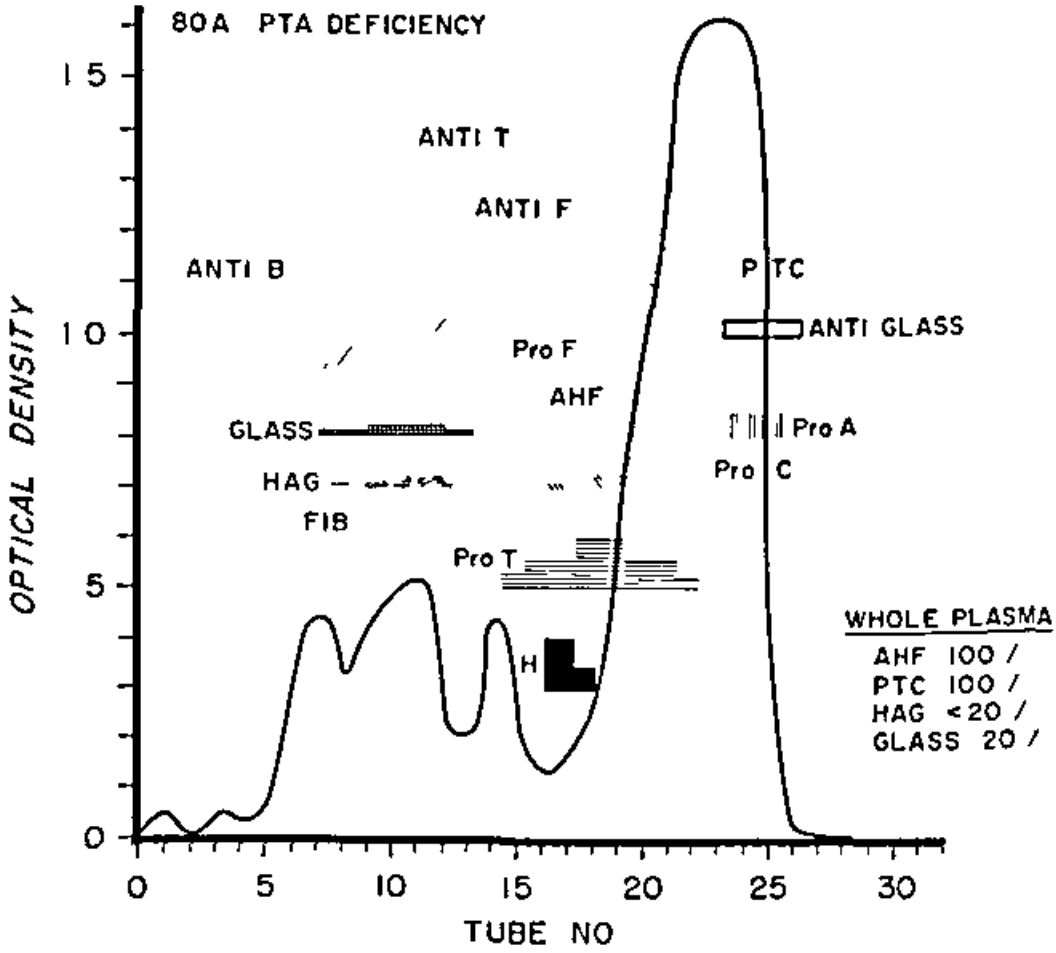


FIG 5 Distribution of Coagulation Factors after Continuous Flow electrophoresis of PTA deficient plasma

In spite of many *in vitro* similarities (abnormal prothrombin consumption and thromboplastin generation both corrected by either serum or adsorbed plasma low glass factor activity) Hageman trait and PTA deficiency are strikingly different in clinical course Hemorrhagic tendency is present in PTA deficiency (12) but completely absent in Hageman trait (8) Thus it must be assumed that Hageman factor is unnecessary for physiological hemostasis or that its action is by passed (? by a tissue component) *in vivo*

The continuous flow fractionation of PTA plasma showed not only a partial deficiency of glass factor but also an inhibitor to this activity present

globulin (1 5 13) By a combination of these methods HF has been freed from other known coagulation factors with the exception of plasma throm boplastin antecedent (PTA) which resembles HF in physical properties and is also closely linked to it functionally (8 14 15)

Whether HF is a distinct substance or a property of a group of sub stances it is by definition something that is present in normal plasma and absent from the plasma of persons with Hageman trait Experimentally HF can therefore be treated as a functional unit and referred to as a factor so long as the operational definition of this term is remembered

It is now generally agreed that HF is responsible for the initiation of clot ting by contact with foreign surfaces among which glass is one of the most active (7 8 10 12 14 15 16 17) In addition to clotting contact with glass induces in plasma a chain of other events which result in the liberation of pharmacologically active substances These include a polypeptide plasma kinin which produces pain and contracts smooth muscle (18) and a globulin permeability factor (19 20) Like coagulation all these reactions are also dependent on the presence of HF (15 21) We are therefore in the fortunate position of being able to study the functions of HF by four independent indicator systems namely coagulation contraction of smooth muscle pain and capillary permeability

In the following pages I shall review the evidence showing that HF is the key component in these plasma/surface reactions and attempt to establish connections between these phenomena

HF in The Initiation of Blood Coagulation

Absence of Contact Activation in HF Deficiency Since Lister (22) showed that coagulation in shed blood is initiated by contact with abnormal surfaces this phenomenon was the subject of many investigations (24 27) Additional information was gained more recently by the use of indirect methods in which test samples of plasma etc were treated with glass and recalcified after addition to or serial dilution in intact normal plasma (indicator plasma) in siliconed tubes (16 28 29 30) This procedure per mitted the study of the effects of contact on incomplete clotting systems such as protein fractions or pathological plasma and gave a rough quantitative estimate of the changes The results of two such independent studies (28 29 30) were in close agreement in that they excluded known clotting factors other than HF and PTA as the components responsible for glass activation The close similarity between the glass reacting fraction of normal plasma and the fraction containing HF pointed to this factor as being the surface sensitive agent (30) Experimental confirmation of this hypothesis was later obtained by applying the indirect activation test to the plasma from patients deficient in the factors in question (8 14 15) Table 1 shows the results of such tests (haemophilia and Christmas disease are included for com

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The Role of Hageman Factor in Plasma/Surface Reactions

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HAGEMAN trait (1) is a congenital coagulation defect probably inherited as an autosomal recessive character (2) Although the condition is very uncommon an increasing number of well authenticated cases have been described (3 10) since the original report by Ratnoff and Colopy (1) and there can be no doubt that it is now a well defined clinical entity The characteristic feature of Hageman trait is the gross coagulation defect *in vitro* in the absence of any haemorrhagic tendency Addition of a very small portion of normal plasma to the patient s blood either in the test tube or *in vivo* by transfusion is sufficient to correct the defect (1 4) This excludes circulating anticoagulants as a possible explanation of the abnormality and indicates a true deficiency of a coagulation factor It is not certain whether Hageman factor (HF) is a single substance or a property shared by a group of plasma proteins (4 11) So far only partial separation of HF has been attained by a combination of various fractionation procedures Thus a concentrate of HF has been obtained by adsorption onto kaolin of 30 40% saturated (NH_4) SO_4 fraction of serum previously adsorbed with $BaSO_4$ and heated to 56°C The kaolin was then eluted with (NH_4) SO_4 and the crude HF precipitated at pH 5 2 (12) HF also appears to be distributed in Cohn s fractions II III and IV By electrophoretic methods it has been identified in the region between beta and gamma

* Working under the tenure of an Australian and New Zealand Life Insurance Medical Research Fellowship

components could react in parallel that is each could be independently activated by contact or in succession one only being directly affected by glass and subsequently reacting with the other factor An experiment designed to test these alternatives is shown in Table 2 When a mixture of HFdp and PTAdp was activated with glass normal clot promoting activity was produced (allowing for the 1 2 dilution of the reacting components) When however these plasmas were treated with glass in separate tubes and mixed afterwards there was no mutual correction Evidently in the presence of the glass surface a time-consuming reaction takes place between HF and PTA and this is in keeping with the second alternative i e that of a successive reaction between glass HF and PTA The more

Table 2

EVIDENCE FOR AN INTERACTION BETWEEN HF AND PTA

	Glass Activated Plasma					
Test	*(a) Normal*	*(b) Hageman Trait*	*(c) PTA Deficient*	*(d) Mixture in Equal Parts of (b) & (c)*	*Normal Intact Plasma*	*Clotting Time (sec)*
1					0 4	770
2	0 2				0 2	155
3		0 2			0 2	9 0
4			0 2		0 2	390
5		0 1	0 1		0 2	570
6				0 2	0 2	240

(Adapted from Biggs *et al* (8) Reproduced by courtesy of *Brit J Haemat*)

Samples of test plasma were rotated for 10 min with an equal weight of ballotini and then added to normal intact plasma in siliconed tubes All mixtures were recalcified in parallel with 0 1 ml of 0 1 M CaCl containing lysed platelets (cf Table 1)

Note the mutual correction of HF and PTA deficiency when the plasmas were mixed first and then activated (test 6) and absence of correction when the samples were activated first and mixed afterwards (test 5)

pronounced defect in HFdp as compared with that of PTAdp suggests that HF and not PTA is the component initially affected by glass surface

Consumption" of PTA in the Contact Reaction When contact with glass is interrupted the clot promoting activity of normal plasma decays Repeated or prolonged exposure of plasma to a sufficient glass surface leads to an irreversible decrease of its clotting ability (29 30) By testing the corrective effect of such exhausted plasma on the clotting of pathological samples it is possible to determine which of the clotting factors have been depleted Table 3 shows such an experiment using the Kaolin clotting time (31) as the test system The results show that normal plasma which has been exposed for 30 min to glass ballotini providing a surface area of about 200 cm /ml has lost all its ability to correct PTA deficiency but that there was no significant loss of HF antihaemophilic globulin or Christmas

parison) It will be seen that HF deficient plasma (HFdp) does not re spond to contact at all PTA deficient plasma (PTAdp) shows a partial defect while the remaining samples behave normally The abnormal results were not due to anticoagulants because the patients plasma added to the indicator system (tests 2 and 6) did not inhibit the response to normal contacted plasma From these data supported by thromboplastin genera

Table 1

INDIRECT CONTACT ACTIVATION TESTS ON PATHOLOGICAL PLASMA (CLOTTING TIMES IN SECONDS)

		Patient				*Normal*			
Test	*Test Plasma*	*Activated*			*Intact*	*Activated*			*Intact*
	Concentration of test plasma in the mixtures with indicator plasma	1/1 *	1/3	1/9	1/3	1/1 *	1/3	1/9	1/3
1	Hageman trait	4000+	1380	1140	1200	205	265	360	1300
2	Hageman trait †					220	260	330	1320
	PTA deficiency								
3	Case 1	720	380	500	900	175	245	350	770
4	Case 2	420	465	645	1200	200	250	375	1360
5	Case 3	480	400	560	780	175	245	350	720
6	Case 3 †					180	255	360	800
7	Haemophilia	2400+	240	300	660	160	250	360	800
8	Christmas disease	1200	185	275	765	135	185	270	750

(Adapted from Biggs *et al* (8) Reproduced by courtesy of *Brit J Haemat*)

Citrated test plasma was rotated for 20 min with 1 g/ml of glass ballotini (0 1 mm diam) and serially diluted in intact normal plasma in stli oned tubes 0 2 ml volumes of the mixtures were recalcified at 37 C with 0 05 ml of 0 1 M CaCl containing lysed platelets (30) Each pathological sample was tested in parallel with normal plasma The differences between the clotting times of various normal samples are mainly due to variations in the strength of the platelet extract used on different occasions

* Undiluted test sample

† Instead of normal plasma a mixture of equal parts of patient s and normal plasma was used in the first dilution tube (1/3) in order to exclude the presence of anti coagulants (see text)

tion studies we concluded that HF is the component which is primarily affected by contact with glass PTA also being required for the evolution of a full clot promoting effect (8 14) The findings of other investigators are in agreement with these conclusions (7 10 11 12 16)

Interaction between HF and PTA HF and PTA are distinct clotting factors since plasmas with these deficiencies are mutually corrective Thus it is evident that at least two components must be concerned in the formation of the clot promoting agent under the influence of contact with glass These

These results cannot be attributed to artefacts because glass coated by HFdp or by plasma heated above 60°C (in which HF is denatured) or by eluate from beads treated with these preparations does not activate HFdp

The adsorbed HF is not consumed in the contact reaction Glass surface after having been coated with HF can be used again and again for the

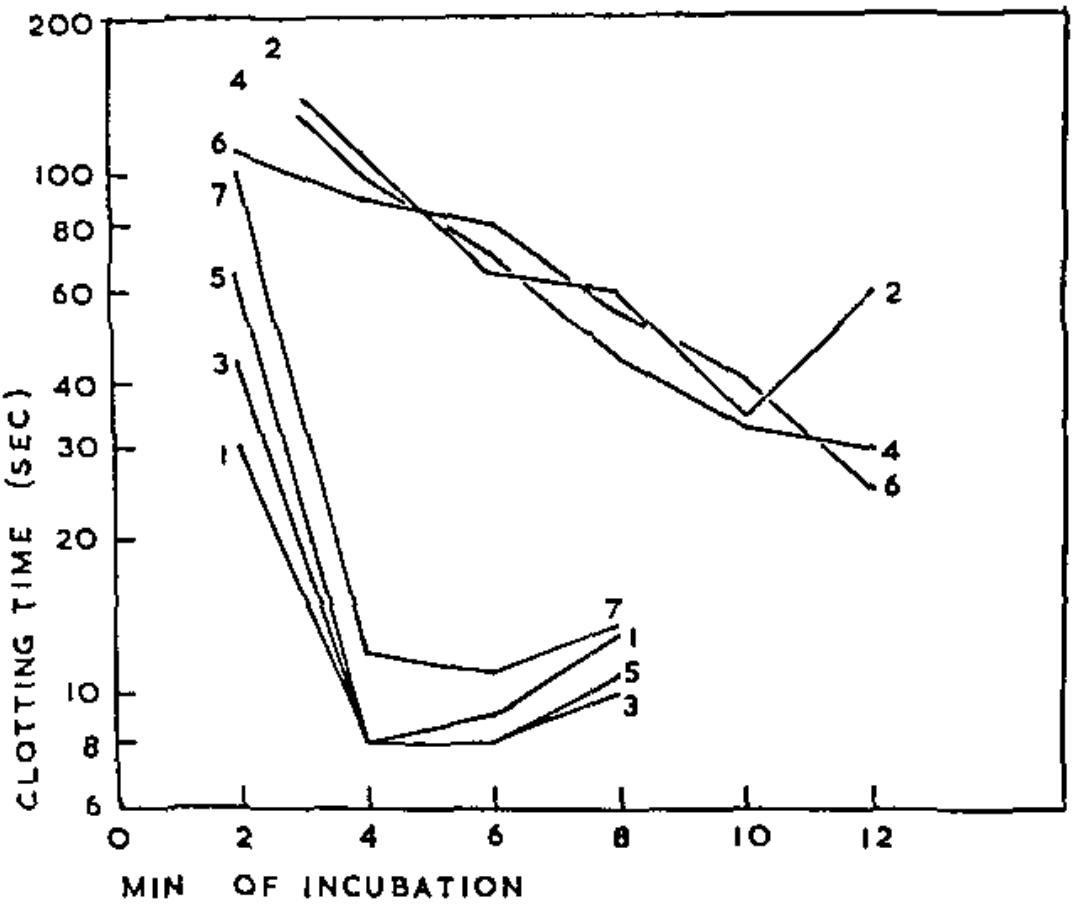


FIG 1 Thromboplastin Screening Test (17 32) in Variously Treated glass tubes [Reproduced in part by courtesy of *Brit J Haemat* (17)] Curve 1) normal plasma curves 2) 7) HF deficient plasma

Treatment of glass tubes 1) and 2) untreated 3) treated with normal plasma and washed 12 times with saline at pH 7 4) as 3) but washed 9 times at pH 11 and then 3 times at pH 7 5) treated with $Al(OH)_3$ adsorbed plasma 6) treated with plasma heated to 60 C for 20 min 7) treated with eluate from ballotini shaken with normal plasma (see text)

activation of successive samples of HFdp (17) which indicates that once it has been adsorbed HF remains on the surface where it acts catalytically This is further supported by the fact that a very small quantity of HF is sufficient to attain the object In the experiments as in Fig 1 normal plasma diluted to 1 100 with saline is as effective as whole strength plasma (17) Evidently HF beyond that necessary to saturate the available surface is without further effect

factor What is still more noteworthy is that HFdp is not depleted of PTA by treatment with glass which is a clear indication that PTA cannot be utilized in the absence of HF The situation is analogous to the lack of prothrombin consumption in haemophilic blood and these results strongly support the view that HF is activated by glass in the first instance PTA only becoming involved at the next stage of the reaction

Attachment of HF to the Glass Surface The observations mentioned above still do not tell us what happens to HF in glass contacted plasma It has been assumed hitherto that HF is somehow activated at the surface and then returns into solution in its active form (12 14 15 16) Our recent work

Table 3

CONSUMPTION OF PTA BY CONTACT WITH GLASS

Exp	*Pathological Plasma (0 2 ml)*	*(Citrated Saline)*	*Added Plasma (0 5 ml)* *Normal* 0 G	30 G	*Hageman Trait* 0 G	30 G
1	PTA-deficient	220 (85) *	115	210	125	122
2	Hageman trait	1000 (90)	110	115		
3	Haemophilia	340 (90)	120	125		
4	Christmas disease	360 (95)	100	98		

Kaolin clotting time (15 31) (sec) on mixtures of plasma 0 2 ml of pathological plasma 0 05 ml of added plasma and 0 05 of kaolin suspension (40 mg/ml) was incubated for 2 min at 37 C 0 1 ml of lysed platelets (approx 5 000/cmm) and 0 1 ml of 0 05 M CaCl were then added in this order

* The figures in brackets denote clotting times of normal plasma tested in parallel 0 G denotes intact plasma 30 G denotes plasma rotated for 30 min with 1 gm/ml of ballotini (see text) and then removed and incubated in siliconed tubes for two hours at room temperature

(17) shows that this view is probably incorrect All evidence leads to the conclusion that the activated HF remains fixed to the surface and initiates further steps of coagulation in this form without itself returning into the plasma This inference is based on the following observations

After a brief treatment of glass surface with plasma or fractions containing HF the glass induces fully normal coagulation in HFdp This property persists after washing with 10 or more changes of saline and it can be detected by the indirect activation test or by any of the conventional tests of clotting function Fig 1 illustrates the phenomenon using a modification of thromboplastin screening test (17 32) The adsorbed material can be eluted from the glass by alkali (pH 10 11) and after neutralizing with HCl it can be reapplied to fresh glass (curves 4 and 7)

correct the PTA deficiency while HFdp is maximally corrected only if glass surface is also supplied (17)

This work is still not completed but the above findings may have a bearing on the assay of purified HF preparations It is probable that purified HF in solution is best assayed by determining its clot promoting effect in the presence of glass The activity in siliconed containers may well be due not to HF but to activated PTA or still other impurities The fact that a similar preparation made from HFdp may be inactive is a necessary but not a sufficient control in these experiments This point may well be illustrated by a more familiar analogy Thrombin for example will shorten the clotting time of haemophilic plasma As a corollary little thrombin can be recovered during spontaneous clotting of haemophilic blood Yet prothrombin is not the factor which is lacking in haemophilia It may therefore be premature to attempt final purification of HF until adequate criteria by which its activity can be measured have been established

HF in the Release of Plasma Kinin

One unsatisfactory feature in the study of the early stages of blood coagulation is that the only observable change fibrin formation is separated from these stages by a complicated chain of intervening reactions which are still only partly understood

In the foregoing experiments an attempt was made to overcome this difficulty by treating these intermediate stages as a functional unit Nevertheless it is obviously highly desirable to get a cross bearing on the inferences drawn from coagulation by using an independent indicator system Such an indicator was provided by the discovery by Keele and his associates of a pharmacologically active substance which appears in plasma as a result of brief exposure to a glass surface (34) This phenomenon was attributed to the release of a polypeptide [pain producing substance later renamed plasma kinin (15)] probably related to kallidin (35) and bradykinin (36) Plasma kinin produces pain when applied to an exposed blister base and stimulates smooth muscle contraction in isolated organ preparations (34)

Activation of plasma kinin like that of the clot promoting activity can be brought about in cell free plasma in the presence of citrate oxalate versene or heparin it is provoked by the same types of surface and is unaffected by treatment with $Al(OH)_3$ and $BaSO_4$ or by heating the plasma to 56°C but prevented by preheating to 60°C (15 34)

Before attempting to draw further correlations between these two sets of reactions it will be necessary to say something about the sequence of changes leading to the release of the kinin

Two Stages in the Release of Plasma Kinin When plasma is brought into contact with glass kinin activity rapidly reaches a peak and then de

Attachment of PTA to the Glass Surface Glass coated with normal plasma has a corrective effect on PTAdp in the thromboplastin screening test (it does not correct haemophilic or Christmas disease plasma) When however HFdp is used as the coating agent the glass does not improve the clotting ability of PTAdp Similarly glass coated with PTAdp (or plasma artificially exhausted of PTA) does not correct PTAdp although it corrects HFdp (17) We interpret these findings as evidence of PTA also becoming attached to the glass surface This attachment is however dependent on the previous adsorption of HF by the surface and it cannot take place in the absence of HF This hypothesis is represented diagrammatically in Fig 2

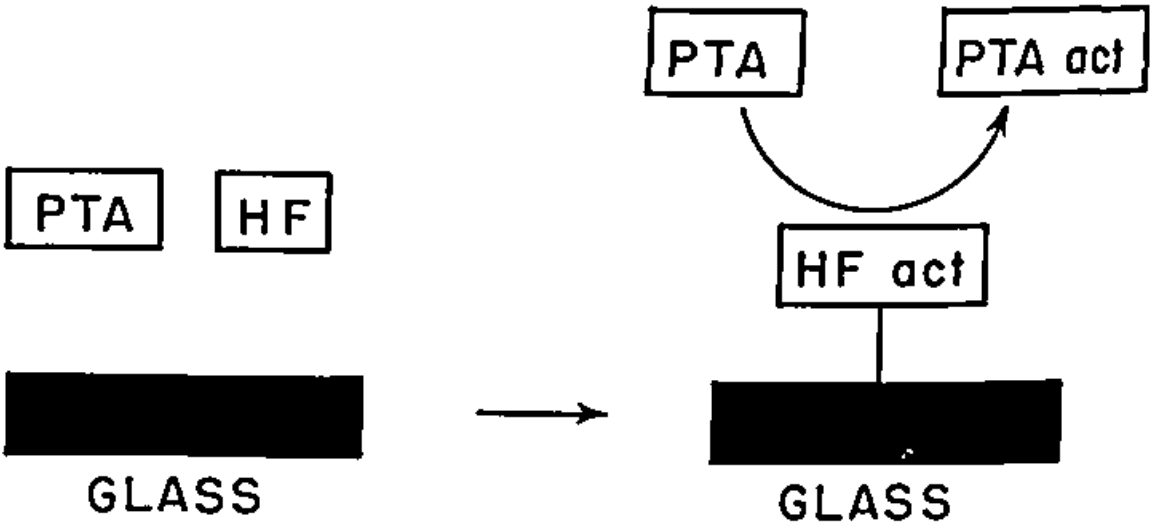


FIG 2 See Text

Clot Promoting Properties of HF Eluate As was stated before the substances adsorbed onto glass can be eluted at a pH 10 11 which agrees with the results of the study of electrokinetic properties of protein coated glass particles (33) This phenomenon is reversed by lowering the pH to about 8 without the loss of the clot promoting effect of the adsorbed components Since adsorption and elution are useful steps in the isolation of HF (12) it is clearly important to know something about the state in which HF is present in the eluate and also about the presence of contaminating substances

Our preliminary observations (17) suggest that

1) HF reverts to the precursor form in solution

2) The active agent(s) in the eluate which accelerate coagulation of plasma in siliconed tubes is not HF but products of the next stage of activation (e g activated PTA) Thus eluate from glass coated with whole normal plasma induces rapid clotting in HFdp both in glass and in siliconed vessels and also corrects the deficiency of PTAdp When plasma exhausted of PTA is used as the coating material the eluate does not

to adequate glass surface (e g 200 cm²/ml for 30 60 min) By using graduated exposure to glass it is therefore possible to remove component B only or both A and B

The products of both stages of the contact reaction (i e activated component A and plasma kinin) are rapidly destroyed by inactivators present in the albumin fraction of plasma (15 37)

The details of experimental evidence for the postulated mechanism of kinin release have been described elsewhere (15) In Fig 3 the interpretation of these findings is presented diagrammatically The scheme served as working hypothesis on which further experiments were based

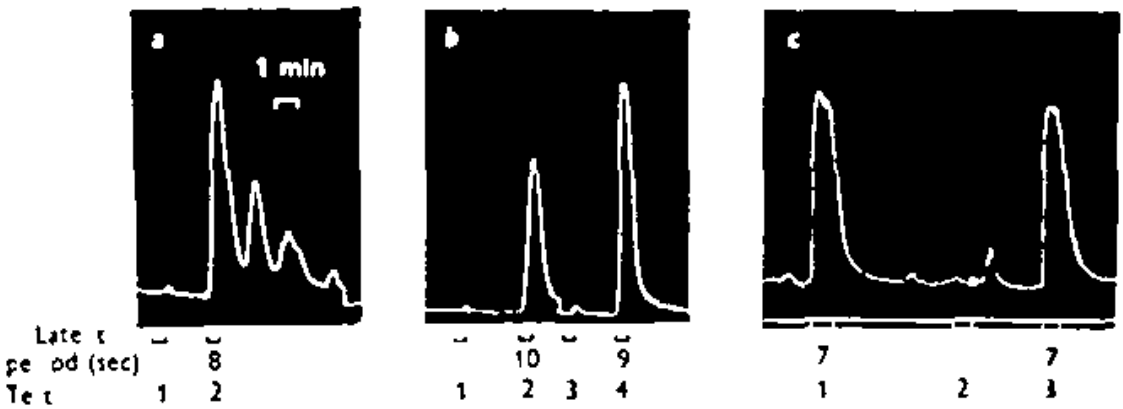


FIG 4 Absence of Surface Activation in HF Deficient Plasma [Reproduced by courtesy of *J Physiol* (15)] Rat uterus

(a) *Direct tests* (0 1 ml applied to the uterus) 1) Patient s plasma rotated for 2 min with glass ballotini 2) Normal plasma treated as in 1) (b) *Indirect tests* 0 1 ml of glass treated (G) samples added to 0 2 ml intact plasma 0 1 ml of the mixture tested after 2 min incubation 1) patient s plasma (G) + normal intact 2) B depleted normal plasma (G) + patient s intact 3) B depleted normal (G) control tested directly as in (a) 4) B-depleted normal (G) + normal intact (c) *Plasma activated* with kaolin (10 mg/ml) for 1 min 0 1 ml tested directly as in (a) 1) Normal 2) patient s 3) normal

Latent period = interval between the application of test samples and the commencement of response

Release of Plasma Kinin in HF Deficient Plasma (HFdp) The analogy between the changes involved in kinin release and the inception of clotting suggested that both are initiated by a common mechanism The behavior of HFdp would obviously provide a crucial test of this supposition Fig 4 (a and c) shows that HFdp did not release kinin when treated with glass or even with kaolin which is more active than glass in these reactions When tested by two stage methods (Fig 4b) HFdp served as a normal source of component B (test 2) but appeared to lack component A (test 1) These observations were further confirmed using the pain test on an exposed blister base as the indicator (15)

clines Exposure of such plasma to fresh glass does not produce more kinin The same final result is achieved by mixing glass activated and intact plasma in siliconed tubes and allowing the mixture to stand for 2 hours or more The disappearance of the capacity to form plasma kinin can be explained by the exhaustion of a limiting factor which is referred to as component B

