HAEMOSTASIS AND BLOOD COAGULATION

THE PHYSIOLOGY OF HAEMOSTASIS

AND

BLOOD COAGULATION

By

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SCOPE AND CONTENTS:

The primary concern and scope of this thesis is with the haemorrhagic diseases which cause serious bleeding, appear with or without trauma, occur spontaneously, or after administration of drugs and can be diagnosed by laboratory data.

By the correlation and utilization of experimental data, the author has attempted to piece together the moiety of the parts in order to explain a logical concept of the mechanism of the whole system of blood coagulation and the endothelial supporting system of the blood platelets in haemostasis.

Preface

Haemostasis is the process by which haemorrhage is stopped whether that haemorrhage has been induced or has risen spontaneously. The term has several applications. Bleeding from a large severed vessel may be stopped by mechanical haemostasis and from a smaller vessel by spontaneous haemostasis involving physiologic mechanisms. Mechanical haemostasis is a surgical problem. However, the haemorrhagic disorders which arise from deficient physiological haemostasis are initially the concern of the laboratory. The correction of such disorders involves the intelligent application of laboratory data and an effective liaison between the research laboratory and the clinician.

The problems faced by the investigator studying the behaviour of coagulation factors in vitro are not the same as the clinician faced with a patient who is bleeding. In a rapidly expanding field of biologic investigation, observations have been empirically directed usually at only one segment of the problem.

The haemostatic process has at least three major components - the reaction of blood vessels to injury, platelet activity and the coagulation of the blood. Each of these three aspects of the problem - especially the last - has been studied separately for years, and there has been much argument about the relative importance of each, but it is only comparatively recently that the extent of their interdependence has been recognized. However, experimental results from the study of 567 cases indicate that the three major components may be considered as independent systems yet each

one component is dependent functionally on the normal function of the other two components.

Summary

Experimental evidence indicates that no longer can the function of capillaries, platelet activity and the coagulation of the blood be considered as separate and independent components of the haemostatic mechanism. Although each component has been elaborately studied separately during recent years, yet each previously considered independent system is functionally dependent on the normal or abnormal function of the other two components.

Methods previously devised to measure the activity of the coagulation factors, individually and collectively, can now be modified to include observations regarding the activity of the three correlated components contributing to haemostasis.

The physiological role of the platelets is assumed to be similar to mitochondrial action in producing energy (ATP) for the coagulation reaction and probably in its function at the normal capillary wall interacts to form ADP which contributes to the integrity of the endothelial surface.

Platelets also behave as surfaces on which adsorption (and, therefore, desorption) of enzymes, substrates and cofactors may take place. In this respect, they are selective and have a specialized function which appears to be different and distinct from mitochondrial function.

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The Concept of Vascular Constriction

It is generally considered that large arteries, and to a much lesser extent large veins, retract and undergo vasoconstriction (see below) immediately on being incised, and that in the case of arteries, this action, combined with a fall in blood pressure, may result in the arrest of bleeding before clotting has had time to occur. In the case of small vessels, and particularly capillaries, there is no general agreement about the importance of vasoconstriction in the control of haemorrhage.

In 1941, MacFarlane (1) made the observation that normal nailbed capillaries disappeared from view on injury even when the venous pressure was raised to 30 mms. Hg, and reappeared between 20 minutes and 2 hours later; in thrombocytopenia and von Willebrand's disease the capillaries did not disappear, but bled continuously. He suggested that the disappearance of capillaries was due to their contraction, and that this was sufficient to arrest haemorrhage and allow the extravasated blood to clot firmly. On redilatation of the capillaries, haemostasis could be maintained by the clot already formed or, in the case of small puncture wounds, by firm adhesion of the wound edges, which might make the presence of a blood clot unnecessary. If clotting was defective, bleeding from larger wounds would recur when the vessels relaxed again.

However, while earlier authors accept that active vasoconstriction plays the main part in the initial phase of arrest of arterial bleeding,

they believe that capillary haemostasis depends to a large extent on the adhesion of the capillary walls at the site of injury.

In 1822, Hayem (2) demonstrated that when the jugular vein of a dog was incised, a mass of agglutinated platelets formed to fill the opening, and apparently to arrest the bleeding. In 1947, Zucker (3) observed the same phenomenon on transection of mesenteric arteries and veins in the rat, and of the venules in the rat's meso-appendix. In 1949, H.D. Zucker (4), while studying the histology of small puncture wounds in human skin, found that the ends of all cut vessels larger than capillaries were occluded by platelet plugs: his sections included very few capillaries but none of these were occluded by platelet plugs. Therefore, he concluded that capillary bleeding was arrested by fibrin formation.

This conclusion on his part is inconsistent with the fact that disorders of fibrin formation do not result in the prolongation of the bleeding time.

Haemostatic action by means of platelet plugs may be mechanical but there is definite evidence that they also act by promoting contraction of the adjacent vessels, through the liberation of vasoconstriction substances. When the muscular wall of the mesentery of a rat was incised, it has been observed (3) that it always underwent vasoconstriction, in both normal and thrombocytopenic animals but, on the other hand, vasoconstriction of the adjacent uninjured vessel appeared to depend on the formation of an adequate plug in the cut vessel, and did not occur in the platelet-deficient animals in which histologically no plugs were formed. It was later demonstrated (5) that the constriction of an injured artery was prolonged when the clot was allowed to remain at the site of the injury.

The early work of Janeway et al (6) demonstrated that serum and platelet extracts contained a powerful vasoconstrictor substance. Later the outstanding work of Rapport (7) confirmed that the serum vasoconstrictor was derived from platelets and he established its identity as 5 - hydroxytryptamine (HT).

It is now known by assay that HT is found in higher concentration in platelets than in any other mammalian tissue. Human platelets contain virtually all the HT of the blood, and are capable of adsorbing HT against a high concentration gradient (8). When blood is clotting, HT is released from the platelets, probably in parallel with thrombin formation (9) and quite independent of fibrin formation (10). HT is a powerful constrictor of muscular vessels, though probably not of capillaries.

The experimental evidence of other workers (11) indicates that platelet HT plays an important role in the haemostatic mechanism in vivo in large vessels but the statistical data correlating platelet counts and bleeding disorders with the HF level provide no evidence that a low HF can be, by itself, a cause of abnormal bleeding nor do these levels correlate with the results of bleeding times and tourniquet tests for capillary fragility.

We must conclude that the substance HT, although responsible for stimulated vasoconstriction in the presence of platelet plugs, forms only a part in vivo of a complex mechanism. We therefore must consider another function of the platelets namely, their role in the coagulation process or their contribution to the formation of the "platelet plugs".

This must first lead us to a general consideration of the coagulation mechanism.

Early Concepts of Blood Coagulation

Experimental work on the coagulation of the blood began four centuries ago and among the various research workers in this field are many illustrious names of men in clinical medicine and in science. Development in this field may be divided into four periods: The pre-classical era, the classical theory era, the prothrombin era and the later era in which a unified concept of haemostasis was evolved.

The Pre-Classical Theory Era (1666-1904)

The observations of Malphigi (12) in 1666 that strands of fibers remained after a clot of blood was washed can be regarded as an attempt to gain an insight into the mystery of the blood clot. Hewson (1772) developed a method for keeping blood in a fluid plate outside the body and so established that the coagulation factors resided in the non-cellular portion of the blood (13). In 1835 Buchanan (14) compared the clotting of blood with the curdling of milk by rennin. In both, he infers, a ferment reacted with a soluble protein to produce a coagulum. Lister (15) used this work in his own studies but the phenomenon of coagulation was not further studied in England until this present century. It remained for Schmidt (16) to formulate a logical sequence of events for the process of coagulation. He formed the hypothesis that thrombin was formed from a precursor (later named prothrombin) which required a zymoplastic agent for its activation. In 1890, indisputable evidence was produced that calcium (17) was essential for coagulation. It remained for Morawitz et al (18) in 1904 to condense the data into two equations:

Prothrombin + Ca^{++} + Thrombokinase-yields-Thrombin

Fibrinogen + Thrombin -yields-Fibrin

During the so-called pre-classical theory era there were very few clinical advances. Haemorrhagic Purpura was recognized as a distinct entity by Werlhof (18) in 1735 and haemophilia by Otto (19) in 1803. However, it was not until a method for the determination of the coagulation time was devised (20) that prolonged coagulation became one of the diagnostic criteria of haemophilia.

The Classical Theory Era (1904-1934)

The concept of coagulation as expressed by the two simple equations of Morawitz was not agreed upon by many investigators. Multiple theories were proposed including the hypothesis that coagulation occurred by the union of two proteins produced by the liver, namely fibrinogen and thrombogene which reacted with a distinctly third agent thrombozyme to form fibrin. The agent thrombozyme was presumed to be derived from extrahepatic vascular epithelium and leucocytes. Chloroform, powdered glass and certain lipoids were considered to aid in the reaction of this "thromboplastic substance". Thrombin was not considered to be a clotting agent but an unsaturated fibrin product produced by the reaction.

One theory (21) stated that blood containing prothrombin combined with an anti-coagulant, heparin. Thromboplastin liberated from platelets and body tissues combined with this anti prothrombin (heparin) thereby liberating prothrombin and allowing it to combine with calcium to form thrombin. Another theory (22) postulated that serozyme served as a precursor to proserozyme which, in the presence of calcium and a rough surface

reacted with a substance (thromboplastin) to form thrombin. Although these theories confused and hindered research in coagulation some of them contained basic ideas which later were substantiated.

Clinical advances in this period included the introduction of a non-toxic anticoagulant, sodium citrate, by Lewisohn (23) in 1915. A new tool as a laboratory aid was introduced by Duke (24) in 1912 when he described a technique for determination of the bleeding time. In 1916 Kaznelson described a specific cure for a specific haemorrhagic disease with the discovery that splenectomy abolished the haemorrhagic diathesis in essential thrombocytopenic purpura. A new therapeutic agent was given to medicine by the discovery of Howell (26) of heparine as an anticoagulant agent.

The practical contributions of this period far outranked the theoretical advances. The conflicting theories of the coagulation process caused the physician of this period to ignore the opinions and contributions of the laboratory physiologist and research worker.

The Prothrombin Era

In 1931 Roderick (27) showed by a quantitative method that prothrombin was diminished in spoiled sweet clover disease of cattle but he failed to define or isolate the responsible factor. It remained for Quick (28) in 1937 to identify the haemorrhagic agent with the development of a simple one-stage procedure for estimating prothrombin activity in the blood and thus a means of assaying extracts of fermenting sweet clover hay. He was further able to develop vitamin K deficiency in chicks by Almquist's procedure and found that the prothrombin was diminished and that bleeding resulted when it reached a critical level. He was able to postulate that

the bleeding in obstructive jaundice was due to vitamin K deficiency resulting from faulty intestinal absorption. The correctness of his suggestion that vitamin K and bile salts be given therapeutically to counteract the hypoprothrombinemia was verified clinically by Warner and his associates (28).