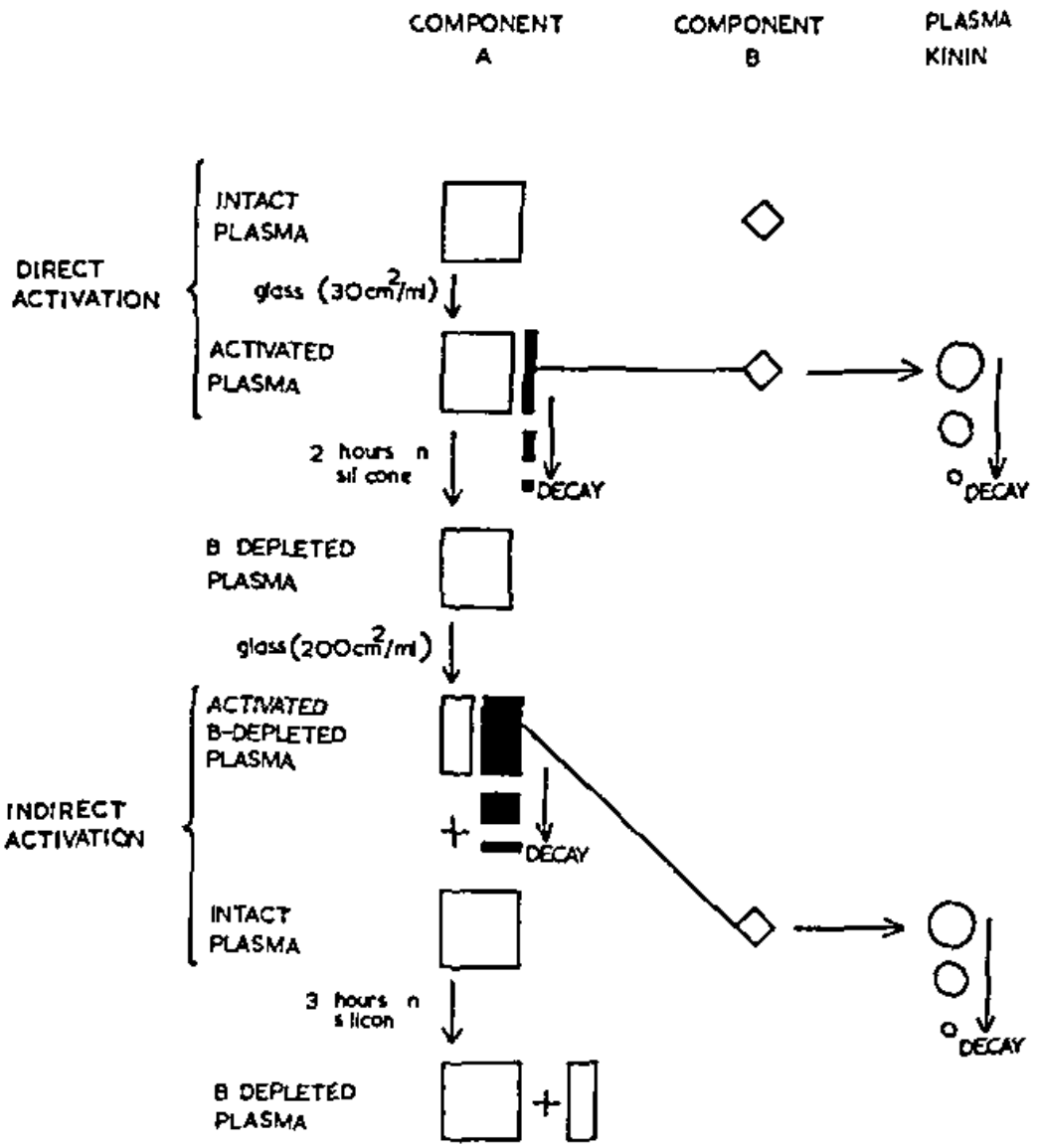


FIG 3 The Postulated Steps in the Release of Plasma Kinin Note the two different ways by which plasma depleted of component B can be prepared The shaded areas represent activated component A

(15) When such B depleted sample is treated with a glass surface and then added to intact plasma new kinin is liberated after a brief interval (in direct activation) Evidently another factor present in B-depleted plasma is activated by contact and then reacts with the component B supplied by the intact plasma This agent is referred to as component A (15) Com ponent A is not as labile as B but it too is exhausted by sufficient exposure

1) Glass surface coated with preparations containing HF induces normal activation of successive samples of HFdp (*Fig 5*) The observations on elution and readsorption of HF already described in connection with blood coagulation also apply to the release of plasma kinin (41)

2) Although neither HFdp nor plasma depleted of component A can be activated by glass a mixture of the two behaves like normal plasma Hence HF and component A cannot be identical but must be complementary As could be expected from similar experiments on coagulation the two plasmas correct each other only when exposed to glass together but not when they are contacted separately and mixed afterwards

3) Glass coated with A-depleted plasma activates HFdp but glass treated with HFdp does not activate plasma depleted of component A

4) Components A and B cannot be removed from HFdp by treatment with glass as they are in normal plasma Even after prolonged exposure to a large surface HFdp can be fully activated provided the HF is supplied on the glass whereas normal plasma after the same treatment can no longer be activated

Thus the behavior of HF in kinin release follows exactly the same pat tern as in blood clotting

The changes involving components A and B on the one hand and PTA on the other are also alike This is however where the analogy between the two systems ends The release of kinin was found to be entirely normal in the plasma from a case of PTA deficiency (15 38) PTA cannot there fore be equated with any of the components of the kinin forming system the two chains of reactions having already diverged at an earlier stage (*Fig 7*)

HF and the Activation of a Permeability Factor (PF) in Plasma

Another effect of the contact reaction is the activation of an agent which increases capillary permeability (20) There are reasons to believe that this is the same as the previously described PF which is activated by dilution of plasma or serum (19 21 40) but their identity or otherwise are not directly relevant to the present argument

The increase in permeability can be measured in subjects blued by intravenously given Evans blue (T 1824) intradermal injections of the test preparations give rise to local blue lesions whose size and intensity are taken as the index of activity

The results of animal experiments (20) suggested that the PF is another product of the chain of reactions which initiate clotting and release plasma kinin It could therefore be expected that observations on HFdp would be similar to those in the other two systems Preliminary testing of HFdp on guinea pigs gave inconclusive results due to species incompatibility the ex

The Site of Action of HF in Kinin Formation The glass activated component A (formerly referred to as contact factor) was implicitly defined as the first detectable *soluble* product of the contact reaction Since HF in its active form was at the time also thought to be present in solution it was a plausible hypothesis that the two were identical (15)

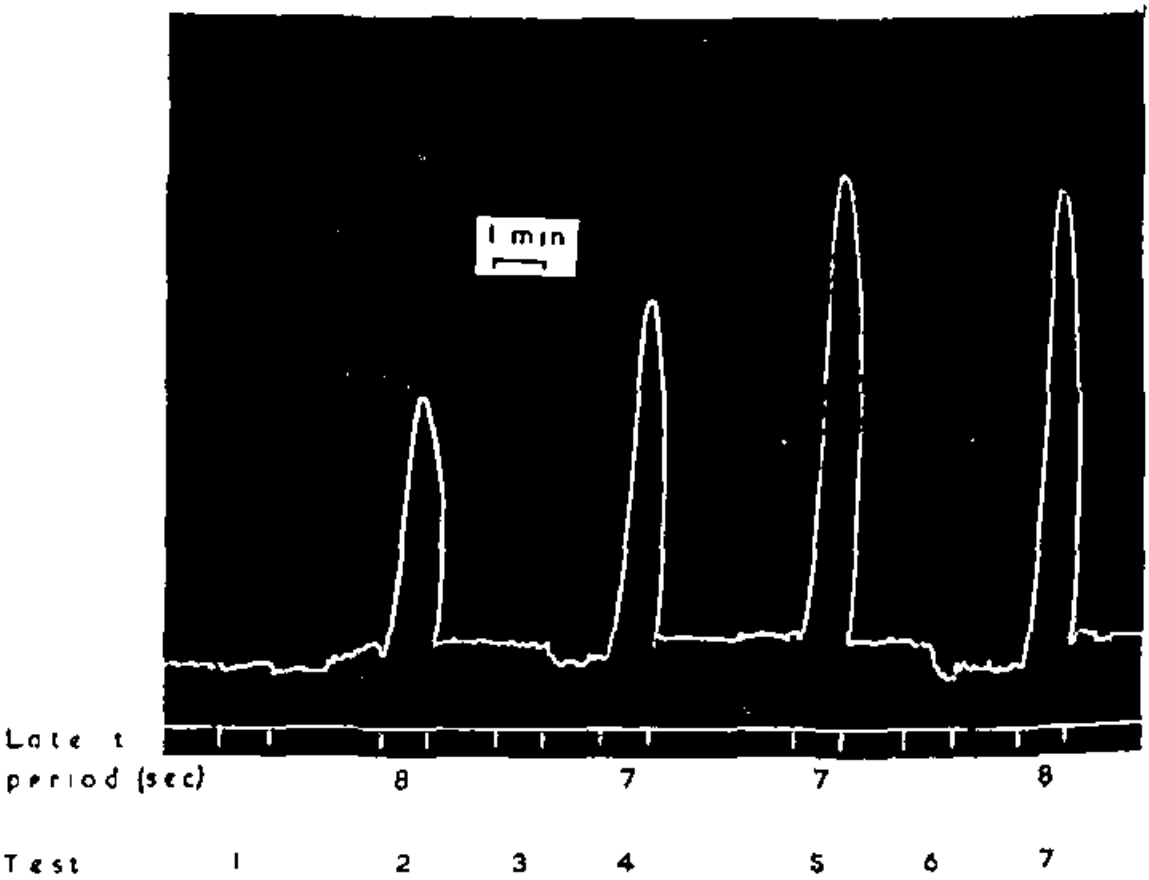


FIG 5 Attachment of HF to Glass Surface Normal Plasma was First depleted of component A by rotation for 40 min with 1 g/ml of glass ballotini (0 1 mm diam) Fresh beads were then treated with this plasma and used for the activation of four successive samples of HFdp The glass treated test samples were tested on the rat uterus as in Fig 4b

Between the tests the beads were washed with 5 10 changes of saline at pH 7 *Test samples* 1) A depleted plasma untreated ballotini 2) HFdp ballotini from test 1) 3) HFdp untreated ballotini 4) HFdp ballotini from test 2) 5) HFdp ballotini from test 4) 6) HFdp untreated ballotini 7) HFdp ballotini from test 5)

Further work however showed that as in coagulation so too in kinin formation HF appears to act only in the adsorbed state (41) It is not identical with component A but activates the latter in the next stage of the reaction Hence the absence of component A activity in HFdp

These conclusions are based on the results of experiments conducted along the same lines as those with blood clotting

cursors for the activation of the PF (39) it is a reasonable guess that HF may be an essential constituent in the early humoral phase of certain foreign body reactions

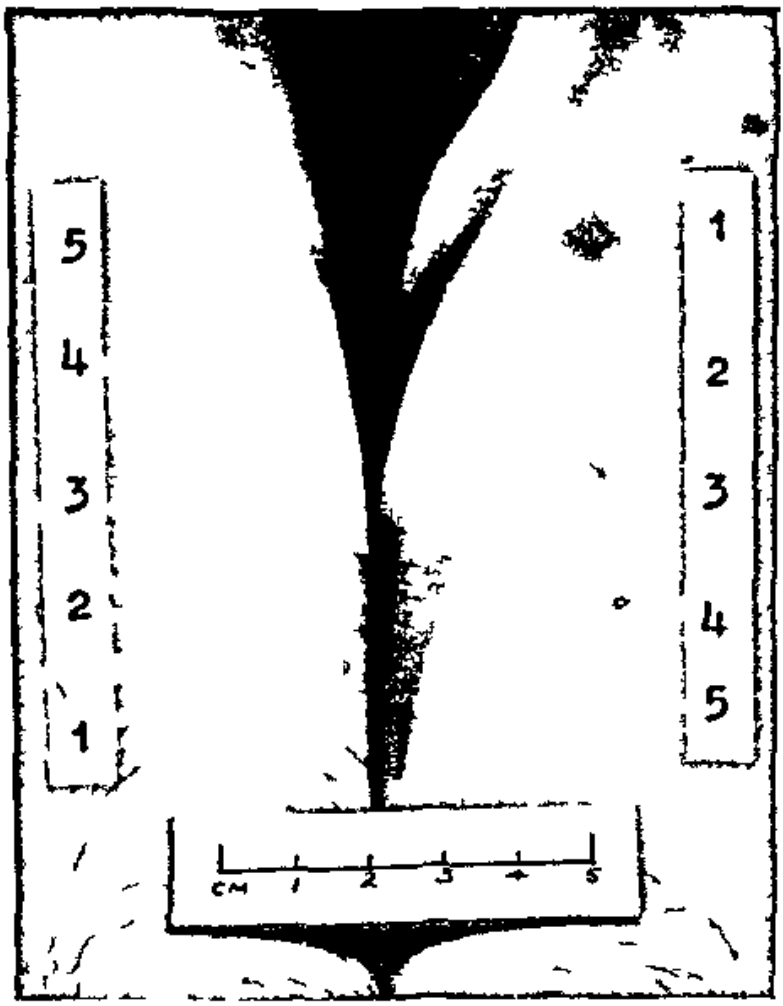


FIG 6 HF and Capillary Permeability [Reproduced by courtesy of *Austral J Exp Biol* (21)] Intradermal injections of 0 1 ml of plasma (diluted 1 4) Photographed 25 min after the administrations Duplicate tests 1) HFdp treated with glass which was coated with normal plasma 2) as 1) but treated with clean glass 3) normal A depleted plasma treated with clean glass 4) mixture in equal parts of 2) and 3) treated with glass together 5) as 4) but without the glass treatment

Dilution with saline was here introduced for convenience only (21) It is not an essential step in the procedure

Relations between the Surface Induced Reactions

In considering the connections between the phenomena described above we must remember that so far these reactions have been studied only by biological indicators and that little if anything can be said about the chemical changes involved It is because of this that the components taking part in them are referred to as factors or agents rather than as sub

periments were therefore repeated on human subjects where this difficulty did not arise (21)

The findings were parallel to those described in the previous section PF was activated by contact with glass in whole or in B depleted plasma and also after heating the plasma to 56°C but not after heating to 60°C

The most pertinent observations are shown in Fig 6 It can be seen that HFdp did not produce any reaction when treated with clean glass but could form PF with glass previously coated by HF Plasma depleted of component A was also inactive As in kinin formation there was a mutual correction when HFdp and A depleted plasma were activated together but not when the two were exposed to glass in separate tubes and mixed after removal from contact with glass

We may conclude from these results that HF and probably also component A are necessary for the activation of the PF and that they interact in the same way as they do in kinin formation HF combining with the glass surface and then activating component A

A possible reservation is that the same procedures which exhaust component A in the plasma also result in the disappearance of PTA The only sure way of excluding PTA as a possible component in the reaction would be to repeat these experiments with plasma from PTA deficient patients However the time relations of the rise decay and exhaustion of PF are more like those seen in the reactions leading to kinin release than in those concerned with clotting (15 20 37) which suggests that the PF is more directly related to the kinin forming system than to blood coagulation

Formation of PF *in vivo* Intradermal injections of finely divided materials which are capable of activating the plasma in the test tube (e g glass silica kaolin) produce a local increase in permeability This effect can be demonstrated in blued guinea pigs or rats or in human subjects For obvious reasons only a small number of human experiments have been attempted these were performed without the injection of blue dye but even so the results were sufficiently uniform to warrant at least tentative conclusions

The injection of kaolin (0 25 0 5 mg in 0 1 ml of saline) into the skin of the forearm is followed by a well defined reaction The original wheal persists or increases during the next 2 hours or so A raised reddened lesion which resembles a positive tuberculin reaction then develops and remains for about 2 days The final stage is a persistent fibrotic nodule

One such experiment was performed on a subject with Hageman trait (15) In this patient there was no trace of the early edematous reaction to kaolin the onset of the second stage (erythema and infiltration) was delayed but the final result (fibrosis) was the same as in normal subjects

These observations must of course be confirmed on other individuals with Hageman trait before accepting them as a specific feature of HF deficiency but since the interstitial fluid contains all the necessary pre

Physical Aspects of Contact Activation

We can only form a tentative picture of what changes take place in HF in contact with the glass surface It seems most likely that adsorption of HF is accompanied by some structural changes in the protein molecules which expose catalytically active groups The observations suggesting that on elution HF reverts to its precursor state support this view

Some authors attempted to explain the action of glass as being due to freeing the HF from inhibition (12 16) This implies that glass surface adsorbs an inhibitor and not HF but an inhibitor on the surface has never been demonstrated and is incompatible with the present findings It is possible that some of the observations which suggested the inhibitor theory may be explained by the presence in the plasma of inert substances which compete with HF for the adsorptive sites on the surface (17) For instance glass bathed with HFdp or with plasma preheated to 60°C loses much of its activity in inducing the foreign surface reactions (17 37) If however the glass has been previously in contact with normal plasma the treatment with these preparations is no longer effective (cf Fig 5) Evidently which ever substance (HF or inert material) gets to the surface first becomes adsorbed and cannot be easily displaced

The second and more fundamental aspect of the problem is why such surfaces as those of glass silica or kaolin are vastly more active than those of organic silanes polyethylene $BaSO_4$ carbon metallic Al and many other foreign materials (15 30 38) while healthy biological surfaces are apparently entirely inactive or even inhibitory

If the initial step of contact reactions is an adsorption (? orientated) of HF then the biological activity of foreign surfaces may depend on their selective affinity to HF or alternatively on a relative lack of affinity to inert blocking substances which compete with HF for the adsorptive sites

Systematic investigation of physically well specified surfaces is yet to be attempted but among the scattered empirical data the following observa tions seem to be particularly significant

1) Gamma Al_2O_3 which is inactive in plasma acquires considerable activating ability after heating above 1200 C (14 38) Though it is known that the heat treatment leads to partial or complete conversion of gamma to alpha form of alumina this cannot be the whole explanation Thus fused alumina (alpha form) is active after ignition but loses this property on grinding (37) which certainly does not alter its crystalline structure

There is a close correlation between these observations and the changes in the electrokinetic behavior of variously treated alumina Freshly ground corundum (alpha Al_2O_3) carries a positive zeta potential in water After ignition to 1200°C the charge becomes negative but it can be again re versed by regrinding (42)

stances A factor may be a single substance or on the other hand it may become resolved into a more or less complex system However legitimate conclusions based on experiments designed to treat a factor as a functional unit should still remain substantially valid when that unit proves to be composed of several constituents

In Fig 7 an attempt is made to summarize the interactions between the various factors involved in the contact reaction This scheme is a further

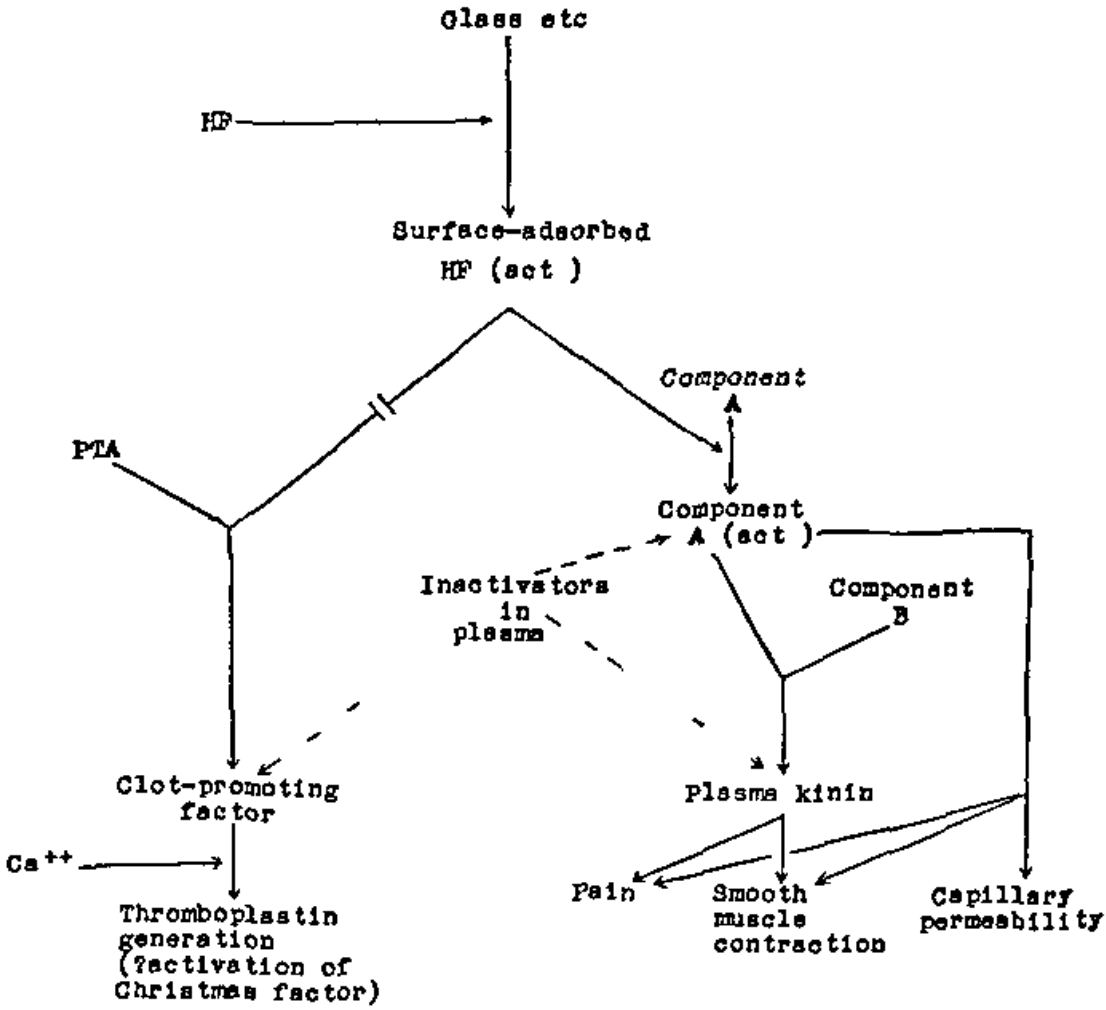


FIG 7 Relations Between the Various Effects of the Contact Reaction Solid lines indicate activation Interrupted lines indicate inactivation

development of the former ones (14 15) The previously employed term contact factor is not used in Fig 7 because the functions attributed to it can now be assigned to at least three components (HF PTA and component A) If the term is to be mentioned at all it can no longer be used in its previous sense but only as a synonym of HF

While it is most unlikely that the present scheme is the final one the key role of HF in this system appears to be firmly established This is important because it provides a short cut for testing any further extension of the hypothesis in studying plasma/surface reactions

We can therefore only guess at the possible consequences of a deficiency with respect to these agents Pain vasodilatation and edema are together with coagulation among the basic features of inflammation (45) These phenomena can certainly be reproduced by the substances which appear in the surface activated plasma But even if these are important physiological mediators of the inflammatory response we must not jump to conclusions that persons with Hageman trait should be necessarily at a disadvantage in this respect The agents in question can be produced in plasma in other ways [e g under the influence of proteolytic enzymes snake venoms bacterial toxins (15)] which do not require the presence of HF The most that can be said is that individuals with Hageman trait are lacking in the initial humoral mechanism of reactions to foreign surfaces Among these silica is probably the most important because of its high potency in activating the HF and its universal distribution in the environment It is conceivable that such a mechanism may play some part in dealing with dust which gains entry into the lungs but in normal circumstances this cannot be of great physiological importance otherwise one would expect persons with Hageman trait to have some pulmonary symptoms Under critical conditions these individuals might indeed show signs of diminished tolerance to silica dust but at present we have no evidence that this is so

Though it may be a little tantalizing that such a specific and potentially useful mechanism as the foreign surface reaction seems at present to be of no great physiological significance it is credible that this reaction represents the evolutionary remnants of what may have been once an important biological phenomenon Perhaps a study of the comparative physiology of these reactions may provide an answer to this question

Summary

Contact with glass and certain other surfaces induces in plasma a series of reactions whose end results include coagulation the release of a pain producing and smooth muscle stimulating polypeptide (plasma kinin) and the activation of a permeability factor All these changes are initiated by the adsorption of Hageman factor (HF) onto the surface and its activation during that process Remaining attached to the surface HF then interacts with PTA to give rise to clot promoting activity and also activates another agent (component A) which takes part in releasing plasma kinin The activated component A is probably identical with the permeability factor or is essential for its formation

Some physical aspects and biological implications of these plasma/surface reactions are discussed

These observations support the familiar concept that the clot promoting properties of foreign surfaces may be a function of their electronegative potential (23 43)

2) Freshly prepared silica sols are practically inactive in the plasma In contrast colloidal silica of approximately 10 millimicron particle size is a very powerful activating substance (37 38)

This suggests that there is a minimal effective unit of surface area beyond which adsorption of HF cannot take place Since HF is probably a globulin it is indeed difficult to imagine how it could be adsorbed by particles which are smaller than the individual protein molecules

Perhaps this is one of the reasons why blood does not readily clot in contact with biological surfaces Healthy vascular endothelium appears to be covered by a gelatinous cement substance (44) whose molecular structure is probably too open to provide adequate units of adsorptive surface for the large protein molecules

In addition to these two conditions (negative charge and sufficient unit area) there may well be other physical qualities which account for the differences between the biological activity of various surfaces If there are this is still a field entirely open for investigation

Some Physiological and Pathological Implications

Coagulation Disorder Since the complete absence of HF in plasma is compatible with a normal haemostatic mechanism we must assume that HF is not concerned in the arrest of haemorrhage On the other hand it has been suggested (12) that abnormal intravascular clotting may be initiated by changes in the intimal surface which may impart to it actions similar to glass If this hypothesis is correct then it becomes highly probable that the reactions leading to thrombosis require HF for their inception Deficiency of this factor might therefore not only be harmless but could be a biological advantage in that it would minimize the risk of thromboembolic disease As a corollary if a suitable antagonist to HF could be discovered it would be an ideal anticoagulant since it would not interfere with normal haemostasis

Unfortunately there is still no evidence that the assumptions on which this speculation is based are true There are too few HF deficient cases to say whether they are immune from thrombotic disorders There are also other alternatives to the above postulated mechanism of thrombosis It is just as likely that intravascular coagulation may be started by the same reactions as initiate haemostasis where HF does not appear to be involved The question is obviously one of great practical importance and should stimulate much further work in this direction

Deficiency in Other Foreign Surface Reactions The functions of plasma kinins and permeability factors in the body are still a matter for speculation

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Part 5

FACTOR X

Editor s Note

In August 1959 the International Committee for the Standardization of Nomenclature of Blood Clotting Factors recommended that henceforth Factor X apply to Stuart factor only It should be understood that the Factor X activity discussed so well by Dr Koller and Dr Spaet in the fol lowing chapters does not refer to Stuart factor *per se* but rather to the special clot promoting activity of serum described as Factor X by Dr Koller and his associates It appears at this time that Factor X activity as originally outlined was due to two or more entities one of which was probably Stuart factor

As newer knowledge and conventions develop our terminology must adapt to changes It is during the transition period that confusion is likely to result So again it is emphasized that the term Factor X as used in this volume is not used in the sense proposed by the International Committee

24

What Is the Present Status of Factor X?