The one-stage method of Quick rapidly became a routine clinical test and involved the determination of prothrombin activity by means of adding a standardized thromboplastin reagent and calcium chloride to oxalated plasma and timing the formation of a clot. As the clotting of blood is slow because of the limited amount of thromboplastin, the addition of an excess of the latter makes prothrombin the limiting factor and therefore the determinant of the clotting time.

The basic equation may be expressed as:

Prothrombin + (co-factors) + calcium + thromboplastin

discovered later

yields thrombin.

This reaction behaves stoichiometrically. Since the co-factors and calcium are above the minimum required concentration and thromboplastin is made constant by adding an excess. The amount of thrombin formed depends on prothrombin which becomes the variable in the equation. Since the concentration of thrombin is determined by the speed of coagulation of the plasma, the clotting time of the plasma becomes the measure of the prothrombin level of the blood.

A two-stage method for estimating prothrombin activity in the blood was developed by Warner et al (28). By the use of this method they were able to demonstrate hypoprothrombinemia after biliary fistula, after experimental liver injury (29), and a case of haemorrhagic disease of the newborn (30).

Two outstanding clinical contributions in this era were the introduction of vitamin K and Dicumerol into therapy. Of great practical importance was the work of the Connaught Laboratories, at the University of Toronto on the purification of heparin that could be safely injected as an anticoagulating agent.

The Unified Concept of Haemostasis

Uncontrollable bleeding which occurs in patients with haemophilia, in congenital hypoprothrombinemia, or in hypofibrinogenemia could not be explained by the so-called classical theory of coagulation and unless a satisfactory explanation of a logical coagulation process can be formulated, no comprehensive concept of the coagulation process is possible.

The deficiencies of the classical theory necessarily forced its replacement by a new and comprehensive hypothesis based on well-controlled experimental evidence. The classical theory had to be replaced because:

1. It failed to define any true function of platelets.
2. It has no proven explanation for the source or specific activity of thromboplastin.
3. It considered the action of thromboplastin and calcium to be catalytic.
4. It was based on the assumption that prothrombin is a specific unit substance.

5. It offered no explanation for the autocatalytic nature of the coagulation reaction.

6. It failed to supply any suggestion as to the mechanism of maintenance of blood fluidity.

7. It failed to correlate coagulation with the complete mechanism of haemostasis as a whole.

Independent workers (31) (32) (33) reached the conclusion that normal plasma contained a previously unknown substance which accelerated the conversion of prothrombin to thrombin by tissue extract. We are indebted to Owren (32) who originally named this substance factor 5(V), which name has now been given official status by the Committee on International Nomenclature for blood-clotting factors (see Chart 1).

Factor V is present in fresh plasma, but deteriorates progressively on storage of oxalated plasma; it is consumed during the course of blood coagulation, and is therefore absent from normal serum.

The next advance was the discovery that normal serum also contains a substance which accelerates thrombin formation in the presence of tissue extract and calcium. Experiments of several groups of workers, particularly Quick and Stefanini (34) indicated that this "serum accelerator" (now known as factor VII), like prothrombin itself is absorbed by inorganic precipitates such as barium sulphate and aluminium hydroxide, and is deficient in the blood of patients with vitamin K deficiency or severe liver disease, or who are receiving drugs of the dicoumarin group.

The discovery of factors V and VII made it no longer possible to use the term 'thromboplastin' to represent both the active principles of tissue extracts and a substance which is capable of converting prothrombin to thrombin in the presence of calcium ions alone. Tissue extracts can no longer be considered as a complete thromboplastin but must undergo preliminary reactions with factors V, VII and calcium before an active prothrombin-converting principle is evolved.

This extension of the classical theory did not help to explain the failure of coagulation in haemophilia for haemophilic blood clots normally in the presence of tissue extracts. Normal blood, collected without contamination by tissue fluid clots rapidly, whereas the clotting of haemophilic blood obtained under the same conditions is much delayed. These considerations, together with the observation that removal of platelets from plasma delayed coagulation led Quick (35) to the conclusion that thromboplastic activity in shed blood developed as a result of a reaction between platelets and anti-haemophilic globulin (now factor VIII), the plasma factor which had previously been shown to be lacking in haemophilia (36).

The concept of intrinsic blood thromboplastin formation was developed and extended by the classical work of Biggs, Douglas and Macfarlane et al (37a, b) who showed that the weakness of the thromboplastic activity of blood was in reality due to a lag phase during which a series of reactions led up to the eventual appearance of an extremely active thromboplastic substance. The preliminary reactions involved not only platelets and factor VIII (AHG) but factor V and one or more other serum factors. The serum factor activity was considered to be due to factor VII, but in

1952 an entity recognized as distinct from factor VIII-deficient haemophilia was labelled Christmas factor or CF (now factor IX), present in normal serum and necessary for blood thromboplastin formation.

Further experiments by the above authors indicated that exposure to glass initiated the process of thromboplastin formation by a process of an action on both CF (factor IX) and platelets. As neither factors VIII, CF (factor IX) nor platelets are needed for thromboplastin formation in the presence of tissue extract then the active principle in tissue appeared to react in an identical way as an intermediate product of the above three blood components.

In 1953, Rosenthal et al (38) described a familial bleeding condition due to a previously unknown plasma thromboplastin factor which they called plasma thromboplastin antecedent (PTA) now known as factor XI.

Ratnoff and Colopy (39) investigated three patients with prolonged coagulation times but without bleeding diathesis and demonstrated that their plasma was deficient in another active principle, which they named Hageman factor (HF), now known as factor XII. Almost simultaneously, Hicks (40) investigated a young girl with a bleeding disorder which appeared as a congenital factor VII deficiency and yet presented no abnormality of thromboplastin generation, the clotting defect being only demonstrable in the presence of tissue extracts. Subsequently it has been confirmed by many investigators that factor VII plays no definite role in blood thromboplastin formation, but is required only for the coagulation action of tissue extracts.