F Koller
Zurich Switzerland

THE name Factor X was proposed by us in 1953 in order to designate a new serum clotting factor (besides Factors VII and IX) This designation is provisional until the International Committee for the Standardization of Nomenclature has decided for what clotting factor the Roman numeral X has to be reserved

The evidence for the existence of Factor X is based on matching experiments with different pathological sera in the thromboplastin generation test (Table 1)

Our conclusions were 1) Factor VII plays no significant role in thromboplastin generation and 2) besides Factor VII and Factor IX a third serum clotting factor must be present in normal serum which is necessary for blood thromboplastin formation

There has been some controversy on this view Biggs Bergsagel Verstraete and Vandenbroucke did not find complete correction of thromboplastin generation when using mixtures of anticoagulant and hemophilia B serum This discrepancy may be explained by the fact that various anticoagulants differ somehow in their mode of action phenylindanedione and tromexan cause a more rapid reduction of Factor IX in the beginning of treatment than marcoumar (Fig 1) For this reason marcoumar serum (in the beginning of treatment) may still show a high Factor IX activity whereas in tromexan serum this factor may already be markedly reduced It is evident that one cannot correct the clotting defect of hemophilia B serum by mixing it with a serum which is also deficient in Factor IX In order to obtain a normalization dicumarol—or marcoumar—serum obtained at the beginning of treatment should be used for the mixture experiments

Table 1

MATCHING EXPERIMENTS *

No	Serum I	F	VII	IX	X	Serum II	F	VII	IX	X	Mixtures F	VII	IX	X	Thromboplastin generation
		%					%				%				
1	Marcoumar (beginning of treatment)	3	+	−		Hemophilia B	100	−	+		50	+	+		normal
2	Marcoumar + Vit K_1	110	+	−			100	−	+		105	+	+		normal
3	Hepatitis mild	100	+	−			100	−	+		100	+	+		normal
4	*idem*	100	+	−		Marcoumar	40	+	−		70	+	−		delayed
5	Hepatic cirrhosis	75	+	−		Hemophilia B	100	−	+		90	+	+		normal
6	Factor X purif adsorpt	7	−	+		Hemophilia B	100	−	+		55	−	+		abnormal
7	Factor X purif adsorpt	7	−	+		Factor IX purif adsorpt	7	+	−		7	+	+		normal

All sera I and II used alone produce an abnormal thromboplastin generation Factors IX and X could be determined only approximately + means normal — means diminished or lacking activity in TGT purif adsorpt means purified by adsorption

In a mixture of 2 sera resulting in normalization of thromboplastin generation Factor IX and X concentrations do not exceed 50% of normal values

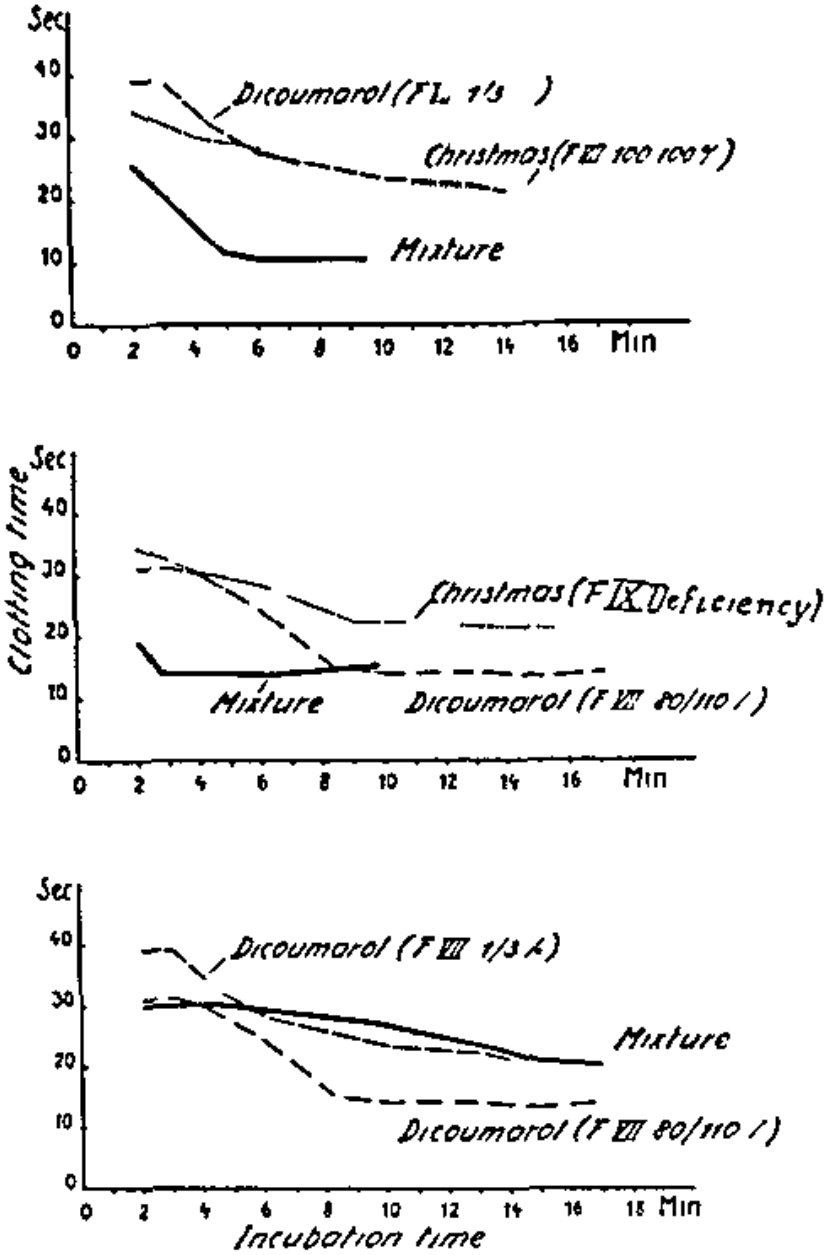


FIG 1 Effect of a Dicumarol Derivative (Marcoumar) on Thrombo plastin generation Dicumarol designates a generation curve ob tained with serum from a patient treated with this Dicumarol derivative Christmas designates a curve obtained with serum from hemophilia B (Christmas disease PTC or Factor IX deficiency) In parentheses the concentration of Factor VII in serum as well as in the corresponding plasma is indicated The first number corresponds to plasma the second to serum

Another disagreement concerned the action of *prothrombin* on the TGT Owing to the defective prothrombin consumption in hemophilia B the serum of these patients contains huge amounts of prothrombin which is converted to thrombin during the TGT The presence of large amounts of thrombin in the incubation mixture produces an acceleration of blood

thromboplastin formation On the other hand marcoumar serum—owing to the lowered prothrombin content of marcoumar plasma—contains less prothrombin than normal serum Owren and Newcomb thought that this fact might account for the delayed thromboplastin generation observed when anticoagulant serum is used There is no doubt that the prothrombin content of serum has a marked influence on the rate of thromboplastin formation However Isenschmid as well as Duckert in our laboratory could show conclusively that prothrombin alone cannot explain the correction obtained in our mixing experiments

The interpretation of the results mentioned above was rendered difficult by the fact that anticoagulants do not cause an isolated clotting defect but a rather complex one Factor X is one of a series of factors influenced by these drugs Is there any evidence for the existence of an isolated Factor X deficiency? Selective clotting defects are usually found in hereditary hemorrhagic diatheses For several years we have been looking in vain for such an isolated defect

Table 2

COMPARISON OF STUART PROWER FACTOR DEFICIENCY AND FACTOR X DEFICIENCY

	Stuart Prower Factor Deficiency	*Factor X Deficiency*
Prothrombin consumption	decreased	normal?
Quick's prothrombin time	prolonged	?
Rate of thromboplastin generation	?	delayed
Amount of thromboplastin formed	decreased	?
Storage stability	Stuart factor is rather stable	Factor X is rather unstable

In 1956 Graham *et al* reported on a congenital coagulopathy which they called Stuart defect named after the name of their first patient A year earlier Telfer had described a Prower defect (Prower being also the name of their first patient) which afterwards proved to be identical with the Stuart anomaly (Bachmann *et al*) We observed in 1956 another case (Delia) a baby with an unusually severe hemorrhagic diathesis who died as soon as the weekly transfusions were discontinued The question arose whether this hereditary coagulopathy was the long awaited case of isolated Factor X deficiency

The Stuart Prower factor has many properties in common with Factor X Both are 1) present in normal serum 2) present in hemophilia B serum 3) present in serum of congenital Factor VII deficiency 4) necessary for blood thromboplastin generation 5) adsorbed on $BaSO_4$ 6) de

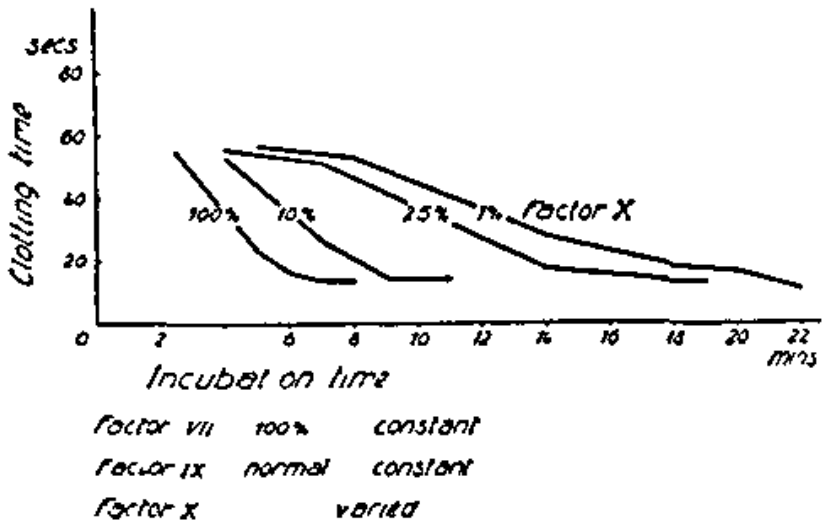


FIG 2 Influence of Variable Factor X Concentrations all other factors being kept constant Clotting time normal incubation time prolonged

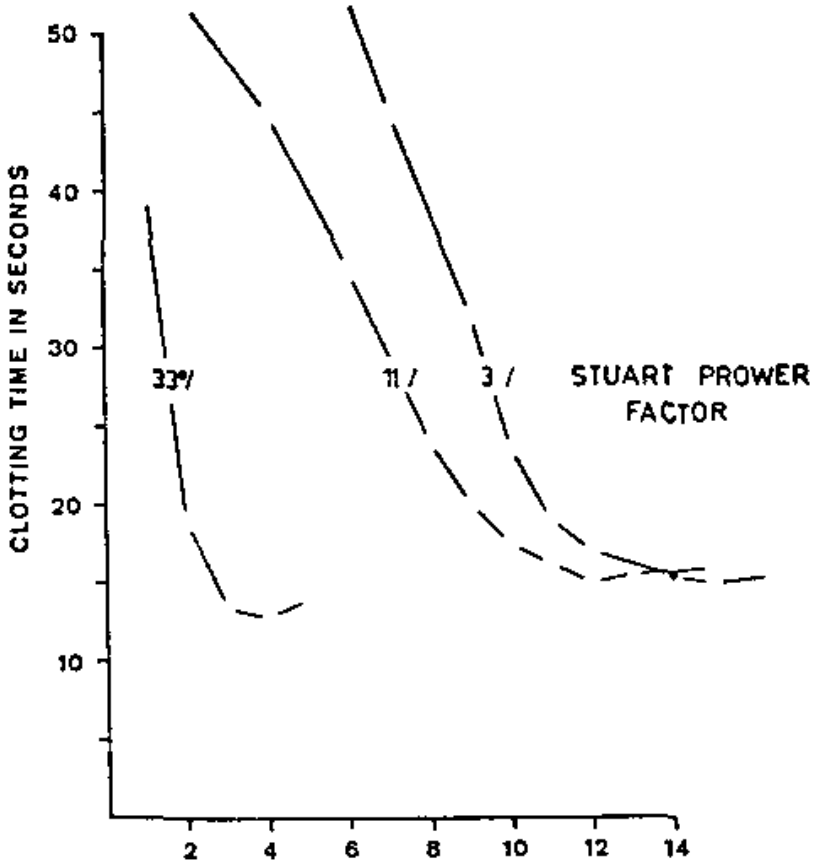


FIG 3 Action of Variable Amounts of Stuart Prower factor Prothrombin constant Factor IX constant (11%) (Duckert Bachmann *et al*)

creased by dicumarol (slower than Factor VII), 7) increased by Vitamin K (slower than Factor VII) and 8) decreased in liver disease There are however apparent dissimilarities (see Table 2) The fact that prothrombin consumption as measured by the prothrombin content of serum is usually found within normal limits during dicumarol treatment (i e in Factor X deficiency) may be explained by the simultaneous decrease of prothrombin *in plasma* under the influence of this drug Serum prothrombin will not be high if plasma prothrombin is already low



FIG 4 Action of Variable Amounts of Factor IX Prothrombin constant Stuart Prower factor constant (33%)

Quick s PT is of course prolonged in dicumarol plasma We attributed this delay to the deficiency of prothrombin and Factor VII There is however no reason why Factor X should not have an effect on Quick s PT too Graham *et al* found a good storage stability of Stuart factor whereas we observed a rather poor stability of Factor X A repetition of these experiments gave however variable results

We stated that Factor X in contrast to Factor IX influences primarily

the formation rate and not the amount of blood thromboplastin (Fig 2) We specified however that particularly in severe Factor X deficiency the rate as well as the amount of thromboplastin formation were decreased On the other hand Stuart Prower factor seems to act also as accelerator pro

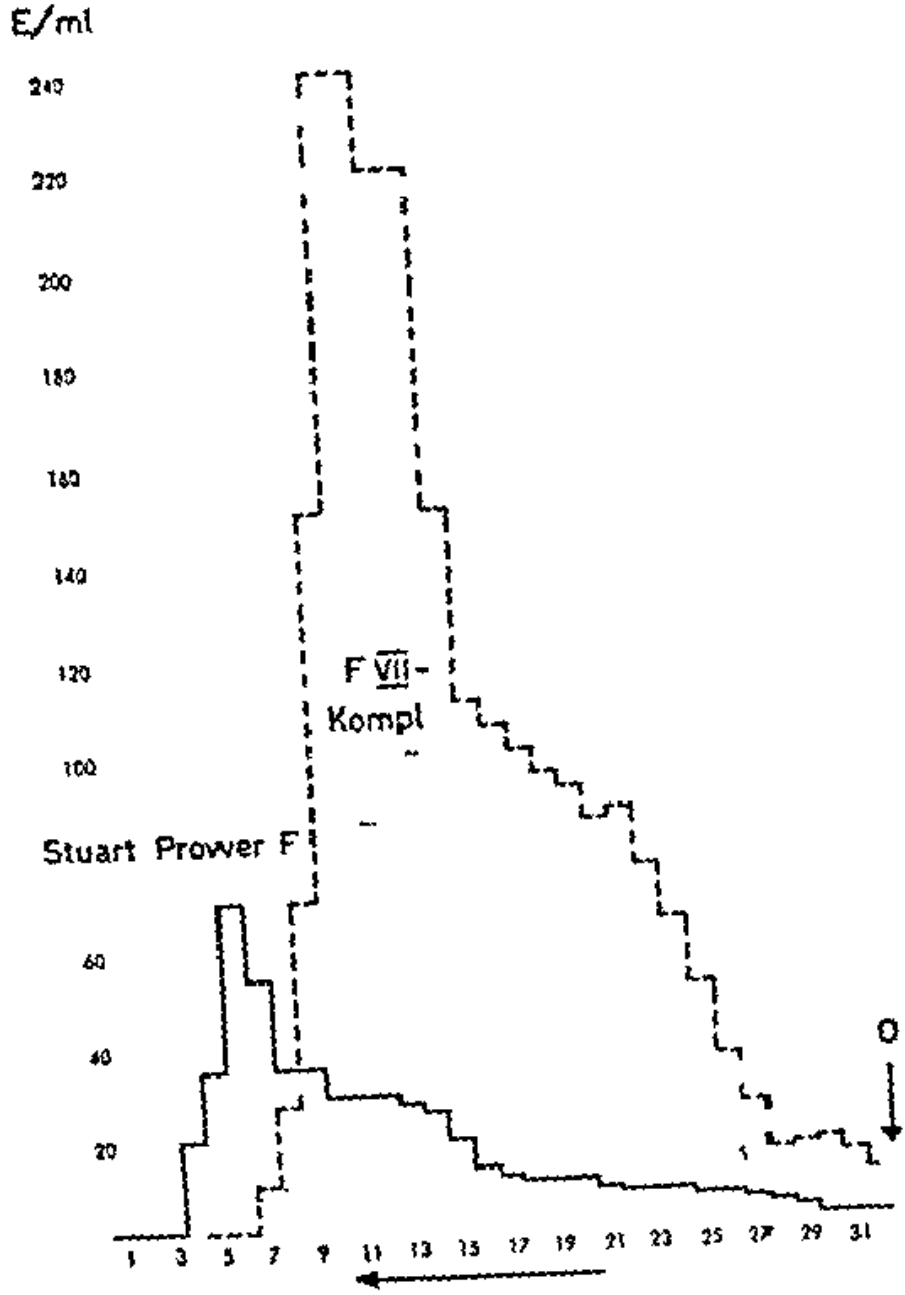


FIG 5 Paper Electrophoresis of Serum Concentrate ($BaSO_4$ adsorbate) The arrow indicates the direction of migration The highest column corresponds to the PAA fraction

vided that the Factor IX level of the incubation mixture is maintained within certain limits (10 35% of normal) (Figs 3 4) We conclude therefore that there is no convincing argument against the assumption that Stuart factor and Factor X may be identical but that there are many

reasons in favor of it We cannot exclude however the possibility that yet another factor exists which possesses all the above mentioned identical properties and in addition behaves like Factor X with regard to stability influence on the rate of thromboplastin formation etc

Recent experiments in our laboratory are of interest in this respect Fisch and Duckert could separate by paper electrophoresis (Fig 5) a fraction from a serum concentrate which shows a high activity in the one stage method for the determination of Factor IX but is unable to normalize the effect of hemophilia B serum in the TGT Therefore by definition this *fraction cannot be Factor IX It has a marked effect on the rate of thrombo* plastin formation which suggested the designation Pre Phase Accelerator (PPA) It is less stable than the Stuart Prower factor on storage and it appears to be absent in hemophilia B serum

In conclusion it may be said that what we described as Factor X is primarily Stuart Prower factor It is possible however that still another serum factor may account for some of the properties ascribed to Factor X

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As reports of Factor X appeared alternative explanations of the phenomenon were offered Newcomb and Owren (13) demonstrated that thrombin profoundly influenced the rate of thromboplastin formation in the TGT and concluded that thrombin acted to convert proaccelerin to accelerin They considered the TGT to be an accelerin generation test The

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The Present Status of Factor X *

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THE concept of blood thromboplastin formation and the development of the thromboplastin generation test introduced a highly sensitive technique which has proved to be invaluable in the study of blood coagulation Yet the very sensitivity of this test has created problems in interpretation resulting from differences in technique significant alterations in results may follow variation in pH ionic strength and modification of reagent preparation (1) In the thromboplastin generation mixture there is simultaneous thromboplastin evolution and inactivation (2) accordingly the activity obtained at any given time represents the net result of both processes The Factor X phenomenon is one derived almost exclusively from thromboplastin generation studies and is accordingly difficult to evaluate Although the Factor X phenomenon is reproducible it is not certain that it corresponds to a distinct chemical entity with a unique role in blood coagulation

Evidence Concerning the Existence of Factor X In 1953 Biggs Douglas and Macfarlane (3) noted that serum from patients anticoagulated with Tromexan ® had diminished activity in the thromboplastin generation test (TGT) This serum defect did not appear to result from deficiency of plasma thromboplastin component (PTC) and it was concluded that Factor VII deficiency was implicated Subsequent studies showed however that Factor VII plays no role in blood thromboplastin formation (1) With the

* This study was supported by Grant No H 2442 (C) National Heart Institute National Institutes of Health USPHS

identification of Stuart factor this problem appeared to be resolved Stuart factor is depressed by the coumarin drugs it influences the one stage pro thrombin time and it is involved in blood thromboplastin formation (4) Thus Stuart factor depression could explain the finding of Biggs *et al* (3) of an abnormal serum reagent in the presence of normal PTC However it was independently noted by Hunter and Walker (5) and Duckert and associates (6) that certain patients under treatment with these anticoagulants had a defect in blood thromboplastin formation associated with an abnormal serum reagent in the presence of a normal one stage prothrombin time (PT) This defect was observed both in the induction period prior to elevation of the PT and following restoration with discontinuan e of the drug or vitamin K administration Previously Koller and his associates (7 10) had postulated the presence of a new serum clotting factor which participated in blood thromboplastin formation The factor appeared necessary to account for the results of mixing in the TGT of sera from patients with PTC deficiency liver disease and Marcoumar ® therapy In line with the system of nomenclature favored by Koller s group the new factor was named Factor X Studies from other laboratories seemed to be confirmatory Biggs (11) mixed sera from various patients in the TGT and on the basis of results with congenital defects and sera of Dindevan ® treated patients concluded that normal serum contained at least 3 factors involved in the TGT PTC Stuart factor and a third factor conforming to the description of Factor X Greig and Tattersall (12) came to similar conclusions using mixtures of sera from patients with congenital defects hepatitis and coumarin drug therapy

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Factor X phenomenon was felt merely to reflect the thrombin content of the system Biggs and Macfarlane (14) cautioned about interpretation of mixing studies and noted conflicting reports as to the assay of PTC from sera of patients with hepatitis or under coumarin drug therapy Bergsagel (15) suggested that the defect in stored serum may represent dissociation on storage of an intermediate product of thromboplastin formation which is present in fresh serum Rabiner and Shulman (16) were able to improve reagent activity of stored serum by freezing and thawing An element of doubt began to invade even the group that first described Factor X Bach mann and associates (17) while rejecting the thrombin hypothesis of New comb and Owren (13) appeared willing to consider the Factor X phe nomenon a combination of mild deficiencies possibly most influenced by Stuart factor

More recent studies have tended to reinforce the concept of Factor X as a unique phenomenon if not a separate chemical entity Spaet and Kropatkin (18) showed that serum stored in the refrigerator developed a defect as a TGT reagent showing no correlation with its prothrombin con tent Such specimens gave completely normal assays of PTC in the TGT and showed normal content of Stuart factor by both TGT and one stage prothrombin techniques These abnormal stored sera behaved in the man ner described by Duckert *et al* (16) for sera defective in Factor X in that they gave an abnormal rate of thromboplastin formation but ultimately produced a normal thromboplastin yield The Factor X phenomenon was evidently masked by a high reagent prothrombin content since hemophilic serum retained its apparent Factor X activity Rabiner and Spaet (19) showed that many patients with parenchymal liver disease presented sera with a defect typical of Factor X deficiency in the presence of normal pro thrombin tests These investigators also were able to produce an abnormal serum reagent in the TGT without depression of plasma prothrombin by the simultaneous administration of dicumarol and vitamin K to normal human subjects A somewhat different line of evidence has appeared based on reactions to injection of rare earth derivatives Hunter (20) observed that intravenous neodymium 3 sulfoisonicotinate produced a clotting dis order principally due to depression of PTC and Factor X Alexander (21) found that intravenous Thorotrast® was followed by changes in serum reagent similar to those in stored serum and has concluded from his data that Factor X is a valid clotting component

Properties of Factor X In many respects Factor X resembles the several other clotting factors which seem to be related metabolically to prothrombin (22) Summarized in Table 1 the properties of these com ponents are quite similar as regards heat lability adsorption onto the in soluble salts of heavy metals and dependence on the integrity of the hepatic pathway of vitamin K metabolism Certain differences in stability and func tions are evident Both Factor X and Stuart factor are less stable than the

Table I

COMPARATIVE PROPERTIES OF THE PROTHROMBIN GROUP OF CLOTTING FACTORS

	Prothrombin	*Factor VII*	*Stuart Factor*	*PTC*	*Factor X*
Activity in Serum	Low	+	+	+	+
Participation in Prothrombin Conversion with Tissue Thromboplastin	+	+	+	0	0
Participation in Blood Thromboplastin Formation	?	0	Affects rate and yield	Affects rate and yield	Affects rate only
Storage Stability in Plasma	Stable	Stable	Stable	Stable	Stable
Storage Stability in Serum	—	Stable	Labile at 37 Stable at 4	Stable	Labile
Depression by Coumarin Drugs	Early	Early	Early	Delayed	Early
Restoration after Coumarin Depression	Early	Early	Somewhat Delayed	Early	Delayed
Adsorption on Insoluble Heavy Metal Salts	+	+	+	+	+
Destruction at 56	+	+	+	+	+
Electrophoretic Mobility	γ Globulin	β Globulin	α and β Globulins	β_1 Globulin	?

other components in serum However serum loses little of its Stuart factor activity when stored at 4°C for two weeks whereas Factor X may virtually disappear from serum under these conditions (23) It is noteworthy that different sera lose their Factor X content at different rates the reason for these differences is not clear At 37°C both Stuart factor and Factor X disappear from the serum rapidly Factor X is considerably more stable in plasma Serum obtained from recalcified plasma of expired bank blood behaves as normal serum reagent in the TGT (23) This finding indicates the presence of adequate Stuart factor as well Other distinctive properties of Factor X include a greater affinity to barium sulfate than the other members of the group and rapid denaturation at 50°C (6)

Clinical Variations in Factor X The Factor X phenomenon is most readily demonstrable following treatment with the coumarin drugs Al though no studies have compared the various members of this drug group the different derivatives reported seem uniformly to produce Factor X de ficiency There appears to be general agreement that Factor X is more sensitive to these drugs than most of the other susceptible factors and several studies indicate that Factor X deficiency persists longest following discontinuance of therapy (5 6) If these conclusions are verified they are of the greatest interest It is a matter of common clinical observation that patients who develop bleeding following coumarin drug intoxication may continue to bleed after restoration of prothrombin to normal levels Per sistent Factor X depression may account for this previously unexplained phenomenon inasmuch as this factor does not appear to be involved in the prothrombin time It would appear that the TGT would be a useful labora tory addition in following certain of these patients

The presence of Factor X deficiency in liver disease has been mentioned by many of the investigators who have studied this factor (6 12 18 19) In other studies such as those by Cowling (24) and Finkbiner *et al* (25) the serum was also found to be abnormal in the TGT but it was not felt necessary to postulate factors other than PTC to account for the defect In the latter study the TGT was not abnormal in the presence of normal prothrombin a finding at variance with the data of Rabiner and Spaet (19) who demonstrated significant Factor X deficiency in many patients with liver disease of mild degree Many of the patients studied had normal prothrombin and presented Factor X deficiency as their only laboratory abnormality although liver biopsy or autopsy confirmed the presence of liver disease A possible reason for the discrepancy in the two studies results from differences in reagent preparation Finkbiner *et al* used serum reagent which had been stored at room temperature from 12 to 24 hours Thus the normal control reagent was probably deficient in both Factor X and Stuart factor making detection of these deficiencies more difficult in test sera Control sera of Rabiner and Spaet were used within several hours of blood collection Spaet and Kropatkin (18) found that serum from a

patient with liver disease had a defect identical to that of stored serum but with virtually normal ability to correct clotting of serum defective in Stuart factor alone Factor X determination may prove to be a sensitive and useful liver function test if the above studies are confirmed Factor X also appeared to be depressed in a patient with hypoprothrombinemia resulting from obstructive jaundice and vitamin K deficiency (11)