During the same year Koller (41) postulated an additional hypothetical serum factor in order to explain his experimental results obtained when he used serum in thromboplastin generation tests obtained from patients receiving dicoumarin drugs. This factor has now been confirmed as the Stuart-Prower factor (factor X) after intensive investigations by Telfer et al (42) of patients who suffer from a congenital deficiency of this substance.

Thus, in the modern concept of coagulation, blood clotting may be conceived as a dynamic process in which positive forces contribute to the coagulation process yet they must be antagonized by negative contrary forces, the latter including substances which behave as natural anticoagulants and agents which remove the formed clot.

Modern theories of coagulation may be more easily understood if the various factors and steps are first considered separately. Because there is less uncertainty concerning the nature of the end products of coagulation than there is relating to those which participate at earlier stages in the process, the various factors will be described more or less in reverse of the order of their formation.

The Clotting Factors

The complexity of the coagulation process has been compounded by the large number of terms, often misleading, which have been applied to the various factors involved. An international committee in 1960 agreed upon a uniform terminology. This terminology will be employed, where possible, throughout the thesis in discussion of the various factors. Synonyms will be found as listed in Table One.

Some Properties of the various Coagulation Factors

The end result of blood coagulation is the formation of a fine network of fibers which enmesh the formed elements of the blood. This is the result of the conversion of fibrinogen to fibrin. Serum is therefore free of fibrinogen. Fibrinogen is the least soluble of all the plasma proteins and is present in plasma in a concentration of about 300 mgms. per 100 ml. It is produced in the liver. Clinical methods have been devised for its measurement based on the fact that it can be separated from plasma by a cold salting-out process by half saturation with sodium chloride or quarter saturation with ammonium sulphate.

N-terminal amino acid analysis of fibrinogen indicates that it may be composed of six polypeptide chains. Species of animals have been shown to differ in the chemical structure of fibrinogen which, in itself may explain the high rate of immunological species specificity of fibrinogen. Human fibrinogen has two tyrosyl residues and two alanyl residues but two chains appear to have no free N-terminal residues.

Table I

International Nomenclature (43)	Synonyms
Factor I	Fibrinogen
Factor II	Prothrombin
Factor III	Thromboplastin
Factor IV	Calcium
Factor V	Proaccelerin (44), Labile Factor (45), Accelerator Globulin (AcG) (46) Thrombogen (47)
Factor VI	See text
Factor VII	Proconvertin (44), Stable factor (45), Serum prothrombin conversion accelerator (SPCA) (48), Auto-prothrombin I (49)
Factor VIII	Antihemophilic factor (AHF), Anti-hemophilic globulin (AHG), Thrombo-plastinogen (45), Platelet cofactor I, Plasma thromboplastic factor A
Factor IX	Plasma thromboplastin component (PTC) Platelet cofactor II, Christmas factor, Autoprothrombin II (49), Plasma thromboplastic factor B
Factor X	Stuart-Prower factor
Factor XI	Plasma thromboplastin antecedent
Factor XII	Hageman factor. Contact factor.

Thrombin is derived from prothrombin. Its activity is similar to a proteolytic enzyme such as the proteinase, trypsin (50). It appears to have the property of an esterase (51) since its activity is inhibited by di-isopropylfluorophosphate.

At first fibrinogen appears to undergo hydrolysis and split into two peptide chains (be prepared for polymerization by enzymatic liberation of two soluble peptides) (52). Fibrin monomers are formed. These are readily soluble in urea and up to this point the chemical reaction is readily reversible. The solid clot, however, which develops is insoluble in urea and may be due to the intervention of calcium ions (53), or thrombin itself may be responsible for the polymerization as well as the original proteolysis (54). N-terminal analysis of human fibrin showed the tyrosyl residues of fibrinogen still intact but the other four chains all had glycyl end groups (55). Both peptides liberated from fibrinogen contain arginine as the C-terminal amino acid (50).

Factor II (Prothrombin)

True prothrombin has been isolated from plasma in a highly purified form which possesses well-defined physical and chemical properties (43). These indicate that it is a glycoprotein which contains 18 amino acids, about 6.5 percent carbohydrate as hexose and, in addition, neuraminic acid and glucosamine (56). Electrophoretic analysis of prothrombin shows migration with the $alpha_2$ globulins with behaviour similar to an euglobulin. prothrombin is found in the blood in a concentration not exceeding 20 mgms. per cent and is absent in normal serum. Purified prothrombin is quite unstable.

The exact nature of the conversion of prothrombin to thrombin is still uncertain. The amino acid composition of prothrombin and thrombin have been shown to be similar (57) and prothrombin can be converted to thrombin by sodium citrate without calcium ions or thromboplastin (58). It has been suggested that the prothrombin molecule is broken into units by proteolysis and at a critical point this is extensive enough to induce esterase activity. Further activation induces clotting activity due to the association of esterase molecules to form a dimer. The clotting activity does not persist and this may be due to the dissociation of the molecule back to the esterase form (59). There is sufficient evidence that the molecular weight of thrombin is only half that of prothrombin, or may be less and that prothrombin loses a carbohydrate fragment in its conversion to thrombin (60).