To date no clearcut cases of pure congenital Factor X deficiency have been reported although two patients have been studied who evidently had congenital Factor X deficiency combined with other clotting disorders One patient reported by Biggs (11) had been previously studied by Newcomb and associates (26) and had a complex deficiency including prothrombin Factor VII PTC Stuart factor and Factor X A patient of Greig and Tattersall (12) presented the findings of combined PTC and Factor X deficiency It is possible that cases of Factor X deficiency are being missed owing to the tendency of many laboratories to classify all patients with an abnormal serum in the TGT as PTC deficiency without further study According to Koller (8 27) Factor X is normally reduced in the newborn

Conclusions

Studies from several independent laboratories have confirmed the presence of a TGT defect seemingly separable from deficiencies involving PTC Stuart factor and other known coagulation components This defect is most pronounced in sera of patients under treatment with coumarin drugs or with liver damage and in stored serum In its properties and its relation to vitamin K metabolism this Factor X phenomenon suggests the presence of a clotting component belonging with the serum factors (22) which appear to be chemically and synthetically related to prothrombin But suggestive as the evidence is it is probably not justified at present to accept without reservation the unique integrity of Factor X as a clotting factor in its own right Available assay methods for all clotting factors are uncertain at best and certain components defy accurate quantitation For example PTC activity increases progressively in a given blood sample on standing in glass (28) and its component serum level is even greater It is still possible that Factor X may merely be a reflection of combined deficiencies none of which is detectable separately Similarly it may represent altered blood thromboplastin intermediates resulting from unidentified changes in known components The final verdict on Factor X will probably come with discovery of a pure and severe defect of it (best studied in several laboratories with different techniques) or chemical purification of a substance which fulfills its criteria

Meanwhile the concept of Factor X presents certain practical advantages It indicates to clinicians the presence of a clotting defect which may appear in the presence of normal prothrombin tests and no evident PTC de

ficiency It emphasizes that disturbance of vitamin K metabolism with coumarin anticoagulants or liver disease leads to hitherto unsuspected alterations in blood coagulability not readily evaluated by routine procedures currently in use

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Part 6

HEMOSTASIS AND HEMORRHAGIC CRISES

26

The Resistance of the Hemostatic Plug in vivo ' in Healthy Men, in Thrombocytopenia and Under the Influence of Indirect Anticoagulants

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IN 1923 *one of us (J R) proposed the theory and then showed in animal* experiments in 1942 that uncontrollable bleeding in the hemorrhagic states due to a coagulation defect or coagulopathy (such as familial hemophilia) was primarily related to an abnormally fragile hemostatic plug The profuse hemorrhages in the hemophilic states (characterized paradoxically by normal or slightly prolonged bleeding times) differ in their pathogenesis then from the hemogenic syndromes (thrombocytopenias and thrombocytopathias) since the latter are chiefly the result of markedly delayed formation of the hemostatic plug either on the basis of numerical platelet deficiency (with normal coagulability) or deficient platelet agglutination (6 7 8)

However de Nicola and de Nicola and Mazzetti (1 2) on the basis of a simple thrombelastographic study believed that the thrombus in the hemophiliac was qualitatively superior to the thrombus in the thrombocytopenic patient that although it was slower in building up its resistance was nonetheless greater However in the usage of Italian authors the word thrombus from all indications is a red thrombus or coagulated clot and not the white thrombus or hemostatic plug that is responsible for the cessation of bleeding from arterioles and venules It is also the coagulated clot that Macfarlane (4) means when he disagrees with de Nicola although he uses a similar technique measuring with a simple

apparatus the force required to separate three surfaces between which blood had coagulated he established that the resistance from the tension of clots in thrombocytopenia is much greater than in normal individuals while the coagulated clots in hemophilia are very fragile Obviously these authors think that their observations are directly relevant to the much more complex process of spontaneous hemostasis

Attempting to approach this problem in pathogenesis in a more direct and less speculative manner we have submitted a new *in vivo* method which permits the study of the resistance of those elements engaged in hemostasis in man—the hemostatic plug

Technique The patient lies on his back with his arms extended along the plane of the bed First the bleeding time is measured on the left fore arm The skin is cleansed with ether and then three cross wise incisions each 2 cm long and reaching the cellular layer of subcutaneous tissue are made on the upper half of the forearm The arm is then placed mid way between pronation and supination to allow the blood to collect at the inner commissure of the incision Every 30 sec blood is blotted off with filter paper care being taken to avoid contact with the wound

The same procedure is repeated on the right forearm to which the un inflated cuff of a Vaquez Sphygmomanometer has been previously attached Three minutes after the shortest bleeding time (BT) the cuff of the Vaquez apparatus is inflated to 40 mm Hg and this pressure maintained until bleeding resumes If bleeding does not recur after 3 min the hemostatic plug is considered effective If bleeding does recur the pressure is released and the BT is measured as described above

In the majority of patients we examined a platelet count Quick time fibrinogen levels prothrombin complex determinations prothrombin con sumption and the TGT were obtained according to approved methods

Results and Discussion

Having established in a previous study that the BT in man obtained as described above is statistically as sound as the modified Duke method (5) (with close correlation between the mean values of 3 BT on the right and left forearms) we then studied the recurrence of hemorrhage under the influence of moderate stagnation in both normal individuals and patients suffering from various morbid states Our first complete results will be published shortly in Thrombosis et Diathesis Haemorrhagica We are presenting in this report our observations made on 1) subjects which were considered normal 2) individuals treated with indirect anticoagulants (Marcoumar Tromexan) and 3) thrombocytopenic patients

The Mean BT of Our Patients In 24 subjects initially considered as normal we recorded a mean BT of 201 sec with a σ of 56 The mean BT was abnormally prolonged with respect to the mean and its σ in two

subjects of this group However these subjects with a pathologic mean BT above 285 sec represented only 8 3% of the total group of normals

Table 1 shows that the proportion of abnormal mean BT is much higher in patients treated with anticoagulants and is especially so in patients suffering from thrombocytopenia being respectively 60 and 100% of all cases with a mean of 356 and 1675 5 sec respectively Five of the 8 thrombocytopenic patients had platelet counts above 30 000 per mm^3 three of them had platelet counts below 4 500 their respective mean BT were 513 and 5 073 sec

Table 1

MEAN BLEEDING TIMES IN PATIENTS TREATED WITH ANTICOAGULANTS AND PATIENTS WITH THROMBOCYTOPENIA

Subject Groups	*Total Number*	*Number of Prolonged BT*	*%*	*Mean BT in secs*	*σ Sec*
Apparently normal	4	2	8 3	201	56
(Indirect) anticoagulated patients	15	9	60 0	356	328
Thrombocytopenic patients	8	8	100 0	1675 5	

Recurrence of Hemorrhages Before adapting the counterpressure of 40 mm Hg we tested other pressures We established that if after the cessation of bleeding a pressure equal to the mean arterial pressure was applied to the arm bleeding resumed in all normal subjects

Table 2 shows the observations made with the counterpressure of 40 mm Hg with which we are concerned

Table 2

RECURRENCE OF HEMORRHAGES WITH COUNTERPRESSURE IN PATIENTS TREATED WITH ANTICOAGULANTS AND PATIENTS WITH THROMBOCYTOPENIA

Subject Groups	*Number of Cases*	*Number of Recurrences*	*Percentage of Recurrences*
Apparently normal	24	3	12 5
(Indirect) Anticoagulated patients	15	7	46 6
Thrombocytopenic patients	8	0	

Among the 24 subjects initially considered normal only three had a recurrence of bleeding with counterpressure One of these had a normal bleeding time and normal coagulation tests until we obtain information to the contrary we must consider him normal from the hemostatic point of view The second patient gave a prior history of hemoptysis His studies showed deficient prothrombin consumption deficient TGT with patient s serum and a 50% reduction in Factor VII and the Stuart Prower factor

apparatus the force required to separate three surfaces between which blood had coagulated he established that the resistance from the tension of clots in thrombocytopenia is much greater than in normal individuals while the coagulated clots in hemophilia are very fragile Obviously these authors think that their observations are directly relevant to the much more complex process of spontaneous hemostasis

Attempting to approach this problem in pathogenesis in a more direct and less speculative manner we have submitted a new *in vivo* method which permits the study of the resistance of those elements engaged in hemostasis in man—the hemostatic plug

Technique The patient lies on his back with his arms extended along the plane of the bed First the bleeding time is measured on the left fore arm The skin is cleansed with ether and then three cross wise incisions each 2 cm long and reaching the cellular layer of subcutaneous tissue are made on the upper half of the forearm The arm is then placed mid way between pronation and supination to allow the blood to collect at the inner commissure of the incision Every 30 sec blood is blotted off with filter paper care being taken to avoid contact with the wound

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The Mean BT of Our Patients In 24 subjects initially considered as normal we recorded a mean BT of 201 sec with a σ of 56 The mean BT was abnormally prolonged with respect to the mean and its σ in two

bocytopenic patients is much more pronounced in anticoagulated patients and is rare in normal subjects

Is the firmness of hemostatic plugs in hemophilia similar to that under anticoagulant therapy?

This seems quite likely on the basis of the behavior of bleeding in hemophilic families and the BT in heparinized animals (7) In this regard however one must always be aware of over simplification Table 3 summarizes our observations on two patients at the beginning of anticoagulant therapy (subjects 1 and 2) and one patient (subject 3) afflicted with hepatic icterus at the onset of his illness and after recovery These findings make one question the role of blood coagulation factors in effective hemostasis especially if one recalls the significance of the vessel wall in the arrest of bleeding (8) and of the vascular effect of anticoagulants (3)

It goes without saying that the problem presented here requires much more investigation We think that our method may be used to advantage in this regard as well as in the control of administration of direct anticoagulants such as heparin Meanwhile it is obvious that more observations will have to be compiled in order to statistically evaluate our results

Summary

1 The authors describe a method for the study *in vivo* of the resistance of human hemostatic plugs This method is essentially based on the relative frequency of recurrences of bleedings in the forearm under the influence of stasis originating after the arrest of bleedings from a standardized counterpressure on the arm

2 Under these conditions the recurrence of bleedings is exceptional in normal people The same may be said if at all possible of thrombocytopenic patients

3 On the other hand the bleeding recurs in an important proportion of cases in patients treated with indirect anticoagulants

4 The significance of these facts is discussed

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The third patient is one of those very rare individuals on whom one cannot perform coagulation tests Hence we cannot be certain about him By eliminating the latter two patients we can state that among 22 normal subjects, only one or a little over 4% resumed bleeding with counter pressure It seems then that a fair conclusion would be that recurrence of bleeding with counterpressure in normal individuals is highly unusual

In 15 patients treated with indirect anticoagulants bleeding occurred in seven or 46 6% of them with a counterpressure of 40 mm Hg Moreover it seemed to us that recurrences were much more frequent with vigorous therapeutic anticoagulation

Though neither our evidence nor the number of patients treated by anti coagulants can be statistically analyzed we still feel that their unusual be havior in our experiments strongly suggests the presence of a decreased resistance of the hemostatic plug in the coagulopathies

Table 3

COAGULATION AND HEMOSTATIC TESTS

	Subject 1	*Subject 2*	*Subject 3*	
			Jaundiced	*Recovered*
Platelets x 1 000/mm³	188	141	378	275
Quick time (%)	95	80	16	100
Prothrombin (%)	120	60	7 2	100
Factor V (%)	82	100	60	100
Factor VII (%)	80	87	15 5	100
Fibrinogen (gm %)	2 6	5 4	2 45	4 8
Residual Prothrombin (%)	2 7	3	4	3
Biggs Test (TGT)				
—absorbed plasma	N	N	N	N
—serum	N	N	Deficient	N
Bleeding time (forearm)	4 25″	6 35	5 05″	4 25
Recurrence of hemorrhage	+	—	+	+

Conversely in the 8 thrombocytopenics in our study there were no recurrences of hemorrhage after counterpressure despite the fact that three of them had platelet counts of 4500 2500 and 2900/mm³ respectively along with mean BT of 58 121 and 59 min even though the counter pressure was raised to levels approaching mean arterial pressure

Recalling that in our earlier tests all the normal patients had recurrence of bleeding with this latter counterpressure we now suggest despite de Nicola that the hemostatic plug in thrombocytopenics is firmer than in normal subjects This remarkable resistance may be the result of its slow formation

It seems to follow then from our initial studies that the tendency for bleeding to recur with our experimental method is non-existent in throm

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Platelet like Activity of Serum in Hemophilia

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IT IS now well known that in hemophilia platelets resuspended in their own plasma are very slowly agglutinated and destroyed (2 6 7 8) Data have been published suggesting that platelet resistance was increased (3 4 10) but when resuspended in normal plasma platelets appear normal in their morphology and function (5 9) Using a rotating agitation with glass Wright (13) has shown that platelets of hemophiliacs and normal subjects have a different mechanical resistance

For a better understanding of the causes of hemorrhage in hemophilia we have tried to find by statistical method whether there was any difference in the various coagulation factors in bleeding periods vs nonbleeding periods

Material and Methods 42 hemophilic patients (34 with AHG deficiency 8 with PTC deficiency) were examined

The presence of a hemorrhagic period was established by the finding of cutaneous or internal bleedings at the time of examination

During 20 hemorrhagic and 22 non hemorrhagic periods 4 tests were generally performed a) clotting time in tubes previously rinsed with saline b) prothrombin consumption by Soulier s (12) two-stage method c) platelet like activity of serum by the method of Alagille and Soulier (1) the results of which are expressed by an index which is the difference in the serum prothrombin time the sixth and the fourth minute after recalcifi cation of a platelet free plasma with the tested serum and d) the serum serotonin level determined by its activity on the atropinized rat bowel as indicated by Quivy (11) Determination of antihemophilic factor was not done

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over all coagulability (coagulation time prothrombin consumption) nor to the physiological test for serum serotonin level

Further work is in progress to study platelets of hemophiliacs in contact and contact free systems in order to understand what part they play in the disturbed hemostasis in hemophiliacs considering their normal coagulation potency (TGT with platelets) whatever the phase of the disease

Although only a few cases have been investigated nonetheless we have been able to observe the beneficial action of prednisone on the coagulation factors in hemophilia Its effect on the deficient platelet like serum activity seems convincing

Summary

A highly significant statistical difference of platelet like activity of serum between hemorrhagic and non hemorrhagic periods in hemophilia has been found It is not related to the clotting time nor to prothrombin consumption The interpretation of this phenomenon is discussed

We are indebted to Dr P Maroteaux for his help with the statistics

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Results The results of the statistical study (Table 1) show that the difference of the average time is statistically valuable for the clotting time ($\Delta = 22\,68 \pm 9\,65$) but not for prothrombin consumption or serum serotonin level (this last study was done in only a few number of cases) However the difference found between hemorrhagic and non hemorrhagic periods in the platelet like serum activity seems to be highly significant ($\Delta = 16\,05 \pm 2\,81$)

Table 1

COMPARISON OF HEMORRHAGIC AND NON HEMORRHAGIC PERIODS CLOTTING TESTS AND SERUM SEROTONIN

	Hemorrhagic Period Mean Values	Number of Cases	Mean Values Difference with Standard Deviation	Non Hemorrhagic Period Mean Values	Number of Cases
Clotting time (in minutes)	48 25	19	22 68 ± 9 65	25 57	20
Prothrombin Consumption (in seconds)	11 6	20	3 17 ± 2 78	14 77	22
Platelet like activity of serum (in seconds)	6 35	20	16 05 ± 2 81	22 4	22
Serotonin levels of serum (in γ/cc)	0 01615	6	0 03766 ± 0 04972	0 05381	11

We thought that delayed clotting time in itself could account for the discrepancy in the platelet like activity expressed in seconds and not in consumed prothrombin Table 2 does not confirm this view Otherwise correlation between clotting time and platelet like activity of serum is very low ($r = -0\,3$)

Table 2

Clotting Time	Platelet like activity in seconds	Number of Cases	Mean value with Standard Deviation	Clotting Time	Platelet like activity of Serum	Number of Cases
>20	13 35	20	D = 4 33 ± 3 91	<20	17 68	19

Due to the small number of cases studied it has not been possible to establish by statistical investigation a relation between serum serotonin levels and platelet like activity of the serum

Conclusions

The platelet like activity of serum expressed in seconds shows a significant difference between hemorrhagic and non hemorrhagic periods of hemophilia This difference is statistically related neither to the tests of

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LIPOIDS AND BLOOD PLATELETS

WITH REFERENCE TO BLOOD COAGULATION AND THE HEMORRHAGIC DISEASES

By

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Im Tatsachlichen grosster Pragnanz

Im Hypothetischen ausserste Vorsicht (Wohlisch 1923)

When theory fancy free
Bids hither yon s the way
Calm fact exhorteth thee
Consider tyro' Stay (Orig)

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EDITORIAL NOTE

The author adopts the American spelling in the text

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(4) Authors are listed alphabetically In case of multiple authorship the second and subsequent authors' names follow in alphabetical sequence
(5) Diphthongs are replaced by separate letters Modified letters (accent etc) when not anglicized (e g ue German u) in the original reference appear in the usual alphabet order but include the modification
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ABSTRACT

By specifically analyzing for the various active principles of plasma platelets tissues and their fractions much new information has been obtained concerning the role of lipids and platelets in blood coagulation and in the hemostatic mechanisms in health and disease Analyzed components are studied in artificial clotting systems especially a two stage thrombin forming system Some 86 cases of bleeding disorders 32 new born normal infants and their mothers and many normal adult bloods have been analyzed with respect to components of the clotting and hemostatic functions

The detailed considerations embodied in the dissertation are encompassed under the following heads

1) the importance of certain lipids especially cephalin

2) the normal need for platelets in plasma clotting

3) the particular significance of a platelet component which has many analogies to cephalin in the thromboplastic system

4) potentiation of the thromboplastic actions of cephalin of platelets and of tissue thromboplastin (to some extent) by a variety of experimental additives Part of this may be explained as a thromboplastin generation through co participation of certain plasmatic components (antihemophilic globulin PTC etc) Part however may be the result of certain proteolytic enzymes particularly trypsin 'disaggregating lipoproteins and thus rendering their phospholipid (and sometimes calcium) available for participation in the clotting reactions

5) possible Ca containing and lipid containing intermediates in the thrombin forming reactions

6) myelin figure formation as an explanation of 'alterations of platelets and certain other formed elements such as thrombocytes megakaryocytes and stromatolytic erythrocytes

7) the multiplicity of factors which platelets may contribute to the blood clotting and hemostatic mechanisms

8) the occurrence of many clinical disorders due to deficiency of platelet functions Thrombocytopenias denote deficient numbers ('counts' and total bulk in body) Thrombocytopathias are deficiencies of specific platelet components e g thromboplastic factor accelerator vasoconstrictor (5 hydroxytryptamine) or retractor factor Such deficiencies can be clinically significant even when the platelet count is normal Bleeding in leukemias uremias etc may often be accounted for in these terms

9) the nature and modes of action of heparin and other 'antithromboplastic inhibitors and of some antiproteases in relation to the mechanisms discussed

10) the 'cephalin availability theory' of the author as a useful working hypothesis to explain the importance of the natural thromboplastic phospholipid Lipid release from platelet tissue or possibly plasma sources may very well be the long obscure 'trigger mechanism' which initiates blood coagulation

11) The supplement reports 11 new experiments in the period 1956-1959 The versatile two-stage method has been modified for the quantitative study of each individual factor in the blood clotting system and adds some

new data concerning thromboplastic mechanisms These data contrib ute further support for the main thesis It is suggested that the term 'prothromboplastic' be used to designate the phosphatide (probably cephalin) which in conjunction with calcium ions and a diverse (exper imentally) group of accessory factors constitutes the mechanism for activation of prothrombin to thrombin 'Availability of the lipid as a trigger mechanism' and its dynamic role in these activator mechan isms seem best explained in terms of a 'phospholipid transfer' hypothesis

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Lipoids and Blood Platelets

with reference to blood coagulation

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PART I

BACKGROUND

FOREWORD

In presenting a dissertation embodying experimental work conducted over a 27-year period (1929 1956) it is necessary to review the knowledge of the field at the commencement of this period and its subsequent progress to the present time in order to evaluate each contribution in the light of contemporary knowledge and theory Several volumes and possibly several authors would be required to review critically and in detail all the extensive literature of the blood coagulation field From the physiological approach alone several thousand titles are referred to in the monumental reviews of P Morawitz [346 347] and E Wohlisch [505 506] and in more recent articles including those appearing in the Annual Reviews of Physiology two of them by the present author [133 140] The purposes of this dissertation may best be served by a selection of those ideas and trends of thought which afford the background for the topics presented

A dissertation more than a definitive scientific publication reflects the interplay of ideas that influence the mind of its author and it is not inappropriate that it indicate to some extent the personal element in research In the period covered Science itself has undergone a change which is deserving of mention This change was gradual in the beginning but it has accelerated in the last few decades of the twentieth century Characteristic of earlier periods was the eminence of individual scientists whose discoveries opened up new fields or special areas within those fields often single handed Students and young investigators came to work in the laboratories of the distinguished authority and thus developed the various 'schools justly adding to the reputation and prestige of the 'master A fine example in our field was the Dorpat school under the leadership of Alexander Schmidt (1860 1895)

The modern scientific era has seen much larger numbers of scientists working simultaneously in the same field Through scientific gatherings of regional national and international scope workers of kindred interest have frequent opportunities for exchange of ideas Thus all are kept fully informed of many lines of progress and of the definition of numerous problems in advance of their solution A not infrequent consequence is that the same solution (or almost) is reached simultaneously by several independent groups of workers perhaps widely scattered over the world An illustration of this may be cited from a field peripheral to that of the present inquiry In 1939 the Journal of the American Chemical Society simultaneously published four papers from different laboratories all of which must share the credit for the definitive solution to the problem of the chemistry of vitamin(s) K Much the same sort of thing is true of the recent

mention These established the clotting phenomenon as essentially a physiological property of the 'coagulable lymph' (Butt 1760 cf. 'coagulated lymph' of de Senac 1749) which we nowadays call plasma Malpighi (1666) and Ruysch (1707) had already noted the 'fibrous' material obtainable from blood clots and clinicians with wide experience of ubiquitous blood letting talked of the 'cruor' or 'crassamentum' appearing after separation of the 'serosity' or serum Much was made of the buffing' of blood in disease This we now interpret as an increased erythrocyte sedimentation rate with the consequent easier observation of clotting sequences starting in the supernatant plasma rich layer The term fibrin which we owe to the French encyclopedists Chaptal [72] and de Fourcroy [99] was not really an advance since they used the crudest methods of preparation of the clot material which they tried to characterize by appearance and elementary analysis Mulder introduced the word protein in 1839 but it has been a long and difficult road still with unexplored bypaths to define individual proteins and among them fibrin and fibrinogen The latter term we owe to Virchow [482]

The 1830 1859 publications of P S Denis are of historical interest Denis' [102] most significant contribution all things considered was the practical preparation of his plasmine ' This was a precipitate obtained from plasma with strong solutions of sodium chloride and other neutral salts Redissolved on addition of water it subsequently underwent coagulation indistinguishable from the appearance of natural fibrin clot

The false impression that fibrin was the result of some 'spontaneous' change in a soluble percursor (fibrinogen) was first questioned by Buchanan [68 69] particularly in his observation that the washings from a blood clot could coagulate certain hydrocele fluids He compared this with the rennet curdling of milk and also did much to try and prove a cellular origin of the blood coagulant Buchanan's work was neglected until republished by Gamgee [183] in support of his confirmatory experiments Gamgee's method of preparing thrombin (as it has come to be termed) from blood clots was further improved by W H Howell [229 232] at the beginning of our current era

2 THE DORPAT SCHOOL

Alexander Schmidt of Dorpat established his famous school in the latter half of the 19th century From the master and his pupils came a large body of work and publications reviewed in Schmidt's two monographs [420 421] in 1892 1895 It is easy from our advanced modern viewpoint to be extremely critical of the crudity of their experimentation and highly theoretical presentations of their ideas It should be remembered however that the difficult biochemical problems of the coagulation field humble us even to this day Then again the 19th century discoveries especially in the field of ferments (or enzymes) aroused such interest that contemporary enthusiasm sometimes got out of bounds Analogies were suggested and their implications tacitly accepted with little of the caution demanded of more recent experimental science Schmidt used a crude technique of storing blood serum under alcohol to obtain his 'fibrin ferment ' which was a very weak

discoveries of several new factors in the blood clotting mechanism Here another consequence is manifest namely some uncertainty be cause of the difficulty in exact identification of the factors in question thus leading to a confusion in nomenclature Such confusion is more troublesome to the casually interested scientist than to those actually working on these problems however

In the coagulation field at least in America perhaps the last of the old school authorities was W H Howell of the Johns Hopkins School of Medicine in Baltimore This eminent scientist and distin guished teacher was quietly continuing his critical experimentation in a modest laboratory which he occupied as Emeritus Professor of Physiology during the period of the 1930's when the present author had the privilege of paying him numerous visits His friendly charm advice and critical discussions and warm personal interest and en couragement were of inestimable value in guiding a new researcher into the complexities of the blood clotting field

On the occasion of the annual meeting of the Federation of Amer-ican Societies for Experimental Biology at Detroit in 1935 a small group of interested coagulationists met together and agreed to main-tain an exchange of ideas By common consensus this 'coagulationist' group has never become formally organized It has however con tinued to meet at each annual meeting of the Federation and the sus tained interest has expanded with each succeeding year so that our mailing list now has over two hundred names There are similar groups of workers in other specialized fields and a very important result of their coordinated interests has been to secure inter society sessions and symposia at such gatherings as the Federation and the International Physiological and Hematological Societies' meetings Other significant contributions to the cross fertilization of ideas among specialized scientific groups are the numerous Research Con ferences which are a distinctive feature of the modern scientific scene Some examples in which American coagulationists get to gether are those promoted by the National Research Council the U S Public Health Service and the Josiah Macy Jr Foundation to mention just a few

With this broad perspective we may selectively summarize the early history of blood coagulation in what we may call the personal era and then proceed to a brief review of its modern status in which due credit for individual investigators need not be denied by present ing this largely from the depersonalized point of view

The topic of the blood platelets will be separately reviewed

CHANGING CONCEPTS IN THE CLOTTING FIELD

A HISTORICAL HIGHLIGHTS

1 EARLY ORIGINS: HEWSON DENIS BUCHANAN

The earliest significant efforts toward an understanding of the coagulation of the blood were made in the 18th century and the Experimental Inquiries of young William Hewson [218] associate of the famous brothers John and William Hunter are particularly worthy of

3 THE WORK OF HAMMARSTEN AND WOOLDRIDGE

Olof Hammarsten [205] in 1883 seemed to simplify Schmidt's theories by suggesting that 'fibrinoplastic substance ('fibrino globulin') was not a component of the coagulant but appeared as a split product of fibrinogen when acted upon by the fibrin ferment However this eminent biochemist went on to considerable success in the purification of both fibrinogen and thrombin We owe to him the application of the 'salting out' technique to precipitate fibrinogen from plasma using ½ sat NaCl or ¼ sat $(NH_4)_2 SO_4$. Weyl Heynsius and others (ref [371]) were then developing the use of neutral salts rather than the earlier acids and alkalies etc for the separation of all kinds of proteins The distinction between albumins and globulins on the basis of solubilities in such salt solutions was made in this era When oxalate or citrate (see later) replaced the older $MgSO_4$ etc as anticoagulants used in separating (centrifugally) the plasma from the blood corpuscles the preparation of fibrinogen and other clotting fac tors by the salting out method was much improved The technique of elementary organic analysis by combustion methods dating back to Liebig (1840) was continued and despite the relative crudity of the materials analyzed did give data on N S P and minerals e g Ca which contributed some information of real value in these early days of protein and other organic chemistry Hammarsten eventually pur ified fibrinogen until he was able to obtain up to 94 percent of its pro tein in the fibrin clot which resulted from treating its solutions with thrombin His thrombin was fractionated from fresh horse serum The conclusion from these careful experiments was that the small quantity of non coagulable protein unaccounted for was merely a minor contamination with what we would now term serum globulins Hammarsten s work on the calcium factor will be reviewed on p 8

L C Wooldridge [508] had considerable justification for some of his claims to priority over the Dorpat school in the investigation of phospholipids ('lecithins) in the blood clotting system He obtained many interesting experimental facts but his tissue fibrinogen theory and many aspects of his work were too unorthodox to gain him wide recognition Even the publication of his obscure works by Horsely and Starling in 1893 failed to shake the widespread acceptance of the thrombin theory

4 THE CALCIUM FACTOR: Early work of Arthus Pekelharing Hammarsten Sabbatini

Alexander Schmidt was never convinced of the need for calcium in the thrombin forming reaction There had been previous suggestions [201 404] for a role of calcium in blood clotting but the significant evidence was provided by Arthus and Pages in 1890 [19] They used oxalates or fluorides to prevent clotting and restored it by simple re addition of calcium salt

Pekelharing [372 373] confirmed this and introduced citrates for the same purpose This worker improved upon Schmidt's methods for obtaining prothrombin Dilution and acidification of plasma with weak

and impure thrombin by modern standards His numerous experiments endeavored to search out all possible factors influencing the clotting mechanism Proplastic' i e fibrinogen containing--liquids such as hydrocele fluid or plasma obtained from blood kept fluid with $MgSO_4$ and other neutral salts (cf Hewson [218]) were clotted by Schmidt's fibrin ferment He obtained good evidence for a precursor of the coagulant The terms thrombin (for fibrin ferment) and pro thrombin (for its precursor) were introduced in his later writings Thus Schmidt has deservedly been called the father of the thrombin theory Many details of his particular theories and terminologies have long since been discarded and need not be reviewed A few words may be said however about some of the experimental facts In attempting to unravel the thrombin problem Schmidt did much to implicate (he thought) both (1) a plasma protein 'paraglobulin ' and (2) tissue or cellular factors which he called zymoplastic substances ' As to the first it did seem rational at the time to look for a proteolytic 'split product' accompanying the postulated enzymatic breakdown of fibrinogen to fibrin

Schmidt and his pupils did a great deal with the tissue materials noted under the second head namely zymoplastic substances ' These were obtained both in aqueous extracts and by means of fat solvents hence chiefly lipoidal in character Heat stability and many other properties were investigated The most significant conclusion about these zymoplastic agents was that they were not thrombin but could participate in the activation of prothrombin

The essential technique whereby Schmidt sought to characterize tissue fractions was first to obtain an alcoholic extract ('zymoplastic substance') then a succeeding watery extract (cytoglobulin') and its acetic acid precipitable fraction (preglobulin') and finally an insoluble residue ('cytin') Of historical interest were the observations (1) zymoplastic substance promoted clotting under a variety of test circumstances some of which led to the conclusion (above) that it could participate in the activation of prothrombin to thrombin (2) the defatted cellular extractives especially preglobulin had considerable clot inhibiting powers e g for mixtures of $MgSO_4$ plasma and fibrin ferment (thrombin) (3) zymoplastic substance however was able to restore clotting in these inhibited mixtures

Schmidt s final summing up of his views on coagulation [421] included the idea of something normally present in blood which could be regarded as an antithrombin This term was actually introduced by Fano (1881) to designate the thermolabile (on boiling) agent which caused incoagulability of blood after peptone injections in the dog as first shown in Carl Ludwig's laboratory by Schmidt Mulheim (1880) Extracts from the medicinal leech contain a substance hirudin which Haykraft [217] showed to have similar 'antithrombic' powers

It was generally known in this era that: (a) the 'thrombic' action of fresh serum progressively decreased on aging even for a few hours (b) thrombin clotted plasma less readily than a fibrinogen solution (c) the weak thrombin preparations of that time had little if any capacity to produce coagulation (thrombosis) on intravenous injection into experimental animals

Even when the blood remained coagulable the clots would soon undergo lysis As little as 1/60 vol of normal dog plasma could prevent this indicating that normal plasma contains a powerful antifibrinolysin This however is reduced or absent in the above cases involving failure of liver function Nolf performed many noteworthy experiments but his theories were heterodox In brief he tried to bring into a dual clotting fibrinolytic scheme: (1) fibrinogen (2) 'thrombogen ' which is possibly equivalent to the orthodox prothrombin although Nolf also identified it with the antifibrinolytic agent (3) 'thrombozyme 'possibly equivalent to Schmidt's zymoplastic substance or Morawitz's thrombo kinase since Nolf asserted that it was produced by certain white cells (thrombocytes and platelets) and by the endothelium of the capillaries Nolf accepted thrombin only as a by product of the reaction between the above three substances and not as the coagulant itself as in the thrombin theory He used the term 'thromboplastic' for a heterogen eous group of agents including wettable surfaces e g ground glass calcium oxalate in colloidal suspension chloroform and similar emulsification which assist in the colloidal reactions The throm boplastic agent intervenes solely to make the reaction possible between these (3 cited) precursors It acts as a catalysator And so also do the alcoholic or aqueous tissue extracts It may be remembered that Freund [174] and Bordet & Gengou [58] retarded blood and plasma clotting by use of paraffined surfaces

7 THE HOWELL THEORY

Around 1910 W H Howell and his pupils were re investigating (a) the *thrombin fibrinogen reaction* (Rettger 1910; Howell 1910) (b) the intravenous injection of thrombin (Davis 1910) and (c) the clotting of peptone and hirudin plasmas especially by glycerinized (Cecil 1911) tissue extracts In a 1911 paper Howell [230] carefully reconsidered the role of thromboplastic tissue extracts and because of their ability to clot the above mentioned inhibitor containing plas mas he concluded in favor of an alternative to Morawitz's theory namely that tissue extracts act only by neutralizing certain clot inhibitors Furthermore he argued for a type of (natural) inhibitor which does not merely antagonize active thrombin but actually prevents the formation of thrombin from prothrombin Hence the new term antiprothrombin' and the specific idea (Howell's thromboplastin) that tissue extracts particularly the thermostable lipoidal fraction act by neutralizing antiprothrombin

In 1912 independent of Zak [514] Howell [231] identified the thromboplastic agent as cephalin in some form of protein combination Lecithin whether from tissues or egg yolk was inactive Howell's cephalin preparation followed the method of Thudichum [464 465] pioneer in the field of brain chemistry Diakonow had separated lecithin from other brain lipoid fractions and shown that its hydroly tic products were glycerophosphoric acid choline and fatty acids such as oleic and margaric (ref [103]) Thudichum found that there were a variety of phosphorylated lipoids (phosphatides; phospholipids) differing especially in their nitrogenous basic groups Retaining the name leci thin for those containing choline the new term cephalin was given to

acetic acid separated this agent by a principle which we would now characterize as isoelectric precipitation Pekelharing however went to undue lengths to identify his material as a 'nucleo-albumin' and suggested that it had a cellular perhaps platelet origin These were not useful ideas and they somewhat lessen the value of his contributions He did insist upon the importance of calcium salts in activating pro thrombin to thrombin (fibrin ferment) but he loses us in his further speculations about its transfer of calcium to the fibrinogen in the act of clotting

Hammarsten [206] provided more convincing evidence of the specific importance of calcium in prothrombin activation while deny ing it any really significant role in fibrin formation By oxalation and 'isoelectric' precipitation of his fibrinogen and thrombin he obtained reagents which interacted to give a fibrin analyzing as little as 0 005 percent calcium This could reasonably be dismissed as a trace impurity (but see Howell's comments [238])

The question raised by citrates which are unlike oxalates in that they do not precipitate calcium was settled at the turn of the century by Sabbatini [417], who showed that they do remove Ca^{++} ions by forming inactive CaCit anion complexes

5 THE MORAWITZ-FULD THEORY

Morawitz [345 346] and Fuld & Spiro [181] clarified ideas concerning the role of tissue factors in the clotting mechanism Fuld's term 'cytozyme' was later adopted by a minority of workers notably including Bordet [55] whereas most European investigators accepted Morawitz's term 'thrombokinase ' The argument was still by analogy with a tacitly accepted enzyme hypothesis and it may be remembered that Pavlov (1899) had just discovered enterokinase to explain the in testinal activation of pancreatic trypsinogen into active trypsin The Morawitz Fuld theory did clearly state that both calcium ions and tissue factor were needed to convert prothrombin into thrombin

Morawitz [345] also gave us the term 'metathrombin' for the inactive form of thrombin in serum believed due to some combination with antithrombin which Morawitz following earlier leads by Alex ander Schmidt thought he was able partly to reverse with an acid and alkali treatment

6 THE HETERODOXY OF NOLF

P Nolf [351] in the later 1900's restudied the phenomena of peptone shock in experimental animals particularly in relation to the liver He not only confirmed earlier French workers (Contjean Gley Hedon and Delezenne) as to the hepatic origin of antithrombin (which Nolf preferred to call 'antithrombosin') but also gave good evidence that the liver produced fibrinogen and antifibrinolysin Nolf was espe cially interested in the fibrinolytic phenomenon pointing out the loss of fibrinogen due to appearance of an active proteolytic enzyme (fib rinolysin) in the blood partly explaining the incoagulability encoun tered not only in peptone shock but also in liver poisoning with phos- phorus (Corin & Anciaux 1894) or with chloroform (Doyon 1905)

of prothrombin calcium and kinase (tissue extract) Howell's 'oppo s e results" were thought to be due possibly to differences in the two prothrombin preparations or in the test substrate (purified fibrinogen vs dialyzed oxalated plasma) Howell failed to mention a much more significan point namely that Mellanby's 'kinase' was a crude aque ous tissue extract whereas Howell's 'thromboplastin' was purified cephalin. Howell cites Fuchs' [177] suggestion that the prothrombin complex might be the same as the 'proserozyme' postulated by Bordet and Delange [56] No further comment is made in the review but I know from personal conversations with Howell that he was unimpressed with this idea Bordet's proserozyme was said to change to serozyme (prothrombin) on contact with wettable surfaces but heparinized plasma or prothrombin solutions are unaffected by the surface of the container Howell [238] did not bring the heparin cofactor into the antiprothrombic thesis but only into the antithrombic action of heparin Thus " unlike hirudin, heparin has no inhibitory effect upon the reaction between thrombin and fibrinogen although " heparin added to plasma or serum, causes the production in these liquids of a true antithrombin by a reaction with some unknown thermolabile constitu ent of the blood." Let us anticipate some of the modern era of our theme to record the reasons for the refu ation of the Howell theory This came about because of failure experimen a ly to verify the essen tial facts which the theory requires Here are the answers to some key questions

1) Are significan amounts of heparin to be found in the blood nor mally? The best answer is that of Jaques [249] who used a reliable chemical assay method which failed to find more than 10 micrograms per 100 ml in normal human bloods This is only about 1/10 the a mount of the heparin needed to keep blood from c otting in vitro Hep arinemia can occur in peptone-shock and other anaphylactoid reac tions [255]

2) Can heparin form a prothrombin antithrombin type of complex? Because of its highly acidic groups heparin can combine with basic groups of many proteins etc (Fischer [162]) Charga [75] obtained evidence of a stoichiometric combination between heparin and prota mine (a basic split-product of salmon-roe protein) It can form a polyanion complex with fibrinogen experimen ally [42] No one how ever has isolated anything like the postulated heparin prothrombin compound. The inhibitory actions of heparin have proved multiple and com lex. They are still insufficiently understood but much that is now known can explain these actions without need for postulating Howell s com lex [425]

3) Does thromboplastin neutralize heparin and so ease prothrom bin? As to the first part of this question let us for the moment iden tify thromboplastin with cephalin (thromboplastic phosphatide) Phos phorylated lipids are acidic in much the same way as the sulfonated heparins Hence a direct neutralization seems improbable chemically There is much more reason and evidence (e g Chargaff [75]) to think of both the phosphatide and the heparin as competing for the basic groups of proteins Cephalin combines stoichiometrically with prota mine [74] Heparin the stronger acid can 'devita- cephalin' from this com lex. "When heparin is made to react with the thromboplastic

those containing ethanolamine In 1915 Howell set his pupil Jay McLean to test in the blood clotting system all phosphatides described in the literature to that time An unexpected result will receive due consideration in a later paragraph The data for Howell's last paper [239] on The isolation of thromboplastin from lung tissue were completed just before his death in 1945 but it was his daughter Dr Janet Howell Clark who wrote it up for publication kindly submitting it for my appraisal before sending it to the publishers This paper showed the progress Howell had made toward characterizing thromboplastin as a protein compound of a phospholipid from which it was possible to remove the protein leaving a residue with marked thromboplastic activity the chemical nature of which has not been determined

Howell [233] used a crude preparation of prothrombin simply obtained by precipitation of plasma with acetone washing with ether drying on a Buchner funnel and subsequently extracting with alkalinized water or saline None of his prothrombins failed to activate with calcium alone In fact many of them activated to thrombin 'spontaneously' and this was enhanced by treatment with $CHCl_3$ and other lipid solvents [71] Howell [229 232] also prepared a thrombin by 8% NaCl extraction of washed blood clots (see p 5) and could purify this by shaking with chloroform until it no longer gave any reaction to chemical tests for phosphorus These data caused Howell to reject the European thrombokinase idea and insist that calcium alone could activate prothrombin Reviewing these matters in his 1916 Harvey Lecture Howell [235] agreed that thermolabile (50° 60°C) aqueous tissue extracts or relatively thermostable cephalin do assist in prothrombin activation but he proposed an alternative explanation namely that under normal conditions the prothrombin is protected from the activating influence of the calcium ions by a combination of some kind with an inhibitory agent or antisubstance and that cephalin exerts its accelerating effect upon coagulation by neutralizing the influence of this inhibitory substance thus liberating the prothrombin so that calcium can convert it to thrombin This is the essential Howell theory

Howell's pupil McLean [326] in the above mentioned study of a variety of relatively crude phosphatides found some that instead of aiding had a marked inhibitory effect upon coagulation These included two preparations of 'cuorin' (method of Erlandsen 1907) and a 'heparphosphatide' (method of Baskoff 1908) In personal conversations with Dr Jay McLean during visits to Columbus Ohio around 1940 he told me how elated he had been to discover antiprothrombin But he had to leave for active service in World War I and missed the follow-up work in which Howell and Holt [242] and Howell [237] established the more important aspects of this anticoagulant action including need for a plasma co factor and identified the agent not as phosphatide but as a sulfonated polysaccharide amine which Howell called heparin In his 1925 Pasteur Lecture [236] and 1935 Physiological Review article [238] Howell clearly identified his antiprothrombin with heparin He did note that Mellanby [330] was not able to obtain any evidence that heparin combines with prothrombin finding that it did not prevent the activation of prothrombin in a mixture

of prothrombin calcium and kinase (tissue extract) Howell's oppo site results" were thought to be due possibly to differences in the two prothrombin preparations or in the test substrate (purified fibrinogen vs dialyzed oxalated plasma) Howell failed to mention a much more significant point namely that Mellanby's 'kinase' was a crude aque ous tissue extract whereas Howell's 'thromboplastin' was purified cephalin Howell cites Fuchs' [177] suggestion that the prothrombin complex might be the same as the 'proserozyme' postulated by Bordet and Delange [56] No further comment is made in the review but I know from personal conversations with Howell that he was unimpressed with this idea Bordet's proserozyme was said to change to serozyme (prothrombin) on contact with wettable surfaces but heparinized plasma or prothrombin solutions are unaffected by the surface of the container Howell [238] did not bring the heparin cofactor into the antiprothrombic thesis but only into the antithrombic action of heparin Thus " unlike hirudin heparin has no inhibitory effect upon the reaction between thrombin and fibrinogen although heparin added to plasma or serum causes the production in these liquids of a true antithrombin by a reaction with some unknown thermolabile constitu ent of the blood Let us anticipate some of the modern era of our theme to record the reasons for the refutation of the Howell theory This came about because of failure experimentally to verify the essen tial facts which the theory requires Here are the answers to some key questions:

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2) Can heparin form a prothrombin antithrombin type of complex? Because of its highly acidic groups heparin can bombine with the basic groups of many proteins etc (Fischer [162]) Chargaff [75] obtained evidence of a stoichiometric combination between heparin and prota mine (a basic split product of salmon roe protein) It can form a polyanion complex with fibrinogen experimentally [42] No one how ever has isolated anything like the postulated heparin prothrombin compound The inhibitory actions of heparin have proved multiple and complex They are still insufficiently understood but much that is now known can explain these actions without need for postulating Howell's complex [425]

3) Does thromboplastin neutralize heparin and release prothrom bin? As to the first part of this question let us for the moment iden tify thromboplastin with cephalin (thromboplastic phospholipid) Phos phorylated lipids are acidic in much the same way as the sulfonated heparins Hence a direct neutralization seems improbable chemically There is much more reason and evidence (e g Chargaff [75]) to think of both the phosphatide and the heparin as competing for the basic groups of proteins Cephalin combines stoichiometrically with prota mine [74] Heparin the stronger acid can 'deviate' cephalin from this combination ' When heparin is made to react with the thromboplastic

those containing ethanolamine In 1915 Howell set his pupil Jay McLean to test in the blood clotting system all phosphatides described in the literature to that time An unexpected result will receive due consideration in a later paragraph The data for Howell's last paper [239] on The isolation of thromboplastin from lung tissue were completed just before his death in 1945 but it was his daughter Dr Janet Howell Clark who wrote it up for publication kindly submitting it for my appraisal before sending it to the publishers This paper showed the progress Howell had made toward characterizing thromboplastin as a 'protein compound of a phospholipid" from which it was possible to remove the protein leaving a residue with marked thromboplastic activity the chemical nature of which has not been determined '

Howell [233] used a crude preparation of prothrombin simply obtained by precipitation of plasma with acetone washing with ether drying on a Buchner funnel and subsequently extracting with alkalinized water or saline None of his prothrombins failed to activate with calcium alone In fact many of them activated to thrombin 'spontaneously' and this was enhanced by treatment with $CHCl_3$ and other lipid solvents [71] Howell [229 232] also prepared a thrombin by 8% NaCl extraction of washed blood clots (see p 5) and could purify this by shaking with chloroform until it no longer gave any reaction to chemical tests for phosphorus These data caused Howell to reject the European thrombokinase idea and insist that calcium alone could activate prothrombin Reviewing these matters in his 1916 Harvey Lecture Howell [235] agreed that thermolabile (50° 60°C) aqueous tissue extracts or relatively thermostable cephalin do assist in prothrombin activation but he proposed an alternative explanation namely that under normal conditions the prothrombin is protected from the activating influence of the calcium ions by a combination of some kind with an inhibitory agent or antisubstance and that cephalin exerts its accelerating effect upon coagulation by neutralizing the influence of this inhibitory substance thus liberating the prothrombin so that calcium can convert it to thrombin This is the essential Howell theory

Howell's pupil McLean [326] in the above mentioned study of a variety of relatively crude phosphatides found some that instead of aiding had a marked inhibitory effect upon coagulation These included two preparations of 'cuorin' (method of Erlandsen 1907) and a 'heparphosphatide' (method of Baskoff 1908) In personal conversations with Dr Jay McLean during visits to Columbus Ohio around 1940 he told me how elated he had been to discover antiprothrombin But he had to leave for active service in World War I and missed the follow up work in which Howell and Holt [242] and Howell [237] established the more important aspects of this anticoagulant action including need for a plasma co factor and identified the agent not as phosphatide but as a sulfonated polysaccharide amine which Howell called heparin In his 1925 Pasteur Lecture [236] and 1935 Physiological Review article [238] Howell clearly identified his antiprothrombin with heparin He did note that Mellanby [330] was not able to obtain any evidence that heparin combines with prothrombin finding that it did not prevent the activation of prothrombin in a mixture

titative in many experiments Yet one finds all sorts of inconsistencies and contradictions For example compare (a) every fibrinogen solu tion which has been tested has coagulated with calcium chloride alone and the statement (b) but a consideration of the properties of kinase and prothrombin and the constitution of plasma point to the conclusion that calcium alone will not clot fibrinogen solutions As 'thrombo kinase' Mellanby used diluted testicular extracts from the bled cock erel after the manner of Rauschenbach (1881) With calcium this readily clotted his 'fibrinogen' and he properly interpreted this as ev idence for prothrombin contamination in the substrate In this early work [327] Mellanby did not separate prothrombin but merely deduced its presence and activation from crude experiments especially varying the concentration of kinase

Mellanby's 1909 thrombin (fibrin ferment) was simply the fluid expressed after complete coagulation of a fibrinogen solution by kinase and calcium chloride ' It was with such reagents that he at tempted to quantitate the thrombin fibrinogen reaction both as to clotting times and fibrin yields He varied (a) calcium and other alka line earth salts (b) neutral salt (NaCl) concentrations (c) acid (HCl) or alkali (NaOH) and (d) oxalate citrate fluoride etc

In 1930 Mellanby [328] presented a purification technique for 'pro thrombase ' which was his term for prothrombin based upon the pre vailing enzyme idea Starting with dilution and acidification patterned after the earlier methods of A Schmidt and Pekelharing and essentially anticipating what we would now call an effort toward isoelectric precip itation he triumphed over many difficulties Thus the precipitate obtained from the diluted plasma is a complex mixture The greater portion of it consists of prothrombase fibrinogen and serum globulin but the pigments of serum cholesterol and thrombokinase (!) are al- ways present in variable quantities The separation of prothrombase from this complex precipitate has presented considerable difficulties Nevertheless by diluting with lime water and bubbling CO_2 through to pH 6 8-7 0 and calcium bicarbonate (thus formed) about 0 0092 per cent he claimed splitting of the prothrombase from the complex The amount of calcium was said to be too small to activate the prothrom base in the time required for filtration and subsequent precipitation by acetic acid to pH 5 3 The final product was dried with acetone (cf Howell see p 10) Admissions of its frequent contamination with thrombin conflict with assertions of no spontaneous activation e g on dialysis Mellanby's product did have considerable potency and in some tests was partly stable to boiling for 5 min at pH 7 8

Addition of tissue extract alone (thrombokinase) to a solution of prothrombase always causes the generation of a corresponding quan tity of thrombase in the course of a few hours This fact holds good even when the prothrombase has been prepared from solutions contain ing no calcium salts i e oxalate plasma and the replacement of cal cium bicarbonate by sodium bicarbonate in the second stage of the pro cess However minute amounts of calcium have a marked effect on the velocity of activation Hence Mellanby balked at any final conclusion thus no definite statement on the place of calcium salts in the reaction may be made until thrombokinase has been isolated in a pure condition

lipoprotein from lungs [87] the phosphatides are split off the protein carrier and a heparin-protein compound results This compound in losing its thromboplastic property has acquired a new one it exerts a markedly anticoagulant effect [83] " The protein moiety of the thromboplastic agent must obviously also be considered Furthermore the plasmatic cofactor (cf [242]) has been shown [67 151a] to intervene in the antiprothrombic as well as the antithrombic actions of heparin and this requires further explanation As to the second part of the question real evidence is still lacking that prothrombin was bound in the first place

4) Can prothrombin be activated by calcium salts alone? This essential corollary to the Howell theory is seriously questioned by more recent work including data presented in this dissertation in which it will appear from experiments with both Howell type and with newer types of prothrombins that cephalin or tissue thromboplastin play a direct role along with calcium in the conversion of prothrombin to thrombin Furthermore wholly new factors [363 364] have now become recognized as equally essential in the highly complex thrombin forming reactions In summary then the Howell theory was too simple and has not stood up to the inexorable pressure of more modern experimental facts

8 J MELLANBY STUDENT AND LEADER IN THIS FIELD

In 1909 J Mellanby working in the Physiological Laboratory at Cambridge (England) as George Henry Lewes' student ' published two [327] noteworthy papers in the field of blood coagulation and in his maturity he returned to this field with further investigations in the 1930's [328 329]

Mellanby first employed fowl plasma as recommended by Delezenne (1897) to prepare fibrinogen by a dilution and acidification technique in principle similar to the techniques of Alexander Schmidt [420 421] He rejected Hammarsten s [205] salting out method because of baneful influences of salts on proteins and stated it is clear that if fibrinogen is a globulin the best method to prepare it is to dilute and neutralize any fluid containing it This procedure will certainly precipitate all the globulins contained in the fluid used but there has not yet been adduced a certain proof that any globulin other than fibrinogen exists in plasma As a matter of fact the majority of experimental results point to the conclusion that fibrinogen is the only globulin contained in plasma [327] This error is not only confounded completely by more modern knowledge (ref [114]) but was untenable even with the data on plasma and serum proteins available in Mellanby's time Furthermore it is inconsistent with many observations in his own work Elsewhere Mellanby cited Pekelharing [373] as identifying prothrombin with 'kinase ' We have noted (p 8) that this authority did believe prothrombin to be a 'nucleo-albumin' of cellular origin but he was very clear that it was a plasma component and quite distinct from the zymoplastic cellular substance of Alexander Schmidt

Credit must be given to the attempts of the Cambridge student to further the knowledge of blood clotting including his efforts to be quan-

1935 [381]) Many including Alexander [6] and ourselves [147] have come to prefer $BaSO_4$ In their 1938 purification of prothrombin by CO_2 (under pressure) elution from the $Mg(OH)_2$ plasma adsorbate and its subsequent transformation to thrombin by means of calcium and lung extract the Iowa workers were assisted by W H Seegers [435 428] After establishing his own laboratory at Wayne University Detroit Seegers and his colleagues further purified prothrombin [424 431 425] and thrombin [433] from bovine plasma to give us the most potent and possibly the purest preparations to date There still remain some questions of trace impurities and it must be remembered that both adsorbents and precipitants yield several other components of the clotting system the presence of which in the final product may call for some critical comment

B MODERN VIEWS ON BLOOD CLOTTING

The following selective outline of present day (1956) knowledge is presented with slight personal bias but is essentially the consensus of the modern 'group thinking' All workers agree that many details and perhaps still undiscovered factors await further exploration and that the present 'working hypothesis' useful as it is especially in many clinical applications yet fails to give an adequately detailed answer to the deceptively simple but truly involved questions: (1) why does blood not clot in the normal circulation and (2) why does it clot (a) in thrombotic conditions and (b) when shed? To unravel the complexities we shall try to analyze the clotting mechanism step by step bearing in mind however the inter relatedness and frequent simultaneity or overlap of the several and successive reactions

1 SKELETON SCHEME OF CLOTTING REACTION

Figure 1 presents an intentionally over simplified scheme It merely indicates the two 'classical' phases of clotting The essential and final (second) phase is the conversion of the plasma protein fibrin ogen into fibrin clot through the physiological intervention of thrombin From one point of view this reaction is colloidal namely a change from 'sol' to 'gel' involving many physico chemical considerations including surface electrical charges depending upon polar groups of the reacting molecules and the 'ionic atmosphere' of the surrounding salt rich medium From another point of view it is enzymatic referring to a specific mode of action of thrombin

Thrombin is not normally present in the circulating blood Indeed there are antithrombic factors naturally presen to inhibit or remove any thrombin which might possibly form in the blood The plasma does contain a precursor protein prothrombin The classical first phase of clotting then is concerned with the conversion of prothrombin to active thrombin This is particularly complex and will require a searching analysis of what our scheme collectively calls 'activators' Possibilities of inhibition of the activator mechanisms are indicated in the scheme and will also require investigation It is questionable whether we can group such first phase inhibitors as 'antiprothrombic' without becoming involved in the old controversies of the Howell theory

Mellanby's 'thrombase' (thrombin) described in a later paper [329] was obtained by slightly modifying the prothrombase method so as to permit conditions favorable to 'spontaneous' activation