Factor III - (Thromboplastin)

The classical theory of blood coagulation attributed the initiation of clotting to a substance, thromboplastin, which, in the presence of calcium ions, catalysed the conversion of prothrombin to thrombin. The modern concept differentiates 'tissue' thromboplastin from 'blood' thromboplastin. The criteria of distinction is that, in the blood, thromboplastin activity arises during the process of coagulation whereas this activity is immediate in certain tissue extracts. Thromboplastin is widely distributed as an intracellular substance in the body and is easily produced in high activity concentration from tissues of the brain, lungs, placenta, thymus and testes. Trauma or injury to tissue cells, particularly of the organs mentioned, demonstrates this activity. It has been further observed in high concentra-

tions in viper venom (61). The term 'tissue thromboplastin' becomes no longer a suitable term and a more specific definition will be elaborated in this thesis.

A potent material was obtained from beef lung (62) which contained a variety of phosphatides, as well as carbohydrate, proteins and ribonucleic acids. An extract has been obtained from brain tissue which contains no protein (63). The species from which the thromboplastin extract is prepared for use in the thromboplastin generation is significant and antigenic dissimilarity between tissue and blood thromboplastins has been demonstrated in many cases by this author.

The most common thromboplastin reagent is prepared from brain or rabbit lung as an ether-soluble phospholipid fraction or "cephalin" with thromboplastic activity. The nature of the phospholipid which contributes this activity is not settled as to whether it is a phosphatidylethanolamine, phosphatidye, serine or lecithin mixture (64). Platelets contain these substances and a phospholipid with thromboplastic activity is present in erythrocytes (65). This author has demonstrated a difference to exist in the thromboplastin activity of normal erythrocyte hemolysates as compared to hemolysates from erythrocytes deficient in glucose-6-phosphate dehydrogenase activity. The nature of thromboplastin in some respects appears to be enzymatic but evidence has been published which seems to oppose this view (63).

Factor IV (Calcium)

For many years the anticoagulant effect of calcium removal from blood has been known. Oxalates precipitate free calcium; citrates suppress ionization and chelating agents as well as ion-exchange resins remove calcium

from the blood thus preventing the formation of a clot. This effect, however, is only partially due to the removal of calcium ions as insoluble salts since an amount of oxalate or citrate calculated to be just sufficient to combine with the ionized calcium will not prevent blood coagulation. No direct evidence exists that the calcium level has been so reduced in the blood of a patient to cause bleeding. Calcium does appear to be required in the formation of urea-insoluble clots (66).

The thrombin-fibrinogen autocatalytic reaction can take place without calcium but acceleration of the reaction is observed in the presence of an organic calcium salt (67). Calcium may be utilized in the formation of a complex with Factor IX activity and has been mentioned as a part of factor VII activity (68).

The role of calcium in many phases of the enzymatic process of coagulation will be elucidated later.

Factor V (Labile Factor)

Factor V is found in normal plasma but not in serum. It is present in plasma treated with aluminum hydroxide and it is not adsorbed with barium sulphate. It is destroyed by heating to 56°C for 30 minutes or by increasing the pH of plasma to 10.5. Stored for 49 hours at room temperature or in the presence of oxalate or E.D.T.A. ions, it rapidly deteriorates whereas citrate ions delay its decomposition. In the thromboplastin generation test a deficiency of this substance will give an abnormal curve similar to deficiencies of other globulin factors. The thromboplastin generation test (TGT) is abnormal with aluminum hydroxide-absorbed plasma; in minor deficiencies of Factor V the TGT curve swings upward into the abnormal

range indicating rapid consumption of the minimal optimum amount of this substance in the substrate. The defect is corrected by normal plasma and by plasma from patients receiving dicumerol (Factor VII deficient), but not by stored normal plasma. The finding of an abnormal TGT together with a deficiency in Factor II differentiates Factor V abnormality from that of Factor VIII deficiency in which the prothrombin (Factor II) is usually normal. In the large number of cases which the author has investigated, it has been shown that Factor V is needed for the production of thromboplastin.

Factor VI was considered to be an active form of this substance (69).

Factor VII (Stable Factor)

Factor VII is present in high concentrations in serum. It differs from Factor V by its stability to heat and to storage and by the fact that it is readily absorbed from serum by means of an asbestos filter but less readily with aluminum hydroxide and barium sulphate.

Factor VII may be formed in the liver (70).

Factor VII defect shows retarded thrombin formation with tissue thromboplastin but this factor per se does not appear to be necessary for thromboplastin generation in the normal blood as the TGT of patients treated moderately with dicumerol or patients with an induced defect of this factor, show normal TGT curves, the deficiency being correctable in the prothrombin complex phase only by the addition of normal serum or normal plasma but not by normal absorbed plasma.

A study by this author of a rare hereditary manifestation of this factor VII deficiency showed in vitro no correction by barium sulphate absorbed plasma, or absorbed serum, or serum heated to 56°C for 5 minutes. The factor of three male siblings (all mongoloids) showed partial correction of the deficiency only when treated orally with vitamin K. The same defect was not corrected by this method in the male children but transfusions of fresh blood offered a transient return to normal optimum levels of Factor VII activity.

Factor VIII

Factor VIII (antihaemophilic globulin) is found in association with the euglobulins and fibrinogen of plasma but is absent in serum. It is not absorbed on aluminum hydroxide, barium sulphate or Seitz filter but can be removed by adsorption on Fuller's earth. Purification to a minor degree can be obtained by separation from fibrinogen by solution followed by heating at 56°C for two minutes. A solubility curve suggests that factor VIII is one of the cryoglobulins.

It has been recognized for a long time that haemophilia is a constitutional anomaly which is transmitted as a sex-linked recessive gene contained on the X-chromosomes of the reproductive cells. The constitutional defect is usually limited to the male but is transmitted from the female through a carrier daughter to a grandson. The sons of an affected female, all outwardly normal, are capable of transmitting the trait as an evident defect to half of their sons and as a recessive characteristic to half of their daughters.