We have cited the foregoing in some detail in order to emphasize the unsatisfactory state of experimentation with the blood coagulation problem as late as the 1930's Fibrinogen had come a long way with Hammarsten [205] and Howell [230] but prothrombin and 'kinase' and even Howell's [232] purified thrombin were far short of critical requirements It is in fact all too evident that most workers preferred to avoid facing the issue of contaminant impurities Most deductions were made as if these did not exist even if at times this meant a considerable twisting of the experimental results It would not seem that it was asking too much by 1930 to ensure that fibrinogen preparations were free from all traces of prothrombin Yet nowhere can we find a product which failed to clot with calcium and tissue extract except possibly in some of the earlier experiments of Howell [230] Most of the time however Howell only used calcium salt in the control test since according to his theory this is all that is needed to activate prothrombin

9 MILLS: PRACTICE VS PRECEPT

C A Mills of Cincinnati was the other notable authority whose personal interest and exchange of ideas played a role in the present writer's entry into the blood clotting field at that time Of his voluminous writings [334 337] little will be said in this review We do recall however Mills' insistence on a critical control of fibrinogen preparations for freedom from prothrombin Mills used cephalin as well as calcium in the preliminary test Unfortunately much of Mills' magnificent conceptual approaches ended in data which must be discredited because of the failure to realize in practice what he preached in theory The critical control was inadequate when most needed to support the interpretation of his experiments The fact was that Mills [338] obtained clotting in his 'prothrombin free' fibrinogen on adding lung extracts or platelets in the presence of calcium This led him back to the heterodoxy of a second type of clotting and even to the nomenclature of Wooldridge's [508] 'tissue fibrinogen'

The refutation of Mills' experiments and conclusions was made in 1934 by the Iowa pathologists Smith Warner and Brinkhous [442] In searching for a suitable fibrinogen these careful workers finally adopted the technique of adsorption with $Mg(OH)_2$ for removal of traces of prothrombin as previously recommended by Fuchs [180] Their second contribution was in the preparation of the lung extract itself Being highly vascular lung is ordinarily full of blood the prothrombin from which can appear in crude aqueous extracts By perfusing the lung with water or saline the Iowa workers were able to obtain a subsequent prothrombin free extract This did not clot the critically prepared fibrinogen even on adding calcium

Prothrombin adsorption techniques were not new Pickering [375] reviewed a variety of reagents which had been tried for this purpose We may list $Ca_3(PO_4)_2$ (Bordet & Delange 1914 [v 55]) $BaSO_4$ (Dale & Walpole 1916 [98]) $Mg(OH)_2$ (Fuchs 1929 [176]) $Al(OH)_3$ (Quick

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2 EXTENDED SCHEME OF CLOTTING AND HEMOSTATIC MECHANISMS

Figure 2 presents a more detailed scheme of the major factors in blood clotting and hemostasis with some indication of interrelation ships For clarity all inhibitor factors are omitted and must be con sidered separately Briefly summarized the following ideas are covered:

1) Hemostasis is a physiological function involving:
   (a) vascular integrity and vasoconstrictor mechanisms
   (b) platelets (and other formed elements) participating in the cell thrombus
   (c) fibrin clot
2) Vascular factors will not be considered except to mention:
   (a) vasoconstrictor role of serum serotonin derived (in part) from 5 hydroxy tryptamine of platelets
   (b) role of damage to vascular endothelium in (i) initiating plate let participation (adherence clumping breakdown) and (ii) af fording site for fibrin deposition
3) Platelet factors (incompletely reviewed) including:
   (a) the above vasoconstrictor (2a)
   (b) a platelet component which reacts with plasma factors in 'thromboplastin generation '
   (c) a platelet 'accelerator '
   (d) platelet retractor factor
   (e) a role of thrombin in the platelet mechanisms
4) Plasmatic thromboplastic factors (AHF PTC etc) are indicated as reacting with platelets (3b) in 'thromboplastin generation '
5) Injured tissue sources of a 'complete' thromboplastin are indicated
6) Prothrombin is the inactive precursor of thrombin in plasma normally activated by Ca^{++} and thromboplastin in the presence of (7) and (8) which are two essential plasma 'co factors '
7) Proconvertin yields convertin in the presence of Ca and thrombo plastin
8) Proaccelerin yields accelerin under the influence of a trace of thrombin
9) Active thrombin formed by the indicated reactions
10) Conversion of fibrinogen into fibrin 'Serum factor' (p 21) should be included
11) Clot-retraction subsequently results from action of a platelet factor (3d)
12) Fibrinolysis and clot resolution result eventually from activation of the proteolytic enzyme system whose components are also in dicated in the scheme
13) Various inhibitors should be included in the concepts outlined above (see pp 29 33)

3 THE THROMBIN FIBRINOGEN REACTION

Most of the early work in this area was poorly quantitative and be set with uncertainties and errors caused by impurity of the reagents used Modern materials are by no means completely free from all

uncertainties but have clearly proved their value in many exacting quantitative tests Particularly significant are data which have been obtained with physical measurements e g opacity rigidity and tensile strength of clots light scattering (differential refractometer) streaming double refraction diffusion viscosity electrophoresis ultracentrifugation and electron microscopy Solubility studies [89] are very valuable in preparative procedures when worked out in relation to the five variables: (1) temperature (2) pH (3) ionic strength (4) protein concentration and (5) content of water miscible organic solvent e g ethanol zinc glycine etc Monumental contributions in these areas of our field have come from the late E J Cohn's Department of Physical Chemistry and Plasma Fractionation Laboratories at Harvard [114]

a) Fibrin yields The modern chemist seeks to follow the course and kinetics of reactions especially enzyme actions by timing changes in the substrate as well as by measuring amounts of the final products This logical approach is beset with technical difficulties in the case of the thrombin fibrinogen reaction The extent to which these may be overcome is illustrated in the selected references [159 348 60] Fibrin yields as measured by the amount of coagulated protein recoverable in washed clots is of course primarily dependent upon the initial amount of fibrinogen available That the thrombin concentration determines only the rate of the clotting reaction but not the ultimate fibrin yield is clearly shown in Figures 3 and 4 These are from experiments with purified fibrinogen and thrombin in the author s laboratory [60] Figure 3 shows effects of varying the relative thrombin concentration a thousandfold but employing a constant amount of fibrinogen Despite clotting time (see below) variations between 18 sec and 30 min the final (10 day) fibrin yields are identical and all 100 percent of the original (coagulable) protein in the fibrinogen These findings are strong evidence for the enzymatic nature of the thrombic action Figure 4 shows fibrin yields with time employing four different thrombin concentrations The linearity of the logarithmic plots (of residual substrate concentrations) within small limits of experimental error indicates a first order reaction [273] again consistent with an enzymatic activity of thrombin

NOTE: The results do not rule out a pseudo first order reaction in which preliminary phases might be slower than the action of thrombin Other things which do not affect the fibrin yield within reasonable limits are (1) temperature (2) pH (3) salt concentration e g NaCl $CaCl_2$ (4) 'fibrinoplastic'* colloids e g acacia (except for minor occlusion effects) (5) minor partial denaturation of fibrinogen ('profibrin' of Apitz 1937) Things which may affect fibrin yield include (a) extremely low fibrinogen concentrations (0 002 0 003 percent) where 'soluble fibrin' i e intermediate polymers of insufficient degree of aggregation forms a significant proportion of the end product according

*Ferguson (1940 [134]) proposed a revival of this old term of Alexander Schmidt in order to indicate agents which shorten clotting times through nonspecific effects best interpreted as adsorptive or similar ways of bringing fibrinogen and thrombin 'reactive groups' together and thus facilitating their interaction

to Morrison [348]; (b) occluded proteins [348]; (c) reversible inhibitors of the type studied by J D Ferry S Shulman et al in the Wisconsin Laboratories [160 438 440] for the same reason; (d) fibrinolytic enzymes if active which are apt to be contaminants of thrombin and some fibrinogen preparations and can destroy fibrinogen and fibrin at approximately equal rates according to experiments in our laboratory [291]

b) Clotting times The earliest efforts to obtain quantitative information about the clotting process were by measurement of the time required to form a visible or a solid clot Because so many factors enter into the determination of any 'clotting time' the whole concept would seem to be fraught with extreme empiricism In fact it is possible to advance the view that there is no such thing as clotting time since the blood does not normally clot in the circulation so that we must merely be measuring the results of highly artificial conditions In actual practice however these conditions can be defined sufficiently to give clotting time determinations real value in following and interpreting the clotting phenomena As a technical point particularly referring to experimental systems of artificially isolated clotting agents most workers prefer to time the first appearance of a definitely visible clot (fibrin strands) rather than a solid gel (tube invertible) Both end points are empirical and depend upon a certain amount of fibrin formation by no means indicative of the ultimate fibrin yield The appearance of successive crops of clots when a weak thrombin is used is a well-known phenomenon Factors known to influence clotting time include:

1) Concentration of thrombin: the stronger the thrombin the shorter the clotting time other things being equal This is a common feature of enzyme reactions Figure 5 shows an experiment from the author's laboratories in which over at least a 10 fold range of relative thrombin concentrations the clotting-times obeyed an 'inverse law' an old idea in the coagulation literature [163] It is perhaps unfortunately very difficult to define the precise experimental conditions for obtaining this result In most experiments the deviation from a linear relationship is significant It may still be possible however to translate clotting times into relative thrombin concentrations ('units' of activity) by use of this type of reference curve (simple plot) obtained under closely similar experimental conditions [20]

2) Concentration of fibrinogen over a considerable range this has remarkably little effect on the clotting time which is not so strange remembering that we time only a partial change in the substrate (see above) The quality of the clot varies however and may affect reading of the end point Particularly with very weak fibrinogens a poor clot and the probability of slower and less complete polymerization (referred to under fibrin yield) may cause longer clotting times

3) Temperature: It has long been known that cooling retards the reaction while warming accelerates it up to temperatures where complications appear because of thermal denaturation of the fibrinogen Thrombin is much more heat resistant but this depends on pH salt content and other considerations Some plasmas and impure fibrinogens may be completely clear when frozen and kept at 20°C

but show clots on subsequent thawing This may indicate that traces of thrombin can act on fibrinogen slowly even in the frozen state but it is just possible that the clotting occurs in the act of thawing

4) pH: There is an ill-defined pH optimum for the thrombin fibrinogen reaction with some dependence upon the experimental conditions It is important that the normal pH of the plasma (about 7 4) is close to this optimum Clotting times are prolonged but only slightly until we approach the extremes of (a) 5 3 in the acid range or (b) 10 in the alkaline region In (b) we run into the 'reversible inhibitor' problem (see (7)) of insufficient polymerization of the fibrinogen [466] This may also be a factor in the acid region [405]

5) Ionic strength: The most important action of sodium chloride and most common neutral salts *is a delay in clotting times with increasing* ionic strength [115] Numerous data on this go back all the way to William Hewson (1770) Polyvalent anions [191] e g ferrocyanides ferricyanides have especially marked inhibitory effects Table I summarizes an experiment of the author's [145] The experiment shows that the inhibitory action of strong neutral salt (4 5% NaCl) *which can be removed by subsequent dilution does not alter* fibrinogen or thrombin and only prevents the appearance of visible fibrin in a mixture of these two agents Confirmation of the fact that clotting times become progressively shorter the longer the thrombin fibrinogen mixture is held with the 4 5% NaCl before dilution is regarded as evidence that the essential step in fibrin formation proceeds independently of the presence of the salt which merely inhibits or retards the gel formation (precipitation) *cf* Apitz [18]

6) Specific ion effects: Calcium is anomalous in that it definitely accelerates the clotting time of thrombin fibrinogen mixtures in a narrow range of low concentrations e g 0 008 0 032 molar in one of our experiments- although this depends on total ionic strength [115 310] By removal of the Ca effect EDTA hinders the fibrinogen fibrin transition [409]

7) Reversible inhibitors: In an extensive recent study by Shulman [438] 42 (out of 80) substances tested markedly delayed or inhibited clotting in thrombin figrinogen mixtures under this author's test conditions This type of inhibition is reversible by dialysis Important conclusions as to the 'polymerization process by which fibrinogen is transformed into fibrin can be drawn from these and other experiments We shall consider this topic in a later paragraph

8) Fibrinoplastic' colloids [60]: The shortening of clotting times by these agents has been mentioned in the proceding section on fibrin yields and the footnote on p 17 suggests an explanation of their mode of action Gum acacia is of some importance in that it is used to stabilize the reactivity of fibrinogen in the Iowa 'two stage' prothrombin (and thrombin) bioassay [443] Protamine has similar effects [134] over a wide pH range We have also noted it with a highly purified lactoglobulin Also see 'platelets (p 51)

9) Partial denaturation of fibrinogen [60]: The present author s view is that the effect of this is due to the same general type of 'fibrinoplastic action as noted under 8) That all of the denatured fibrinogen is included in the fibrin clot may be evidence of an adsorptive or occlusion phenomenon The Wohlisch [504] 'denaturation theory' of clotting is now largely discredited

10) Adsorptive removal of thrombin: Compared with whole blood or plasma clotting (see later) the surface of the container has little effect on the thrombin fibrinogen reaction An exception is seen in the case of very weak thrombins where adsorption onto glass etc re moves part of it from solution and hence lengthens the clotting time This can be minimized by coating the tube with non wettable silicone [60] It is a common experience that poorly washed glassware thus contaminated with a trace of thrombin can clot fibrinogen solution stored therein later

c) Nature of fibrin formation Progressing from the uncertain ex periments of Apitz [18] Laki and his colleagues [278 274] produced evidence of a step wise process in the formation of fibrin from fibrin ogen under the influence of thrombin The latest interpretation of these and other data was well brought out in the presentations and dis cussions at the 1951 Conference of the Josiah Macy Jr Foundation [274] J T Edsall (representing the Cohn laboratories) J D Ferry (formerly of the Cohn team but now at Wisconsin) K Laki (now at Bethesda Md N I H Laboratories) D F Waugh (Massachusetts In stitute of Technology) were the chief authorites at this meeting with the present author contributing a minor role The modern concept of fibrin formation is as follows:

Fibrinogen is a fibrillar protein whose long filamentous molecules approximate 38 A in diameter and 700 A in length (usually) with a mo lecular weight close to 500 000 (perhaps 330 000) In solution these large molecules moving with Brownian movement exhibit consider able swirling and 'steric hindrance' During clotting however they line up 'like logs in a stream' and unite end to end and also side-to side until the 'polymerization aggregates' are large enough and hydro phobic enough to separate from the watery solution as visible fibrin Fibrin filaments or needles were seen to appear during clotting under the ordinary transilluminating microscope by Ranvier (1873) and Schimmelbusch (1885) Stübel in 1914 and Howell in the same year demonstrated them very clearly with the dark-field microscope Howell [234] discussed the problem of the fibrin gel formation in 1916 Electron microscopy first used for observing fibrin by Wolpers and Ruska [507] in 1939 has been used to support the modern views by Schmitt [422] by Hawn & Porter [212] Hall [204] Kaesberg & Shulman [264] and others Even the electron microscope does not re solve the fibrinogen molecules and the simplest polymers or protofi brils but it does show variations in meshwork density and the fibrillar composition together with cross striations of the fibrils which appear uniform under the ordinary (including dark field) microscope Con sideration of clotting conditions (see above) in relation to electron mi croscopy and many physical measurements (see earlier) affords strong support for the modern concepts Thus under inhibitory conditions much smaller aggregates are formed and resemble the normal early or lag phase in which there is yet no visible clot but viscosity bire fringence opacity and other data indicate some intermediate polymer ization Under some conditions the fibrin polymer is reversible undergoing disaggregation and resolution e g in urea guanidine lith ium bromide (strong solutions) etc [307] Irreversible and urea insoluble clots have recently been shown to depend on two things

namely (1) calcium [266] and (2) a special 'serum factor' by Laki & Lóránd [277] Lóránd [304 305 307] and Shulman [439] An earlier indication of this was found in 1944 by Robbins [405] in studying the somewhat similar problem of solution' of fibrin in acid (0 03% HCl) or alkali (0 5% Na_2CO_3) These data assist in explaining some of the peculiar actions of calcium when present in the thrombin fibrinogen (+ serum factor) mixture (see above) the 'serum factor' being a common contaminant of 'purified' fibrinogen (but see p 65)

It is suggested below how a limited attack on the fibrinogen molecule activates polar groups which enable the altered substrate molecules to aggregate in the polymerization processes The electrical charge distribution on fibrinogen molecules has been studied and there is good evidence that Coulomb forces play a role in the preliminary phases of aggregation Salt inhibitors (see ionic strength) are to be interpreted (Ferry [157]) as 'shielding' of the polar groups by the charged 'ionic atmosphere' contributed by the salt Further stages involve van der Waal forces (e g adsorption etc) and perhaps specific chemical bonds (currently debatable) [158] At all events the final steps do not involve thrombin Like other enzyme systems thrombin is not truly a part of the fibrin end product It is a practical fact however that a considerable amount of thrombin is adsorbed onto the fibrin clot [434] Indeed upon this depended the success of early methods of obtaining thrombin by extraction from washed blood clots

d) Thrombin This is by no means the simple proteolytic enzyme suggested by the 19th century coagulationists It is unlike trypsin [458] Most of the alleged evidence for its proteolytic powers [219 380] can be dismissed as due to contaminant fibrinolysin [149 462] or possibly as a certain degree of solubility of fibrin [247] However Sherry and his associates [437] have recently shown that fibrinolysin free thrombin can hydrolyze certain bonds in some synthetic polypeptides e g TAME (tosyl arginide methyl ester)

Lóránd [307] has contributed some provocative new ideas He uses Seeger's citrate thrombin (see p 23) and (a) bovine fibrinogen or (b) human fibrinogen [308] to show:

1) non protein N is liberated pari passu with the clotting action of thrombin;
2) the glutamic acid missing from the fibrinogen after the action of thrombin can be identified in a 'fibrino peptide' which has been isolated and characterized [309 306];
3) in bovine fibrinogen thrombin is thought to split arginine glycine bonds [30]; (NOTE: It does not do so in the case of insulin according to Middlebrook)
4) alanine replaces glutamic acid in the N terminal amino acid residues of human as compared with bovine fibrinogen but the general process is the same;
5) electrophoretic studies show a characteristic pattern and the paper strip method permits characterization of the split products The pattern with thrombin is quite different from that produced by fibrinolysin

Lórand concludes that

a) the fibrino peptide' is a by product which does not participate in the fibrin polymerization but does account for the

small quantity (about 2%) of nitrogen not recoverable in fibrin even in optimal clotting of the highly purified fibrinogen now available

b) the fibrinogen molecule activated by this action of thrombin now contains some 20-30 acidic groups which can very well account for the first phases of polymerization to fibrin;

c) with prolonged action of strong thrombin e g 3 4 mg thrombin acting on 150 mg fibrin for 24 hours at 37°C complete fibrinolysis can occur (Guest and Ware [203]) associated with further small N P N increase [307] Hence

d) fibrin is just a transitory stage in the continuous degradation process of fibrinogen by thrombin '

In this special way therefore we may now return to a proteolytic concept of the action of thrombin on fibrinogen

e) Other coagulants, etc, Properly purified fibrinogen solutions are quite stable and are never 'spontaneously' coagulable Whereas thrombin is the coagulant always required under physiological circumstances some other means of coagulating fibrinogen are available experimentally Examples are

1) crystalline papain [112] a proteolytic enzyme from the paw paw (papaya) fruit Unlike thrombin (usually) papain readily goes on to digest the clot

2) staphylocoagulase [186] a product of the reaction of a pro staphylocoagulase from broth cultures of certain micrococci with a plasma factor [187] which is apparently unrelated to prothrombin

3) certain snake venoms e g 3 species of Bothrops and 3 or 4 of Crotalus according to Eagle's studies [111]

True fibrin clotting should be distinguished from a variety of fibrinogen precipitations (which are reversible) and denaturations (most of which are not) The pseudo clot with ninhydrin [82] is a case in point Some of the author's [155] dark field observations of (a) thrombin (b) papain and (c) ninhydrin clots are shown in the accompanying photomicrographs (Figure 6): (III) and (IV) show the nondescript appearance of ninhydrin clots compared with the fibrillar gels formed with (I) thrombin and (II) papain Laki [275] has recently confirmed the last

4 THE CONVERSION OF PROTHROMBIN TO THROMBIN

It is with this area of the clotting field that the major investigations of this dissertation are concerned The best modern prothrombins do not change into thrombin 'spontaneously (but cf [425]) Among the activators mentioned in the schemes of Figures 1 and 2 are (1) calcium (2) thromboplastic factors (3) 'accelerator' factors (4) 'convertor' factors Noncommittal terms are used in approaching these problems with an open mind

a) Purity of prothrombin preparations, Prothrombin is difficult to purify The early preparations of Schmidt Pekelharing Hammarsten Mellanby Howell Eagle [110] and others were undoubtedly too crude when reviewed in the light of modern advances [425 276 491] Nevertheless they gave us many of the basic ideas concerning the activation

of prothrombin e g by calcium ions and thromboplastic (thrombokin ase) factors with possibilities of inhibitors which have merely been confirmed with the modern 'purified' prothrombins Contamination with (a) traces of activators (b) traces of inhibitors (c) fibrinolytic enzyme (or precursor etc) (d) perhaps other factors may still need critical consideration even in the best modern preparations

b) Nature of prothrombin [425] Prothrombin is a specific pro tein accompanying the globulin fraction(s) of the plasma proteins Its quantity in the blood (15 mg/100 ml) [425] is very much less than that of fibrinogen (280 mg/100 ml) In fact unlike the latter it does not show an independent peak in plasma electrophoresis [491] Seegers and his colleagues have prepared the most potent hence presumably the purest prothrombin to date and their work provides the most reliable data on its characterization [425] Seegers believes that most of the difficulties in obtaining a biochemically pure prothrombin come from some activation and other marked alterations in the molecule during the purification storage and experimental proce dures The purest preparation examined by Lamy & Waugh [280] was monodispersed in the ultracentrifuge but with some boundary spread ing Its physical constants led to computation of the molecular weight at 62 700 and the shape of an ellipsoid molecule of 119 × 34 A Chemical analysis yields a trace of S (cystine and methionine) some 4 3 percent polysaccharide carbohydrate [431] and 3 75 percent ash Perhaps because of 'derivatives' (Seegers) prothrombin solutions show more than one electrophoretic component but the major compon ent constitutes 70 90% of the total [432] The thrombin yield may de pend upon changes during storage in the dry (lyophilized) state but generally averages 23 000 units* (two stage assay) per mg tyrosine or 1400 units/mg dry weight Highest: 32 300 35 800 *

c) Biophysics of thrombin formation [280]

(1) 'Biothrombin' is a convenient term [280] for the thrombin ob tained from purified prothrombin in the conventional manner i e addi tion of calcium salts tissue thromboplastin accelerator globulin (pro)convertin etc Maximal transformation to thrombin is obtained only when the prothrombin concentration is relatively low For physi cal studies concentrations of prothrombin of the order of 0 5 percent must be used (about 10 100 times as concentrated as used to obtain maximal activation) Under these conditions not more than 50% of the 'maximal' thrombin yield can be secured With the (lung) thrombo plastin allegedly removed by high speed centrifugation subsequent ultracentrifugation shows a single symmetrical peak having an average sedimentation constant S ~ 4 8 corresponding to that of the original prothrombin Some boundary spreading suggests polydispersity how ever The specific activity of the material at the peak was 30 000 (thrombin) units/mg tyrosine

(2) 'Citrate thrombin : distinguishes a new product which Seegers et al [432] obtained in 1948 simply by incubating purified prothrombin in 25 percent sod citrate Several other salts (but not all) could be used and the reaction could be modified by certain diphenyl sulfones [432] It is extremely difficult to evaluate the biological significance of this very artificial method of thrombin formation However it must have biochemical significance Refractive index ultracentrifugation

and electrophoretic studies on 'citrate thrombin' give evidence of an initial splitting of prothrombin into two similar (molec wt : 34 000) fractions but on dilution there appear 'aggregates' and 'disaggregates' and continuing changes over a 24 hour period [280]

(3) 'Trypsin thrombin': is also discussed in the cited reference [280] and will be mentioned again later (p 25)

These modern data are difficult to analyze but do seem to indicate that prothrombin can split up into a number of molecular fragments some of which can re aggregate probably into new agents Autoprothrombin I has been identified with proconvertin [491 425 13] while autoprothrombin II has properties resembling PTC [429] In the formation of both 'biothrombin' and 'citrate thrombin' small but significant amounts of CCl_3COOH soluble carbohydrate and tyrosine compounds are liberated confirming the idea that thrombin forms from prothrombin as the result of some 'splitting' process [427] These very latest ideas cannot yet be integrated with the older data on mechanisms of prothrombin activation from the viewpoints which are presented in this dissertation

d) 'Prothrombinogen' (?) In 1949 Quick & Stefanini [396] claimed to distinguish between *'free' prothrombin and a precursor form for* which they suggested the name 'prothrombinogen' They compared their experiments with older data of Nolf [351] and Bordet [55] and concluded that their prothrombinogen differed from Bordet's 'prosero zyme' in only a few particulars Only a part of the plasma prothrombin is believed by Quick to be in the precursor form and Quick & Hussey [389] (cf [154]) have recently attempted to explain the normal 'prothrombin time' of infants (see our Table LII) on the basis of a lack of prothrombinogen Few contemporary coagulationists consider that Dr Quick has enough factual evidence to support his shrewd ideas (see Macy Foundation Conferences) but Ware [491] is now inclining toward the prothrombinogen idea The present author [154] remains unconvinced

e) Calcium in relation to the conversion of prothrombin into (bio)thrombin Older plasma clotting experiments by Nordbo [352] and McLean & Hastings [325] extended the earlier idea of Sabbatini [417] that clotting depends upon calcium ions There is a definite optimum of calcium required approximately that available in the blood under physiological conditions Not merely the calcium ion concentration but the actual ion 'activity' (in terms of the Debye Huckel theory) requires consideration Nordbő particularly studied this and further pointed out that the 'available' calcium ions in a mixture of plasma oxalate (or citrate) and added calcium salt depend upon a time consuming process of attaining an equilibrium of the different forms (free Ca^{++} inactive (CaCit) anion and protein bound Ca etc) It is because of this time factor that a several fold excess of citrate or oxalate must be added to prevent clotting and conversely that much less than the stoichiometric equivalent of calcium to anticoagulant need be added to restore clotting (cited [125])

Protein-bound calcium is present in the blood plasma normally and in prothrombin and other materials prepared from it unless very modern techniques of complete decalcification (e g by ion exchange resins) are used to obviate this A number of workers (cited [125])

have continued to suggest that (protein)-'bound' calcium can play a role in clotting Even Quick [383 394] still retains the idea that pro thrombin is a 'calcium compound ' There is little point in arguing about this since the weight of evidence supports the conclusion that ionized calcium must participate at some point in the conversion of prothrombin to thrombin A possible reconciliation of the two view points will be suggested from data presented in this dissertation Under all ordinary conditions of activating purified or even crude pro thrombins omission of ionized calcium salt or the presence of suf ficient excess of citrate or oxalate fails to result in thrombin forma tion by thromboplastin (and adequate accelerator and convertor fac tors) The special conditions under which thrombin may be formed apparently in the absence of ionized calcium include (1) Seegers' [432] 'citrate thrombin' formation (see above) which we shall not attempt to explain; (2) experimental mixtures such as those of Milstone [339] (see p 48) and of recent workers [40] with the thromboplastin generation test (see p 48) which are best explained as intermediates' containing bound Ca (which however was ionized in an earlier phase)

f) Thromboplastin and the activation of prothrombin This topic will be explored in much detail in the body of this thesis Aqueous (saline) tissue extracts of many kinds are powerful activators of purified (and crude) prothrombins in the presence of calcium salts and adequate amounts of 'accelerator and 'convertor' factors Puri fied cephalin seems to have thromboplastic actions but much weaker especially with Seegers' and similar prothrombins It is now gener ally regarded as an 'incomplete' thromboplastin Platelet suspensions also act as an 'incomplete' thromboplastin So does a red cell lipid fraction [105] However when platelets or cephalin are incubated with several other plasma factors (including AHF PTC etc see later) a complete' thromboplastin is 'generated ' Modern workers are still trying to find full explanations for these experimental facts (see p 47)

g) 'Thromboplastic enzyme.' The coagulant property of weak solu tions of pancreatic trypsin which some earlier observers erroneously concluded to be a 'thrombin like' action was clearly shown by Eagle & Harris [112] to be the result of some role in the activation of pro thrombin in experimental test systems Some of the author's exper iments with crystalline trypsin will be mentioned in this thesis More recently Schultze & Schwick [423] have examined extensively the con ditions under which trypsin transformation of prothrombin to thrombin can be carried out Lamy & Waugh [280] report some ultracentrifuge studies (see 'trypsin thrombin ' p 24)

The author [141] spent several years trying to establish a similar role for the natural plasma tryptase (fibrinolysin or plasmin) and possibly to give it an important role in the initiation of ordinary blood clotting [136] However the final outcome [476] was evidence that trypsin inhibitors could prevent the proteolytic actions of fibrinolysin without removing the 'thromboplastic' effect quite contrary to the findings with trypsin Pending possible reopening of this line of inves tigation it must be concluded that any thromboplastic effects of fibrin olysin preparations must be due to some unidentified impurity which they contain Ratnoff Hartmann & Conley [402] also investigated the

relationship between proteolytic activity of plasma and blood coagulation and in a later paper Ratnoff & Colopy [401] suggest that they may have been dealing with 'Hageman factor' (see later under Hemophilia)

h) 'Accelerator' factors in prothrombin conversion Many modern workers were fully aware that calcium and thromboplastin did not provide a sufficient answer to the question of prothrombin conversion The most significant step however came from the very extensive studies of P A Owren [361] on a human patient suffering from a peculiar bleeding disorder which he named 'parahemophilia' [362] The new factor which this patient was finally shown to lack was called 'factor V' and later 'proaccelerin' by Owren [364] Quick [384] had earlier evidence for a 'labile factor ' Another lead was the 'thromboplastin cofactor' of Fantl & Nance [119] Owren [361] also provided evidence for conversion of his 'factor V' (proaccelerin) into a 'factor VI' (accelerin) Ware & Seegers [493] prepared the new agent under the name 'accelerator globulin ' by which it is now widely known or 'AcG' for short They also found differences between 'plasma AcG' (proaccelerin or factor V) and 'serum AcG' (accelerin or factor VI) the latter being formed from the former by a trace of thrombin Stefanini & Dameshek (p 20 of ref [453]) list other possible synonyms

The present author and his colleague Dr Jessica H Lewis have studied (1) cases of congenital hypoproaccelerinemia [297]; (2) differences between proaccelerin and accelerin in the activation of prothrombin (cf [492]); (3) destruction of AcG by fibrinolysin [299]; and (4) its role in performance of the two stage prothrombin assay and in determining prothrombin consumption in clinical cases [153] In summary

(a) (pro)accelerin is essential for the conversion of prothrombin to thrombin by calcium and tissue thromboplastin;
(b) a trace of thrombin converts the precursor form (proaccelerin) into the active accelerin;
(c) AcG has more effect upon the thrombin yield than upon the rate of activation which is somewhat delayed when AcG is very inadequate however;
(d) satisfactory assay methods are now available both by a 'one stage' (specific) technique [395] and a 'two stage' [493] including modifications (see pp 58 59) developed in the author's laboratory for application to clinical cases of hemorrhagic disorder Plasma serum and platelets may be tested by these techniques;
(e) serum except from the ox cat or rabbit [349] is apt to be devoid of this factor because of lability and due to the fact that it is used up during clotting;
(f) AcG levels fall after hepatectomy [322] or liver injury [461]

i) 'Convertor' factors in prothrombin activation The discovery of this factor also owes much to the first clearly identified clinical case of B Alexander and colleagues [9] They named the factor SPCA (serum prothrombin conversion accelerator) and obtained evidence for a precursor (pro-SPCA) [11 8] Owren was on the track of this additional factor [360 366] but it was Koller (et al [271]) who correctly reinterpreted Owren's data and named it 'Factor VII ' It is probably

the same factor which (a) Warner Brinkhous & Smith [497] suggested as a prothrombin 'convertibility' factor and (b) Mann et al [319] called co thromboplastin We prefer to adopt the names proconvertin (for the precursor) and convertin (for the activated form) as suggested by Owren [364] There are other suggested synonyms (p 20 of ref [453])

Graham & Hougie [198] report that mixture of the bloods of Alexander's original case R [9] and ours R. S. [298] results in mutual correction of the coagulation defects Together with other experimental findings this suggests that there may be more than one factor involved under the terms SPCA proconvertin factor VII stable factor etc Calcium and thromboplastin convert proconvertin to convertin and abolish the lag phase in prothrombin activation to thrombin [295]. Serum often contains much unaltered proconvertin as well as some convertin the total activity being much elevated

In deficiency of proconvertin* prothrombin conversion is very slow and inadequate Restoration by partly purified preparations from human [9] or bovine plasma (our laboratories) completely restores both rate and yield of thrombin formation Dr Lewis and the author [298] reported two cases of severe congenital 'hypoproconvertinemia' including Stuart in whom the prothrombin consumption was reduced This had not been observed in earlier case reports [9] due, we believed to their being a milder degree of this defect cf [198] We have two more recent cases of VII lack (unpublished) in whom the prothrombin consumption was within normal limits Our congenital 'hypoproconvertinemics' did not respond to vitamin K_1 unlike the milder acquired hypoproconvertinemias accompanying hypoprothrombinemias in (a) vitamin K deficiency of infants (b) liver disorders or (c) after the drugs dicumarol tromexan phenylindanedione etc (author's data) confirmed by Naeye [350] The one stage [101] assay method is satisfactory for clinical use [298] We [298] have also had some success with a two stage technique which enabled us to compute a 'proconvertin' index (see p 51) *Stuart factor was not separated however

ß) Plasma factors in thromboplastin generation While tissue thromboplastin may very well play a significant role when blood is shed over injured tissues it cannot explain the good clotting which occurs in blood which has been obtained directly from a blood vessel e g by venepuncture with every precaution to avoid tissue contamination The necessary thromboplastin is generated in the blood itself by a complexity of reactions involving (1) platelets (2) several plasma factors which are present normally but deficient in hemophilia and some recently identified 'hemophilia like conditions (3) some of the other prothrombin activators The platelet factors and the thromboplastin generation test will be discussed in a later section

(1) Hemophilia and related problems Only a few key points will be reviewed from the extensive recent literature in this field and most of the older work will be excluded

(a) Hemophilia A Notwithstanding efforts to account for the hemophiliac s clotting difficulty on the basis of some inhibitor e g the 'antithromboplastin' idea of Tocantins et al [470 474 475] the major accumulation of evidence points to a specific clotting factor present

in the plasma of normal persons but deficient in the hemophiliac Patek & Stetson [>68] and later other workers in F H L Taylor's group at the Thorndike Memorial Laboratory of the Boston City Hospital contributed convincing evidence for this antihemophilic factor (AHF) which they called 'antihemophilic' globulin (AHG) Koller [270] lists it as 'factor VIII' AHG accompanies fibrinogen etc in the Harvard Laboratories' plasma 'Fraction I' It is somewhat unstable and difficult to isolate and purify Bidwell [46] in England claims a good bovine plasma fractionation but the problem needs further solution [488]

AHF is utilized or consumed in the clotting process With other factors it participates in thromboplastin generation from platelets (or cephalin) (p 46) In the natural clotting of hemophilic blood or recalcified hemophilic plasma the 'prothrombin consumption' is very defective Following this lead of Dr Brinkhous's we have also developed a method depending upon the prothrombin consumption (1 hr at 37°C) in recalcified mixtures of 'substrate' (known severe hemophilic) and normal (vs test) plasma for quantitative estimation of plasma AHF levels These tests can also be modified to assay anti AHF [152] By changing the substrate (known cases) they also assay PTC and anti PTC etc

We shall say nothing about the important familial aspects of hemophilia except to mention the very interesting work on canine hemophilia [197]

(b) PTC deficiency (Hemophilia B) In February 1952 Dr Lewis and the author [294] were investigating a 'bleeder' who seemed to be made worse instead of better by blood transfusions Our tests immediately showed that he had a circulating anticoagulant (see later) but by preparing plasma fractions free from this inhibitor an underlying clotting defect was disclosed which was definitely not hemophilia By withholding transfusions the anticoagulant gradually diminished as shown by the tests illustrated in Figure 7 clearly revealing evidence (the long recalcification clotting times in the last tests) of the basic clotting defect In April 1952 Aggeler and colleagues [5] in California published the first clearly identified case of the new 'hemophilia like' disease which they named PTC deficiency (plasma thromboplastin component) Using their techniques we quickly confirmed that our case was the identical disorder but with a new type of inhibitor complication [294] A few months later Biggs Douglas Macfarlane and colleagues [51] published an English case and from the name of of the patient coined the term 'Christmas disease' Koller's name for PTC is 'Factor IX' [270]

An earlier lead was provided by a number of observers particularly the Argentinian Pavlovsky [370] namely that the mixing of two 'hemophilic' bloods from certain cases resulted in correction of the clotting times both in vitro and in vivo Very interestingly some of the classical 'hemophiliac' families (e g Von Tenna) extensively studied in Europe have turned out to be not what has recently been accepted as hemophilia but the similar PTC deficiency (or Christmas disease) To avoid the confusion which has resulted Dr Koller [270] of Switzerland has proposed a revision in nomenclature namely (1) Hemophilia A (the usual type); (2) Hemophilia B (PTC-deficiency);

and (3) others (see below) Another interesting recall is the obtaining of an 'antihemophilic globulin' from serum (rather than plasma) by the Dutch workers Bendien and Van Creveld [39] a year or two ahead of the Boston (Thorndike) group [368] Unlike AHG PTC persists in the serum since it is not completely consumed in clotting The present author suggests therefore that the Dutch workers were really dealing with hemophilia B and that their 'globulin' was a PTC preparation

(c) PTA deficiency (Hemophilia C) R L Rosenthal and co workers [413 412 414] have uncovered cases allegedly lacking a third plasmatic thromboplastic factor (PTF) which they call plasma thromboplastin antecedent (PTA) We have confirmed the defective prothrombin consumption in one of their cases

(d) 'Fourth factor' deficiency (Hemophilia D)? Spaet Aggeler & Kinsell [446] encountered another type of case with a possible fourth plasma component but did not rule out an inhibitor of PTC

(2) 'Hageman Factor.' Ratnoff & Colopy [401] observed three patients with prolonged bleeding times but without significant hemorrhagic symptoms Their studies suggest yet another factor the chief action of which is failure compared with normal plasma to accelerate the clotting of platelet poor plasma obtained for normal blood centrifuged at high speed without anticoagulant They gave it the name 'Hageman factor after the first patient and obtained correction of the defect with a fraction prepared from normal $BaSO_4$ adsorbed heated serum

(3) 'Factor X.' Duckert Koller and colleagues [108] investigating sera from various pathological conditions in the thromboplastin generation test concluded that still another factor must be recognized ('Factor X') It is said to be deficient particularly in sera from cases with hepatitis or persons under treatment with a dicumarol derivative (Marcoumar) X is now used for Stuart factor (see SUPPLEMENT)

5 INHIBITOR PROBLEMS

a) Inactivators of thrombin Quoting Seegers [425] the following mechanisms participate in the removal or neutralization of thrombin (the comments are those of the present reviewer):

1) Thrombin may be adsorbed on fibrin Fibrinolysin can release it [268]
2) Large amounts of thrombin are neutralized by antithrombin This antithrombin does not require heparin for its action This is the classical antithrombin of serum or plasma [505 506] It acts over a period of time in a progressive manner [190] and is limited in degree i e a given amount of antithrombin can neutralize up to a definite limit of thrombin Morawitz [344] used the term metathrombin for the inactive product which thrombin forms with the antithrombin of serum His claim for partial reactivation by treating serum with acid and alkali was confirmed by Gasser [184] Astrup & Darling developed a method of assaying antithrombin [21] which they later [22] distinguished from the 'thrombin inhibitor' which heparin forms with some labile plasma component (co inhibitor)

They state [22] while normal antithrombin seems to combine with thrombin to form an undissociable compound the compound between thrombin inhibitor and thrombin as well as the compound between heparin and thrombin coinhibitor seems to be highly dissociable That fat solvents can inactivate antithrombin was noted in the early literature chloroform especially being used for this purpose [351] although it has the disadvantage of also destroying thrombin [476] Seegers et al [433a] have done many experiments with ether especially to remove this antithrombin from defibrinated plasma Sternberger [456] used alcohol and has obtained some very provocative results [457] Fibrinolysin will not work [476]

3) Heparin co factor together with heparin interferes with the reaction of thrombin and fibrinogen This will be discussed further in a subsequent paragraph (p 31)

4) Antithrombin accelerator activity arises during the clotting process and also neutralizes thrombin Seegers' [425] new agent (AA = antithrombin accelerator) refers to the anti thrombin demonstrable after ether extraction of defibrinated plasma To it is attributed the disappearance of thrombin formed therein after activation of the prothrombin by calcium (lung) thromboplastin and (platelet) AcG the latter reagents not being the source of the alleged AA factor Further (Seegers [425]): There is an inhibitor of the antithrombin accelerator action which we call antithrombin accelerator inhibitor AA inhibitor is removed by $BaCO_3$ adsorption and is recoverable in the citrate eluate These new ideas of Seegers are highly provocative particularly in regard to his suggestion that AA inhibitor is proconvertin or associated with proconvertin in partially purified materials

b) Heparin [242 382 151a 151 22] it is now generally agreed that little if any heparin is present in the circulating blood normally [249 61] The protamine titration method [250] of measuring 'heparin-like' activity in plasma is subject to questions of interpretation but does agree with anticoagulant tests e g prolongation of clotting times of whole blood [15] or recalcified plasma routine in author's laboratory p 61 when heparin is known to be present This is true both in vitro and in vivo In vivo however heparin may be removed from the circulation by (a) renal excretion [254] (b) metabolic alteration or (c) a heparinase enzyme [248 252] Chemical extraction and assay of the metachromatic color reaction of heparin with azure dyes are the most convincing methods [342] They have recently been used in conjunction with paper chromatography [36] Wilander's work [501] concerning the origin of heparin from the tissue basophils or Ehrlich mast cells is now generally accepted These mast cells appear to break down and release significant amounts of heparin (proved conclusively by chemical extraction) in certain pathological conditions particularly those involving an anaphylactoid type of reaction [255] They also yield histamine [195] which probably forms a complex with heparin [403] Rocha e Silva [407] implicates a fibrinolytic enzyme activation and finds histamine release to be an accompaniment Hyperheparinemia can cause bleeding disorders [448]

c) Heparin co factor (heparin complement) [242] Fitzgerald & Waugh [164] have recently fractionated co factor from Cohn's plasma 'Fraction I' Their tests extend the work of Astrup & Darling [22] and do not support Klein & Seegers' [268] idea of nterference with the thrombin fibrinogen interaction (also Glazko & Ferguson's [190] 'im mediate' antithrombic action) but rather point to a definitely time consuming reaction They conclude that heparin plus cofactor inacti vates clotting by removing thrombin by direct combination Thus the reaction is 'progressive' and 'limited' Further complications arise since reaction velocity specific capacity of cofactor (units T/mg C) and extent of reversibility (using protamine) are functions of cofactor concentration These authors [164] suggest that cofactor does not occur as such in plasma but that heparin cofactors in effect repre sent altered normal antithrombins

Some of the author's work with heparin and 'cofactor' etc will be presented in this thesis We shall not review the chemistry of the heparins nor the modern work on paritol treburon thrombocide sul fonated dextrans and other synthetic heparin like substances of cur rent interest particularly in the search for a cheap heparin substitute

d) Specific inhibitors of individual clotting factors These factors being protein in nature for the most part antibodies to individual clotting factors considered a priori are a possibility which recent investigations are beginning to support with experimental facts Such inhibitors are particularly significant when they can be demonstrated and shown to contribute to the bleeding tendency in clinical cases [453]

(1) Antiplatelet factors causing thrombocytopenia and inter fering with clotting and hemostatic functions are discussed on p 37 Normal and pathological factors which specifically inhibit one or other of the various components of the thrombin forming system are begin ning to receive serious consideration as follows:

(2) Anti AHF and anti PTC The present author and associ ates [152] presented a paper at the September 1955 Meeting of the American Physiological Society and a definitive publication will ap pear shortly By use of the prothrombin consumption test on specif ically deficient substrates (lacking AHF or PTC respectively) to which mixtures of normal and patient plasmas were added we assayed the anti AHF and anti PTC inhibitors in eight cases with hemorrhagic dis orders The routine tests on these cases showed prolonged clotting times but normal prothrombin time (Quick test) In five cases with primary hemophilia the anticoagulant could not be demonstrated by simple clot delay on mixing with normal blood or recalcified plasma but required the specific anti AHF test One hemophiliac bled to death in 18 days following a tooth extraction despite a record 400 trans fusions with whole blood plasma and antihemophilic globulin We could demonstrate removal by the potent inhibitor of the transfused AHF faster than it could be supplied In all five hemophiliacs the acquired inhibitor was specifically anti AHF whereas in the two PTC deficients it was anti PTC The eighth case of idiopathic ' in a female and showed a very high titer of anti AHF as well as an apparently high titer of anti PTC The last might be questioned on technical consider ations

Inhibitors of the above type have been reported in a very small mi nority of cases of hemophilia PTC deficiency certain allergies; some

systemic collagen diseases [175 453]; and questionably associated with pregnancy History of previous transfusion therapy can usually be elicited In one of our PTC cases the evidence was very clear that transfusions led to the recurrence of high titers of the inhibitor In this case and in the 'idiopathic' female electrophoretic analysis revealed an abnormal increase in serum gamma globulin This is further evidence that the inhibitor is acquired by some unusual type of 'immunity reaction' However the result in causing transfusions to become harmful instead of helpful complicates the clinical control of the bleeding problem

(3) Anti(pro)convertin (Factor-VII inhibitor) Wagner et al [487] have obtained certain fractions from normal dog plasma which specifically inhibit active convertin Jurgens [259] has prepared a similar agent from normal human serum and suggests that it has certain properties in common with plasma and serum antithrombin

(4) Anti(pro)accelerin (Factor-V inhibitor) has been questionably reported by Horder [223] in a case with a bleeding disorder It is said to be 'lipoid like' and to cause the factor V deficiency which explains the bleeding syndrome in the case cited *

(5) Antithromboplastin (A) in the sense of a circulating inhibitor of added tissue thromboplastin in cases with a bleeding tendency is suggested in several accounts referred to on p 209 of the new text by Stefanini & Dameshek [453] The present author suggests that the positive identification of a genuine antithromboplastin must fulfill certain criteria:

(a) it must specifically inhibit added tissue- i e 'complete' thromboplastin e g in the one stage prothrombin time (Quick) test

(b) there should be no alternative explanation possible on the basis of inhibition or lack of some other factor in the thrombin forming system

Hougie [226] has already pointed out some of the interpretive difficulties For instance if the inhibition is demonstrable only at high dilutions of the added thromboplastin it might be due merely to lack or inhibition of the plasmatic thromboplastic components (AHF PTC etc) In fact the test might be no more in essence than the performing of the partial thromboplastin test of Langdell et al [282] These authors indeed use it to assay the lack of AHF When Fantl & Nance [120] investigated an Australian woman and reported inhibition of human brain thromboplastin but not that of other species they might simply have been observing the influence of dilution on two different reagents which did not have true equivalency of thromboplastic potency in the first place

Among the several cases cited [453] the patient tested by Chargaff & West [81] and later restudied by Conley et al [92] shows that many turn out to be anti AHF anti PTC etc AcG controls are important also

Antithromboplastin (B) in the sense of Tocantins et al [474] refers to an alleged plasma factor for which the following claims are made:

*Ferguson et al described an unequivocal case of anti AcG (Proc Soc. Exp. Biol. & Med. 95 567 1957)

1) it is a constituent of normal (platelet free) plasma
2) it inhibits added cephalin or tissue thromboplastin but requires a special series of minimal plasma dilutions in order to demonstrate and assay its effects
3) it is less effective in the presence of wettable surfaces or after the blood has clotted
4) it is somewhat species specific and does not affect the thromboplastic action of Russell's viper venom
5) it can be concentrated in certain plasma fractions and in lipid rich tissue extracts Both these points were confirmed by Overman [358 359]
6) it is increased in cases of hemophilia [475]
7) it is also increased in post irradiation hemorrhage [471]

Dr Tocantins is a very able and careful worker but there is now considerable doubt as to the significance of the data under discussion One group [196] raises many objections including questions as to the control of ionic strengths in the limited dilution experiments

e) Summary of the inhibitor problems and their clinical significance From the foregoing brief review it is evident that the inhibitors are a little known and difficult area in the coagulation field More knowledge is needed because of their importance in occasional clinical cases Valid information has begun to accumulate as the result of the devising of specific testing techniques In some cases the diagnostic question is settled The therapeutic goal is elusive however Adrenal cortical hormones failed in an extensive trial on two of our clinical cases The relationship of inhibitor appearance to transfusions necessitates a searching review of this form of therapy There is need for more rapid advance in the difficult field of fractionation of the individual clotting factors from the blood in practical preparations which can be used for rational replacement therapy

BLOOD PLATELETS

A HISTORICAL

Several careful microscopists in the early nineteenth century must be credited with the discovery of the blood platelets although they were ignorant of their true nature and functions Most of these workers are cited in a 1934 review of the history of haematology by Sir Humphrey Rolleston [408]

Alexander Donne (1842) [106] termed them globulins' (a term still used in the French literature) but probably confused them with fatty particles of the chyle Addison (1841) possibly Wagner (1842) and certainly Andral (1843) [17] observed them in commencing clotting of the blood Andral thought them to be 'fibrin molecules ' as did Fr Simon G Zimmerman (1848 60) [516 517] also cited Gerber's 'free nuclei' and Fr Arnold's 'Elementarkorperchen as well as Simon s term 'Kugelchen Zimmerman used Simon s technic of receiving blood into potassium ferrocyanide and delayed clotting enough to permit investigation of the supernatant still more or less turbid fluid He also could receive the blood into neutral or carbonic acid salts In slowly clotting bloods he clearly noted the 'little bodies

(kleine Korperchen) before fibrin appeared and described the latter as forming homogeneous masses or threads often radiating from the 'little bodies' or in some cases from leukocytes He concluded in favor of the idea that these little bodies already exist in the circulating blood " As they resisted ether and warming Zimmerman showed they were not fatty globules but an organic (vesicular) formation (*organische Bildungen mit Inhalt gefullte kleine Blaschen*) Zimmerman also studied birds and amphibia and decribed the nucleated cells (distinguishable from leukocytes but confused with erythrocytes) which were termed 'thrombocytes' by later writers [100] He discussed their formation in terms of Schwann's cell theory" and suggested a lymphatic origin

In 1865 Max Schultze again noted them in human bloods as did Vulpian (1872) and Riess (1872) who thought they were remains of disintegrated leukocytes In 1874 William Osler [357] gave another classical description which distinguished them from bacterial micro organisms In 1877 Hayem [213 214] called them 'hematoblasts' or the 'third' (formed) element of the blood and insisted that they were precursors of the erythrocytes a view which he maintained as late as 1923 [216] While much of the confusion of platelets with the other formed elements may be blamed upon poorly controlled observation in vitro two more recently identified phenomena may be suggested as sources of error: (1) living leukocytes can lose fragments during observation (dark field) on glass slides in a shed blood drop [122]; (2) erythroplastid [116] formation can occasionally be observed under similar conditions from certain erythrocytes or their precursors

Figure 8 shows one of a series of 14 photomicrographs taken by a former colleague (Dr P H Ralph) over a 2 hr period in an observation of an erythroplastid forming from a bone marrow erythroblast in a case of Hodgkin's disease

Bizzozero's [54] work was most significant He described platelets in the circulation in the mesenteric vessels of rabbits and guinea pigs and later in the intact bat's wing He demonstrated their adhesive quality their participation in thrombi and their role in the coagulation of the blood

Lowit [311] tried hard to prove platelets to be mere artifacts e g leukocyte fragments but Bizzozero's data settled the controversy Eberth & Schimmelbusch (1886) gave a classical description [113] of experimental thrombosis including the role of the platelets and of fibrin in the transilluminated mesentery

J H Wright [510] identified the origin of the mammalian blood platelet from cytoplasmic fragments of the megakaryocytes of the bone marrow He also showed the phylogenetic relationship to the thrombocyte of the lower animals which serves similar physiological functions in these orders Dr W H Howell showed me his beautiful tissue preparations obtained for a 1937 paper [241] in which he stated: platelets represent a solid secretion from a unicellular gland the megakaryocyte This is a good way to emphasize the conception of a dual nature of platelets namely partly like a cellular element and partly as 'amorphous' material contributing to the chemistry of clotting etc

1 PLATELETS IN THE BLOOD UNDER NORMAL AND THROMBOTIC CONDITIONS

Many workers have confirmed and extended the observations of Bizzozero [54] and Eberth & Schimmelbusch [113] Several labora tories have shown fine cinematograph films of the circulation in small vessels including platelet observations e g those of (1) M Knisely [269] (formerly of Chicago now at Charleston S C); (2) the research team at the Hoffman La Roche Swiss laboratories; (3) the Boston Uni versity group under G P Fulton and B R Lutz [41]; (4) Jean Hughes at Liege

In vivo platelets are tiny little discs which appear lenticular or 'batonnet like'* when viewed edgewise Endothelial injury causes leukocytes and platelets to 'marginate' from the axial stream to the 'skimmed' plasma layer closest to the blood vessel wall Platelet plugs can be seen to form and seal off microscopic holes in an injured vessel particularly in venules The platelets adhere individually and to other platelets They also break away one by one or in clumps of varying size These may be arrested temporarily or more perman ently further along the course of the circulation

In observations on the formation of cellular thrombi in the frog mesentery on pricking the capillary endothelium with a microneedle (Kite Chambers micromanipulator) the author [127] noted the sig nificance of rate of blood flow In slower rates of flow all the corpus cular elements enter into the thrombus formation and the red cells being the most numerous largely preponderate In more rapid rates of flow it is the stickiest elements which have the best change of re maining adherent Hence the distinction between 'white' (thrombocyte or platelet) thrombi and 'red' or 'mixed' thrombi long recognized by pathologists Hayem's (1889) term clou hémostatique' and the German 'Blutstillenden Keil' are highly descriptive (p 82 of Quick [383]) Wharton Jones (1851) Mantegazza (1869) and especially Zahn (1872) [513] preceded Bizzozero [54] in demonstrating the importance of white thrombi for controlling hemorrhage after vessel injury

2 VISCOUS METAMORPHOSIS AND PLATELET ADHESIVENESS

The thrombotic platelet mass later evinces a hyaline or granular appearance for which the term 'viscous metamorphosis was coined by Ebert & Schimmelbusch [113] and later used by J H Wright and G R Minor [511] At one of the Macy Conferences [509] Helen P Wright reviewed this topic and described her quantitative approach based upon rotating the platelet containing fluids in gla s tubes for