Assays of plasma factor VIII by the method of Bentley and Krivit (71) can distinguish at least four grades of factor VIII deficiencies: (1) classical, in which factor VIII is 0 percent, (2) moderate in which factor VIII is less than 3 percent of normal activity, (3) mild, in which factor VIII is about 15 percent and low grade, in which factor VIII is about 33 percent of normal activity. Normal activity values of factor VIII may vary from 65 to 136 percent. Grades (3) and (4) are not detectable by partial thromboplastin generation tests but by strict quality control, using various dilutions of plasma, can be detected by the thromboplastin generation test (p. 22) or TGTR* procedures. Low levels of factor VIII actively can be demonstrated in female carriers.

Factor IX (Plasma Thromboplastin Component)

Like factor VII, factor IX is present in properly prepared serum and can be absorbed by aluminum hydroxide or barium sulphate. It can be precipitated by 45 to 50 percent ammonium sulphate and removed by a Seitz filter, thus distinguishing it chemically from factor VIII. Factor IX is stable on storage but labile to heat for an extended period of time. It is found in the euglobulin and pseudoglobulin protein fractions.

A deficiency of this factor is a constitutional anomaly which is inherited and transmitted as a sex-linked, recessive Mendelian trait and is detectable in the serum component by the thromboplastin generation test or TGTR.* This defect may occur, like factor VIII, in conjunction with a vascular defect (Von Willebrand's disease) (72).

* TGTR - Reagents supplied by Warner-Chilcott Laboratories, Morris Plains, N.J.

It is evident, however, that in certain infectious diseases, the activity curve of this factor may be altered qualitatively when hypogammaglobulinemia is inherited as a sex-linked anomaly. Eight cases investigated by the author showed a direct relationship between gamma globulin and factor IX activity when both deficiencies were inherited on the X-chromosome.

Factor X

The Stuart-Prower factor (factor X) has been recognized through its deficiency in certain inherited and acquired disorders. It appears to be inherited as a highly penetrant but incompletely recessive autosomal characteristic. The heterozygotes are found to be only mildly affected but technically manifest abnormal findings in the prothrombin complex and in the thromboplastin generation test (43). The latter observation distinguishes this deficiency from that of factor VII.

Factor X is absorbed onto aluminum hydroxide, barium sulphate, Seitz filter and tricalcium phosphate. It is stable at room temperature for several days but can be destroyed in serum by incubation for 5 minutes at 56°C.

Factor X is involved in the extravascular as well as the intravascular process of coagulation and plays a role as a substrate factor in the conversion of prothrombin to thrombin (43).

Factor XI Plasma Thromboplastin Antecedent (PTA)

This is a stable factor present in plasma and in serum. Maximum precipitation of the factor occurs at 25 to 33 percent ammonium sulphate

saturation of normal plasma. Electrophoretically it is located in the B_2 globulin fraction. The activity of factor XI increases when the plasma from factor XI-deficient subjects has been stored. Sufficient time of storage of hitherto defective plasma will eventually correct the factor XI deficiency in the same patient.

Factor XI deficiency can occur in either sex and appears to be transmitted as an autosomal dominant trait with a high degree of penetrance and variable expressivity. Thromboplastin generation is markedly abnormal and this test proves to be the most reliable for the detection of this deficiency (73). The defect can be corrected by plasma absorbed with barium sulphate and by serum, since factor XI is present in both. It can be distinguished from factor VIII deficiency which is corrected by normal absorbed plasma but not by serum, whereas factor IX deficiency is corrected by normal serum and not by absorbed plasma in the TGT.

Factor XII

Factor XII is a factor lacking from blood affected by the homozygous expression of an autosomal gene. The defect may be detected by a simultaneous prolongation of coagulation in both glass and silicone tubes, abnormal thromboplastin generation and decreased prothrombin consumption. The remarkable feature is that a deficiency of this substance is usually not associated with haemorrhagic manifestations (74).

Factor XII electrophoretically migrates between the beta and gamma globulins and is present in serum as well as plasma. It is poorly absorbed by barium sulphate, resists heating to 60°C for 15 minutes and is

present in 25 percent and 50 percent ammonium sulphate fractionated normal plasma precipitates. Factor XII is best activated in vitro by contact of blood with foreign surfaces (75). Glass, acetone, chloroform, versene and dilution have all been shown to be agents which release peptides from plasma and behave as activators of factor XII (76).

The Platelet Factor

The formation of the platelet plug is the key event in normal haemostasis. At first the plug is permeable to the blood, but soon it becomes impermeable and bleeding ceases. This change in permeability is thought to be due to the process of 'viscous metamorphosis'. This process consists first in the clumping of the platelets, then swelling of the mass and finally the release of granular material in the form of phospholipids, into the surrounding plasma. The phosphatides may be extracted from washed platelets by the method described by Folch et al (77) and separated on salicic acid columns following the method of Phillips (78). This results in four major groups of phospholipids (cephalins, lecithin, sphingomyelin, and lysolecithin). The cephalins which contain the most active thromboplastic phospholipids are normally eluted from the salicic acid column in a single fraction. The specific activity of each fraction can be determined if, prior to chromatography, the platelets have been incubated with high specific activity P_{32}.

The work of Westerman and Jensen (79) has shown the specific activity for inositol phosphatide to be 98.1 CPM/microgram, for sphingomyelin 3.5 CPM/microgram, for lecithin 1.6 CPM/microgram. Phosphatidylserine, phosphatydlethanolamine and phosphatidic acid were essentially inactive during the three-hour incubation period. Plasma phosphatide P_{32} activity remained at low levels throughout the incubation period. Further elaboration supports the concept that the individual phosphatides are functionally distinct.

Platelet viability, clot retraction, and viscous metamorphosis have been proven to be dependent upon intact intraplatelet glycolysis and adequate A.T.P. production. The precise means by which energy production is related to platelet function and viability has not been proven or ascertained but from previous studies of the interaction of the so-called platelet factor 3 in the thromboplastic process it may be postulated that the phospholipid platelet surface is the site of energy, dependent processes which are necessary for cell viability and function.

It is essentially true that thrombocytopenia is associated with prolonged bleeding times and increased capillary fragility. Furthermore, the question of the association of increased capillary fragility or permeability with most cases (but not all) of immunologic thrombocytopenia begs for clarification. The paradox of normal bleeding time, normal clot retraction but prolonged coagulation time of blood associated with increased capillary fragility has not been explained in the presence of normal platelet counts. Both clinically and experimentally, complete dissociation between thrombocytopenia and purpura have been noted. "In immunologic thrombo-

cytopenia both thrombocytopenia and purpura are usually found suggesting that the same agent is capable of damaging both the platelets and the capillary wall" (80). It has been suggested that platelets and vascular endothelium are antigenically similar.

When antiplatelet serum is given intravenously, a thrombocytopenia is produced: when it is injected into the skin, a local area of spreading haemorrhage is produced, without a reduction in circulating platelets. On the other hand, anti-endothelium serum produces purpura without thrombocytopenia (80).

While platelets and endothelium may qualify as "shock organs" for the immune reaction, they do not necessarily react simultaneously with the same intensity, or respond equally to therapy.

Materials and Methods

Thromboplastin Generation Test (TGT)

Principle:

When a suspension of platelets, deprothrombinized plasma, and serum is allowed to react in the presence of calcium ions, each contributes to the generation of thromboplastic activity in the incubation mixture. The alumina plasma contributes factors V and VIII, whereas serum contributes factors VII and IX. Factory X also enters into the reaction, and this is supplied by the serum.

If all three fractions are prepared from normal blood and allowed to react, the thromboplastin rapidly developed in the mixture will clot recalcified plasma in from 10 to 14 seconds. Once maximal activity is obtained, the reaction is stopped, and the thromboplastic activity of serial dilutions of the reaction mixture is determined the results of which, when plotted as a thromboplastin dilution curve, can be expressed as percent of normal thromboplastin activity (Fig. 1).

The localization of a defect in coagulation is achieved by substituting (one at a time) into the normal system the three fractions prepared from the unknown blood sample. For example, substitution of unknown alumina plasma for normal plasma, or unknown serum for normal serum, allows a comparison of the activity of these functions with the results obtained with the corresponding normals. Likewise, substitution of an unknown suspension of platelets for the normal can determine whether platelet function in the unknown blood is normal or deficient.

Reagents:

1. Aluminum hydroxide gel (prepared by the method of Miale and Wilson (81).
2. Simplastin* for standardization of aluminum hydroxide suspension.
3. 3.8% sodium citrate.
4. 0.85% sodium chloride with barbitol buffer (see Appendix A).
5. 0.025 M. calcium chloride.
6. Barium sulphate, Merck, U.S.P.
7. Sodium oxalate 0.1 M.

Equipment:

1. Water bath thermostatically controlled, 37°C with racks.
2. Siliconized glassware (Siliclad, Clay Adams)
   a. Heavy duty Pyrex conical centrifuge tubes, 12 ml.
   b. Syringes, 20 ml.
   c. Glass pipettes graduated at 0.1 ml. and 0.2 ml. (U.S. Bureau of Standards - certified).
3. Stop watches and large timer.
4. Pyrex test tubes (not siliconized) 12 x 75 x 13 x 100 mm.

Method:

1. The collection of blood from known normal controls and from the case to be studied was carried out to meet all the essentials of controlled procedures (Appendix A).

* Warner-Chilcott Laboratories, Morris Plains, N.J.

2. The citrated specimens of the normal controls and the patient were handled as follows, using siliconized tubes throughout and carefully labelling each step.

a. The blood was centrifuged at 1500 r.p.m. for 10 minutes.

b. The supernatant "platelet-rich" plasma, using siliconized pasteur pipettes, was aspirated into siliconized conical centrifuge tubes.

c. The plasma was centrifuged at 3000 r.p.m. for at least 30 minutes and the volume of the plasma recorded.

d. The "platelet-poor" plasma samples were aspirated into separate siliconized test tubes, labelled, and stored at 4°C. These were used to prepare aluminum hydroxide or barium sulphate absorbed plasmas and as substrate plasmas.

Additional modifications:

e. The platelet sediments remaining in each tube were washed at least twice with veronal buffered saline (Appendix A) using 2 ml. portions of the saline. After adding the saline, the sediment was thoroughly mixed using a siliconized glass rod, and the specimen was centrifuged at 3000 r.p.m. for 10 minutes. After the second or third wash, the platelets were resuspended in an amount of buffered saline equal to the amount of plasma as measured in step c. The suspension was chilled, labelled and stored at 4°C.

f. The platelet-poor plasmas obtained in step d were processed as follows:

(1) To a conical test tube was added the amount of $Al(OH)_3$ which would give the proper adsorption. To another conical tube into which oxalated platelet-poor plasma was pipetted, 100 mgms. of

barium sulphate was added for adsorption for use in the TGTR* which was used as a cross-check for the original method. Both tubes are centrifuged at 3000 r.p.m. for 3 minutes after at least 10 minutes of incubation at 37°C. The plasmas were separately decanted and the "prothrombin time" determined by the use of Simplastin.** If the prothrombin time of the adsorbed plasma was between 2 and 3 minutes, the material was ready for use. If the time was less than 2 minutes, the adsorption technique was repeated.