*Few workers have bothered to check the term batonnet as originally used by Aynaud [24] N Rosenthal in H Downey s Hand book of Hematology [411] says baton like and the latest note of the Boston workers [41] uses bayonet like Actually Aynaud compared this platelet appearance to the little stick with tapered ends which is used in the childish game that the English call tip cat the Americans mumbly peg and the South Africans kennetjie

definite time periods and comparing the platelet count (hemocytometry) with a vaselined tube control She reduced 'platelet adhesiveness' by heparin and chlorazole dyes in vitro and by dicumarol in vivo Conversely adhesiveness was increased after operations or parturition at periods when the platelet count was also elevated and many young forms were circulating Similar findings were reported for the thrombocytosis following injections of adrenalin or of pyridine Splenectomy gave similar results but now there was little further effect from pyridine Moolten & Vroman [343] with an improved technique failed to confirm Helen Wright's suggestion of an increased platelet adhesiveness in hemophilia Her idea in this disease is also contrary to observations of many other workers Dr Wright's interpretations included the following (with the present reviewer's parentheses):
the factors which appear to maintain the various (formed) elements in suspension are first their surface charges (p 174 of Tocantins [469]) which are of the same (electrical) sign; and second their constant motion both as a mass and relative to one another 'It is believed moreover that the surfaces of the formed blood elements carry variable amounts of adsorbed water and that this helps to render them stable in suspension as well as augmenting the viscosity of the blood

their surface reactions may be in part dependent on some of the metabolic activities of the cell The platelet surface may be modified by substances normally (fibrinogen globulin) or abnormally (immune globulins of anti platelet sera) present in the blood Adhesiveness must also be related to alteration in the wettability or stickiness of injured vascular endothelium [419] It may be recalled that Roskam [415] collected many data leading to the suggestion that platelets are surrounded by a firmly adsorbed protein film to which many of their properties may be due That this is not necessarily fibrin or fibrinogen however is indicated by the normal platelet adhesiveness etc in a case of congenital afibrinogenemia studied by Pinniger & Prunty [376] and confirmatory observations by Alexander et al [10] and others [296] cited [7]

3 PLATELET 'AGGLUTINATION' (NORMAL)

According to Aynaud [24 25 26 27] platelets suspended in plasma may be agglutinated by a wide variety of substances e g gelatine egg albumin peptone gums lecithin heavy metals dyes and surface tension lowering agents such as ricin sod taurocholate and saponin The peptone plasma experiments were particularly interesting as showing that platelet agglutination could be independent of clotting Tocantins [469] reviewed many of the older papers dealing with platelet agglutination under various experimental conditions and in numerous clinical disorders A number of modern workers have restudied some of these problems on well washed (? plasma free) platelets especially aided by the silicone technique (see pp 67 68)

Copley & Robb [93] observed that washed platelets could be agglutinated by adding plasma or serum especially the latter and also by 'purified' preparations of globulin prothrombin thrombin placental tissue juice and plasma with a high concentration of heparin (!) This last finding was unexpected and in conflict with the data of C H Best

and colleagues [45] These Toronto workers inserted a glass chamber between an artery and vein in an experimental animal and observed platelet agglutination and adherence to the flat surfaced glass chamber microscopically The cinematograph film of these observations was re exhibited at the Macy conference (1949) Heparin caused a great reduction or even absence of observable platelet agglutination Dr Best and others [161] believe that some heparin preparations contain impurities which could explain the findings of Copley et al

4 PLATELET ANTISERA PLATELET GROUPS AND THE THROMBOCYTOPENIA PROBLEM

Following the 1905 suggestion of Marino Bedson [33 34 35] injected rabbit platelets into guinea pigs and obtained a platelet antiserum which caused thrombocytopenia and endothelial (capillary) damage when injected into 'non sensitized' animals Ackroyd [1] has recently reviewed the problem of "platelet agglutinins and lysins in the pathogenesis of thrombocytopenic purpura He includes:

a) the 'abnormal splenic secretion' theory of Frank (1925);
b) the efforts of Troland & Lee (1938) and others to obtain spleen extracts ('thrombocytopen') allegedly capable of reproducing the human thrombocytopenic condition in experimental animals;
c) the demonstration by Evans Duane et al (1949 51) of a 'thrombocyte agglutinating factor' in the sera of some ITP (idiopathic thrombocytopenic) patients;
d) several reports that transfused platelets survive for shorter periods in ITP cases;
e) Harrington's [207] convincing experiments in which by injecting himself with plasma from an ITP case he lowered his platelet count to dangerously low levels and was fortunate to recover;
f) confirmatory repetition of this extremely carefully conducted by Stefanini et al [454];
g) Tullis' [478] finding that 9 out of 18 sera from ITP cases caused agglutination and lysis of normal platelets in the presence of complement;
h) demonstration by both Harrington's [208] and Stefanini s [455] groups that platelet agglutinins could occur in some patients after pregnancy or transfusions; or
i) even in some individuals who have never been pregnant or transfused [455]; and
j) the confusion due to demonstrations [451 502] of fall in platelet count following transfusions of normal blood etc

This whole problem is beset with technical and interpretative difficulties but the following conclusions represent current thinking

1) antiplatelet agglutinins and lysins occur in humans sometimes without but usually with thrombocytopenic purpura;
2) there are platelet 'groups' or 'types analogous to those encountered with red cells;
3) 'acquired' cases of thrombocytopenia often have a demonstrable cause of agglutinin production Ackroyd [1] has made some very clear cut studies of drug induced (sedormid; quinidine)

thrombocytopenias showing that both drug and specific antibody are needed for the platelet destroying action;

4) complement is involved in the platelet lysis as it is in hemolytic and bacteriolytic immunity reactions

5 IN VITRO 'ALTERATION' OF BLOOD PLATELETS

In 1873 Ranvier [397] observed the microscopic changes of platelets and formation of fibrin in a drop of shed blood He described alterations in the shape of the platelets and in "the refraction of their granulations ' However his most significant observations were: (1) fibrin needles are often seen first at the edges of platelets or platelet debris; (2) platelet 'granulations' occur at the intersecting nodes of the fibrin network

Aynaud (see p 36) made a classical series of studies of platelet alterations in vitro which have largely been neglected by more recent workers in this field However they formed the chief background for the present author's observations [124] which are embodied in this thesis With extraordinarily skillful use simply of paraffined equipment the centrifuge and the microscope Aynaud described the sequence of alterations from the original disc or batonnet (v p 35) form to the spiculated 'stellate ' swollen and granular (disintegration) forms The controlling factors studied by this worker include (1) surface (2) temperature (3)dilution of plasma (with citrate saline) (4) the decalcifying (citrate etc) anticoagulants and (5) many experimental additives (see previously)

Fonio & Schwendener [171] used $MgSO_4$ plasma to observe various platelet alterations some of them bizarre

Zucker [518] confirming unpublished observations in the present author's laboratory agrees that even siliconed surfaces which are our best non wettable surface to date fail to prevent but merely retard morphological changes in the platelets held in citrated plasma The decalcifying ion exchange resins e g Amberlite or Dowex '50' do not help In fact most recent workers find these unsuitable for platelet recovery and prefer the strong Ca binding 'chelating' agent EDTA (sodium ethylene diamine tetra acetate also called Sequestrene or Versene) ref Tullis [479]

$CaCl_2$ was not found to have much influence on the swelling etc shown by washed (x3) platelets in buffered saline (Milstone [340]) Whether or not thrombin can produce platelet alterations as claimed by Zatti [515] and Fonio [169] is still an unsettled question (Zucker [518]) but is probably true and this may account for some new facts such as low serotonin assays in severe clotting disorders (Dr J H Lewis the author and M B Zucker unpublished data)

B PLATELET FUNCTIONS

For the purposes of this thesis it is not necessary to review some platelet functions (e g metabolism; 'platelet loading'; etc [469]) Included are those concerned with hemostasis and allied functions

1 RELATION OF PLATELETS TO HEMOSTASIS ETC

As noted previously platelets play a key role in thrombosis and embolism besides acting as a defensive mechanism against blood loss Pure platelet or 'white' thrombi are relatively rare and the usual thrombus is 'red' or 'mixed' with the bulk and numbers of erythrocytes preponderating Moreover clotting is initiated about the time (Erkelens' thesis [118]) that platelets adhere and begin to disintegrate Hence a thrombus is also rich in fibrin which forms a supportive meshwork and buttressing that adds greatly to the hemostatic effectiveness In the clinical 'bleeding time test' (e g methods of Duke [109] or Ivy [244]) defects of platelet or vascular function rather than of clotting function are causative of prolongation of the bleeding The bleeding time is not infrequently normal or nearly so in cases of hemophilia for instance Conversely the whole blood 'clotting time' test (e g Lee-White method [286]) is often normal in thrombocytopenics and primary vascular diseases

Since hemorrhagic disorders [453] can accompany (1) vascular abnormalities (2) platelet deficiencies quantitative (thrombocytopenic) or qualitative (thrombocytopathic or thrombasthenic) or (3) coagulation inadequacies it is evident that all three normally share in the defensive hemostatic mechanisms

Vascular anomalies are beyond the scope of the present thesis Just how important the contributions of the first two factors may be is emphasized by known cases of afibrinogenemia [7] who lack the essential clotting mechanism These cases do have bleeding problems but often manage much better than say a severe hemophiliac May it not be that certain factor(s) of the thrombin forming mechanisms are also necessary for some platelet functions?

Serotonin, Marjorie B Zucker [518] performed experiments on the formation of the hemostatic plug which appears after cutting a (small) blood vessel in the rat Noting a frequent vasoconstrictor effect at the time of platelet deposition Dr Zucker investigated this additional phenomenon and used an ear vessel perfusion technique for bioassay of the 'platelet vasoconstrictor' factor The consensus of a number of workers [367] on this problem is that disintegrating platelets liberate 5-hydroxytryptamine [399] which reacts with some plasma factor to become 'serum serotonin' (probably 5 hydroxy tryptamine creatinine sulfate) previously recognized by Rapport et al [400] We have some unpublished data showing low serum serotonin (platelet vasoconstrictor) values in cases with hemophilia and other severe clotting disorders The suggestion (p 38) is that thrombin (or some factor in the thrombin forming system) is needed for platelet 'release' of 5 hydroxytryptamine

2 THROMBOCYTES

The thrombocytes serve the hemostatic functions in lower animals e g birds fish reptiles amphibia etc much as the platelets do in mammals [185 189] That they are not quite as efficient in some ways

is suggested by the relative ease of preparing incoagulable or very slowly clotting plasmas from the thrombocyte-possessing bloods by careful collection and centrifuging perhaps even without use of anti coagulants This is very difficult in mammals even with use of anti coagulants because of the ease of platelet breakdown There are species differences however and equine blood has been used by a number of investigators [24]

3 PLATELETS AND CLOT RETRACTION

a) Clot retraction In 1951 O E Budtz Olsen [70] of Cape Town published an outstanding monograph on this subject with a very extensive bibliography His theoretical approach may be quoted verbatim: ' The blood clot consists of a three dimensional network of fibrin threads ' The serum is held in this network by capillary forces When the serum artificially is pressed out of the meshes the network collapses and does not again expand when immerged in serum due to adhesion between the individual fibers This can be seen by manipulation of the network under the microscope The capillary force holding the serum in the clot might conveniently be expressed by this formula:

$$\text{Force:} \quad \frac{2\gamma \cos \theta}{d\,g\,r}$$

where γ: the surface tension of the serum
θ: the angle of contact of the serum with the fibrin which is an expression of the wettability of fibrin
d: the density of the serum
g: the force of gravity
r: the width of the mesh

which is simply the formula for any fluid in any capillary tube Consideration of the force of gravity by this author is a new contribution

When the clot is placed in rigid structures like glass tubes gravity tends to draw the serum out of the fibrin network against the capillary forces holding it back; an artificial 'clot retraction' may thus be produced To avoid this Budtz Olsen designed an ingenious and simple method (' the suspended clot method") Under constant (37°C) temperature conditions 5 ml of blood (or test mixture) are floated free from the influence of gravity in an inert oil mixture The serum expressed during retraction of the clot floats to the surface of the denser oil mixture and can be measured ' It is shown that the size of the meshes in the fibrin network and therefore the capillary forces holding the serum in the clot depends mainly on the concentration of fibrinogen in the plasma It unavoidably follows that clot retraction is inversely proportional to the fibrinogen concentration 'of major influence on clot retraction is the volume of red and white cells in the blood These cells play a passive obstructive role and it is demonstrated experimentally that an almost linear inverse relationship exists between the volume of packed cells and the clot retraction ' correction can be achieved by simply adding the haematocrit to the observed clot retraction ' ' Rigid control of the temperature is essential in all work on clot retraction

Character of the clot its firmness rigidity and elasticity depend nearly exclusively on the fibrin concentration and the occurrence or absence of fibrinolysis They are completely independent of platelets Fibrinolysis and clot retraction are independent processes Clot retraction is due to a special function of platelets 'viscous metamorphosis leads to the formation of long strands or pseudopodia of cytoplasm connecting individual platelets or platelet masses These strands subsequently contract producing a patchy fusion of platelets throughout the clot As the platelets are adherent to the fibrin network of the clot the contraction of the cytoplasmic strands and the merging of the platelets result in a contraction of the whole fibrin clot with a squeezing out of the serum and a few of the red and white cells enmeshed in the fibrin network

From the delicate platelet mechanism producing clot retraction it would be expected that the force exerted by the process is very small By a simple experiment (p 46 [70]) this assumption is shown to be correct the force (about 19 mm H_2O) is so insignificant that it becomes extremely unlikely that clot retraction serves any physiological purpose in mammalian life It can certainly not draw together the walls of even the smallest vessels

In the crab clot formation and retraction take place in the complete absence of fibrin and are entirely cellular functions It is suggested that the process is a redundant phylogenetic relic of no importance in higher animal life

A critical review of Budtz Olsen's work will not be attempted here but the present writer would raise some questions e g :

(1) Platelets don t retract clots formed from fibrinogen with thrombin but only with 'thrombin forming' coagulant mixtures The present author s experience is otherwise

(2) Clot retraction cannot draw together small vessel walls Tocantins [468] who is cited by Budtz Olsen collapsed cellophane tubing by clot retraction

(3) Fibrinogen concentration is the chief determinant of size of fibrin mesh and hence of capillary forces There are many discrepancies between the ineffectiveness of certain agents in modifying Budtz-Olsen s clot retraction measurements and data of physico chemical and electromicroscopy studies which indicate they can greatly alter the fibrillar composition of the fibrin clot

(4) Can fibrinolysis be critically ruled out in connection with clot retraction? (See below)

(5) Accepting Budtz Olsen s worthy critical review of Glanzmann s (1918) [188] case data on hereditary hemorrhagic thrombasthenia there are contrary to the South African s opinion undoubted cases of platelet anomalies (thrombocytopathias) in which these elements are deficient in some retractor factor (? Glanzmann's retraktozyme') Such platelets can be isolated and compared with normal platelets shown unable to induce clot retraction in normal platelet free plasma [453] Such a case will be described in the present thesis

b) Role of platelets, Budtz Olsen [70] refers to Thackrah (1819) and others whose work clearly indicates that the fact of deficient clot

retraction in association with certain purpuras was known at the beginning of the nineteenth century Hayem [215] in 1896 was the first to correlate poor clot retraction in purpura with definite reduction in the number of blood platelets Among workers who have experimentally reduced platelet counts by centrifuging plasma and carefully correlated with clot retraction data we may mention Tocantins [467]

LeSourd & Pagniez [289] Bordet & Delange [57] Opitz & Matzdorff [356] and others [70] have recorded inability to obtain clot retraction with platelet 'extractives' but only with 'intact' platelets In recent electron microscopy studies Bessis & Burstein [44 43] observed adherence of fibrin filaments only to speculated 'altering' platelets and not to 'disintegrated' forms Glanzmann's [188] suggestion of a 'retraktozyme' (enzyme-like factor in platelets) has therefore not gained acceptance until recently when Fonio [170] claimed separation of the outer 'hyalomere' from the central 'granulomere' of platelets by differential centrifugation and the identification of 'retraktozyme' with the hyalomere fraction Very recently 1956 Magalini & Stefanini [316] claim isolation of a 'retractin' both from platelets and from blood free tissues Their best method is to disintegrate and extract with ether water (65:35) and then deposit in the cold at -20°C Thromboplastic factor is eliminated by heating for two hours at 56°C and subsequent storage at 20°C Tests for vasoconstrictor factor (5-hydroxytryptamine) are also negative They conclude: 'it could represent a lipoid substance'

How do platelets bring about clot retraction? The weakness of the force involved in clot retraction is stressed by Budtz Olsen as by previous workers While it is usually sufficient to detach the fibrin strands from adherence to glass this may not be the case in some instances Fibrin is more adherent to collodion [220] and Tocantins [467] observed failure of retraction in such vessels However clot retraction is not significantly better in non wettable siliconed tubes Budtz Olsen reviews much of the older literature (e g Fuchs [179]) on the influence of the containing vessel's surface on clot retraction Although he cites their paper in another connection (temperature effect) he doesn't include a very interesting experiment of Lampert & Ott [279] which the present writer has frequently reproduced for the the benefit of his students Thus if blood is allowed to clot in a wide test tube paraffined down <u>half</u> of its side the retraction of the clot will subsequently be away from the glass and <u>to</u> the paraffin That fibrin is less adherent to paraffin than glass was shown by Fuchs (1931) and many workers have used paraffined vessels in clotting experiments because of its 'non wettability' It is difficult to explain the Lampert & Ott experiment except on the basis that the glass in some way 'activates' the retraction producing factor

Observing certain similarities between retraction e g of plasma clots containing platelets and thrombin fibrinogen clots which do not normally retract but can be made to withdraw from the glass tube and 'clump' when known to contain <u>very small</u> amounts of trypsin or fibrinolysin Ferguson & Erickson [149] revived the idea [219] that clot retraction may be related to fibrinolysis This suggestion is not easily dismissed by the arguments of Budtz Olsen whose experiments give no heed to the <u>quantity</u> of fibrinolytic enzyme It may also be necessary

in the most recent work [316] to present conclusive evidence to rule out all possibility of traces of active fibrinolysin being present or formed in the test systems The present author does not insist on 'the fibrinolytic theory ' but merely believes that it should be convinc ingly ruled out

This being so it is appropriate to consider some peculiar 'retra- tility' of platelet 'processes ' This will be done in the present thesis especially in terms of lipid water phase relations It can be stated with fair certainty however that platelets do not extend and witndraw true 'pseudopods ' comparable to those in leukocytes etc Budtz-Olsen's data are very weak on this point This author however may be referred to for a good comparison between physical 'syneresis' of certain gels and the 'clot retraction' phenomenon The differences are numerous and support the conclusion: the fundamental mechanism of the two processes is probably entirely different

4 CLOTTING FACTORS IN BLOOD PLATELETS

a) Need of platelets for blood clotting There was much doubt about the blood platelets being essential for normal blood clotting until Brinkhous [64] using the newly introduced siliconed surface glassware of Jaques et al [251 288] showed that plasma recalcification clotting times and prothrombin consumption (utilization during clotting) were delayed the more the platelet count was reduced by centrifugation Successful experiments in obtaining a non clotting platelet free plasma will be cited (pp 67 68) in the body of this thesis The thromboplastin generation test is discussed on p 46

b) Erroneous ideas In the earlier literature about what the plate lets can contribute to the blood clotting mechanism appear the following:

1) platelets act as a complete coagulant' for fibrinogen (a) in the presence of calcium by allegedly providing a tissue fi brinogen (Wooldridge [508] 1886; Mills [338] 1930); (b) in still earlier ideas of a similar kind platelets were not sepa rated from the white corpuscles but only from red cells (Mandl 1842; Buchanan 1843 [68] cited by Anderson [16] 1844)
2) platelets contain prothrombin (Morawitz [346] 1905 Bayne Jones [32] 1912; Christie Davies & Stewart [85] 1927; Fuchs [177] 1930; Howell [238] 1935 cited by Tocantins [469] 1938) Disproved by Bordet [55] Mills [338] and Eagle [110]
3) platelets directly activate prothrombin through their zymo plastic substance (A Schmidt [420 421])
4) abnormal 'stability of platelets is the cause of hemophilia (Sahli [418] 1905; Minot & Lee [341] 1916; Morawitz [347] 1925; Howell & Cekada [240] 1926; Christie Davies & Stewart [85] 1927; Fonio [169] 1932) See present thesis Hayem [216] 1923 suggested that whatever is responsible for the stability of hemophilic plasma also accounts for the stability of platelets in that plasma

c) Platelet 'thromboplastic factor (prothrombin 'activator') Bor det & Delange [57] 1912 implicated platelets as one source of their

tissue clot accelerating principle or 'cytozyme' and extracted a thermo stable lipid material soluble in alcohol ether and chloroform but little soluble in acetone which agreed with Howell's [231] 1912 iden tification of the thromboplastic factor as cephalin Because cephalin was less potent than crude platelet (or tissue) extracts Howell [231] and Rumpf [416] believed the true thromboplastin to be a cephalin protein (i e lipoprotein) complex ' Eagle [110] also noted that the lipid extract from platelets had only about 1/10 of the original acti vator potency Chargaff Bancroft & Stanley Brown [78] prepared platelets from horse blood and isolated their various lipids A phos phatide fraction was found to contain a potent activator They con cluded: present methods for the separation of phosphatides are still much too crude to permit the isolation of compounds in a state even approaching purity It may very well be that a cephalin contain ing highly unsaturated fatty acids and therefore more soluble in ethyl alcohol is the real activator of blood clotting ' As in their later work on lung thromboplastin Chargaff et al [78] also noted that the 'defatted' material after platelet extraction was clot inhibitory In 1945 Chargaff [77] subjected normal human oxalated plasma to high speed (150 min at 20 000 r p m) centrifugation and lengthened the recalcification clotting times 3x or more In the following year fur ther studies [81] added information that the 'pellet' sediment from such centrifugation had thromboplastic properties similar to those of the 'macromolecular lipoprotein similarly obtained from lung ex tracts Flynn & Standley [167] reported to the Macy Conferences (1949) that their confirmatory observations also included microscopic examination of stained films which clearly showed that the pellet obtained by high speed centrifugation of human plasma is composed of platelets and occasionally erythrocytes Chargaff was mistaken then in identifying a 'plasma thromboplastin ' and was really just con firming Brinkhous' (1939) observations of the effects of platelet re moval on plasma clotting

In 1935 the present author knowing only of a short note of Hauro witz & Sladek [211] 1928 containing lipid analyses of platelets vis ited the Research Laboratory of the Children's Fund Detroit Michigan where a group of workers under Dr I G Macy were performing some excellent lipid (and other) fractional analyses of erythrocytes After preliminary work on some horse platelets prepared for the author by the Mulford laboratories the Michigan group completed a study on the lipid distribution of human platelets in health and disease [117] shortly after the appearance of Chargaff's work Sixty eight percent of the total platelet phospholipid analyzed as cephalin and it was com puted that if this were all liberated it could furnish 100 ml of blood with some 5-10 mg of cephalin This could be very significant in the light of the quantitative clotting data of Spagnol [447] and of Ferguson [129]

d) Platelets and the activation of prothrombin Eagle's [110] data were as follows:

(1) platelets (like cephalin) can participate in the activation of prothrombin (cf [328]) to thrombin Since Eagle's prothrombin acti vated slowly (about one hour) with $CaCl_2$ alone he concluded that platelets and cephalin accelerate coagulation by increasing the rate of

thrombin formation without affecting the quantity produced How ever Eagle may not have tested sufficiently small amounts *His weak* est cephalin (0 0001 cc of 0 1% suspension) does not quite reach 100% activation in his Figure 3 [110]

(2) Prothrombin prepared from hemophilic plasma and com pared with a similar preparation from normal plasma showed in the presence of a concentrated platelet suspension considerably slower thrombin formation but a normal (100%) eventual yield With calcium *alone Eagle claimed ultimately normal' thrombin yields* (actually his curves Fig 1 ref [110] show 80 90%) but very slow rates partic ularly in the hemophilic Simple plasma recalcification clotting times showed that hemophilic platelets were as good as normal platelets in accelerating clotting in a series of strengths of the platelet suspen sions in both (a) normal plasma and (b) hemophilic plasma However the stronger platelet suspension (both types) worked less well on hemo philic plasma and the weakest platelet strength did not work at all In comparable experiments cephalin speeded up the clotting of normal recalcified plasmas but had so little effect on the hemophilic (Table III of [110]) as had previously been shown by Mills [333] that Eagle dis missed it as unaccountably ineffective

Addis [3] had demonstrated in 1911 that hemophilic plasma was not deficient in prothrombin but that its conversion to thrombin was delayed which led him to the hypothetical suggestion of some quali tative defect in the hemophilic prothrombin Addis prepared 'pro thrombin' by a method similar to that later used by Eagle and Howell [238] pointed out that both studies could be subjected to the same criticism namely that their prothrombin contained thromboplastin and that this might be what is lacking in hemophilia Using his acetone method Howell with Cekada [240] could not find any difference in quantity or reactivity between the prothrombins of normal and hemo philic blood *Howell prepared a 'plasma thromboplastin and found less* of it in hemophilic plasma Purified preparations (after glycerol ex traction) were said to be protein free (ninhydrin and biuret reaction) but Howell could not obtain enough material for conclusive analyses and turned to the study of lung thromboplastin also known to be able experimentally to restore normal clotting times in hemophilic plasma independent of platelets Howell did think that his plasma thrombo plastin might come from platelets and he was able to prepare active thromboplastic material from platelets both normal and hemophilic (p 19 of ref [240])

Fonio [169] by cold centrifugation in paraffined tubes was able to obtain hemophilic plasma which clotted very slowly (4 8 hours at room temperature) but rapidly on adding washed platelet suspension from normal blood or less rapidly with washed hemophilic platelets

Other early workers including those we have dismissed under *erroneous ideas argued for more stable' or less active* platelets in hemophilia but it is not profitable to go into this any further All recent work supports the conclusion that it is not the platelet which is at fault in hemophilia but some plasma factor (AHF) which works with platelets to generate thromboplastin' for participation in the ac tivation of prothrombin to thrombin (see p 46)

e) Platelets and prothrombin consumption In 1939 Brinkhous [63]

tissue clot-accelerating principle or 'cytozyme' and extracted a thermo stable lipid material soluble in alcohol ether and chloroform but little soluble in acetone which agreed with Howell's [231] 1912 iden tification of the thromboplastic factor as cephalin Because cephalin was less potent than crude platelet (or tissue) extracts Howell [231] and Rumpf [416] believed the true thromboplastin to be a cephalin protein (i e lipoprotein) 'complex ' Eagle [110] also noted that the *lipid extract from platelets had only about 1/10 of the original acti* vator potency Chargaff Bancroft & Stanley Brown [78] prepared platelets from horse blood and isolated their various lipids A phos phatide fraction was found to contain a potent activator They con cluded: present methods for the separation of phosphatides are still much too crude to permit the isolation of compounds in a state even approaching purity It may very well be that a cephalin contain ing highly unsaturated fatty acids and therefore more soluble in ethyl alcohol is the real activator of blood clotting As in their later work on lung thromboplastin Chargaff et al [78] also noted that the 'defatted' material after platelet extraction was clot inhibitory In 1945 Chargaff [77] subjected normal human oxalated plasma to high speed (150 min at 20 000 r p m) centrifugation and lengthened the recalcification clotting times 3x or more In the following year fur ther studies [81] added information that the 'pellet sediment from such centrifugation had thromboplastic properties similar to those of the 'macromolecular lipoprotein similarly obtained from lung ex tracts Flynn & Standley [167] reported to the Macy Conferences (1949) that their confirmatory observations also included microscopic examination of stained films which clearly showed that the pellet obtained by high speed centrifugation of human plasma is composed of platelets and occasionally erythrocytes Chargaff was mistaken then in identifying a 'plasma thromboplastin ' and was really just con firming Brinkhous' (1939) observations of the effects of platelet re *moval on plasma clotting*

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