(2) The remainder of the plasma was labelled as substrate plasma, and stored at 4°C until the test was set up. Substrate plasma from the normal control was used which had a normal "prothrombin time" to qualify it as suitable for measuring thromboplastic activity.

3. Serum was obtained from the tubes of clotted blood which had been incubated at 37°C for two hours. The clot was rimmed and centrifuged at 2000 r.p.m. for 10 minutes. The serum was aspirated and labelled "normal serum" and "patient serum".

4. To prepare $BaSO_4$-adsorbed normal plasma for differentiating a factor IX from a factor XI deficiency or to be used as a source of factor VIII in the TGTR*, 2 ml. of plasma was added to 100 mgms. of barium sulphate, the "prothrombin time" checked as in 2.f(1), incubated for 10 minutes and centrifuged at 3000 r.p.m. for 15 minutes. The adsorbed plasma was de-

* TGTR - Warner-Chilcott Laboratories, Morris Plains, N.J.

** Simplastin - Warner-Chilcott Laboratories, Morris Plains, N.J.

canted into a clean tube. This same adsorbed plasma was only used as a source of fibrinogen to measure prothrombin consumption (Appendix A). The reagent was standardized for use when no clot was formed within ten minutes after 0.2 ml. of Simplastin was added to 0.1 ml. of the adsorbed plasma at 37°C.

5. Preparation of final reagents:

a. The normal platelet suspensions were prepared as outlined in step 2, e.

b. The patient's platelet suspension was prepared as outlined in step 2, e. If the platelet count was below normal, the platelet suspension was adjusted so that it contained the same concentration of platelets as the normal suspension.

c. When a patient demonstrated a defect of one of the coagulation factors V, VIII, IX, X, XI or XII the patient's platelets were incubated for 4 minutes in the reagent containing a normal amount of the factor in question, then resuspended by the method described in step 2, e.

d. The normal control alumina plasma (step 2f(1)) was diluted 1:5 with buffered saline.

e. The patient's alumina plasma (step 2f(1)) was diluted 1:5 with buffered saline.

f. Normal control serum (step 3) was diluted 1:10 with buffered saline.

g. The patient's serum (step 3) was diluted 1:10 with buffered saline.

Note: Further dilutions of plasma and serum were made as required.

Performance of the Test

a. Pipet 0.1 ml. of normal substrate plasma into each of six plain glass tubes, 12 x 75 mm. in the water bath at 37°C. Six tubes were used for testing each incubation mixture.

b. The following incubation mixtures were prepared, one at a time, and the test was performed with each.

(1) 0.3 ml. of normal 1:5 alumina plasma
0.3 ml. of normal 1:10 serum
0.3 ml. of normal platelet suspension

(2) 0.3 ml. of normal 1:5 alumina plasma
0.3 ml. of normal 1:10 serum
0.3 ml. of patient's platelets

(3) 0.3 ml. of normal 1:5 alumina plasma
0.3 ml. of patient's 1:10 serum
0.3 ml. of normal platelets

(4) 0.3 ml. of patient's 1:5 alumina plasma
0.3 ml. of normal 1:10 serum
0.3 ml. of normal platelets

(5) 0.3 ml. of patient's 1:5 alumina plasma
0.3 ml. of patient's 1:10 serum
0.3 ml. of patient's platelets

(6) 0.3 ml. of normal 1:5 alumina plasma
0.3 ml. of patient's 1:10 serum
0.3 ml. of patient's platelets (as in step 5c)

c. Two tubes, each containing 5 ml. of 0.025 M $CaCl_2$ solution are placed in the water bath.

d. 0.3 ml. of 0.025 M $CaCl_2$ was added to the incubation mixture to be tested and the master timer was simultaneously started.

e. Beginning one minute after the $CaCl_2$ is added to the incubation mixture, 0.1 ml. of the recalcified incubation mixture plus 0.1 ml. of 40 M $CaCl_2$ solution are added to tube 1 of the substrate plasma. Start a stop watch at the same time. At 1-minute intervals the procedure is repeated with substrate tubes 2, 3, etc. starting a separate stop watch for each tube. The time of the first appearance of a clot in each tube is recorded. Usually during the second or third minute, a clot will form in the incubation mixture. This is easily removed with a wooden applicator stick. Formation of the clot does not interfere with the results, but if not removed, it will clog the pipets used for transferring the recalcified incubation mixture.

f. If a serum defect is present that is not caused by deficiency of factor VII or X (as evidenced by a normal "prothrombin time" in the original blood), it is necessary to determine whether it is a deficiency of factor IX or factor XI. This is done by setting up <u>two</u> more incubation mixtures. In one mixture, 0.15 ml. of patient's 1:5 serum plus 0.15 ml. of normal 1:20 serum is substituted for 0.3 ml. of patient's serum. In the other, 0.15 ml. of 1:5 diluted $BaSO_4$-adsorbed plasma and 0.15 ml. of 1:5 patient's serum is substituted for the 0.3 ml. of patient's serum. TGT's are per-

formed in each. If the defect is corrected by normal serum and by $BaSO_4$ plasma, it indicates a deficiency of factor XI. If the defect is corrected by normal serum, but not by $BaSO_4$ plasma, it is caused by a deficiency of factor IX.

g. In testing for anticoagulants the TGT is repeated, adding to the normal incubation mixture serial dilutions of the patient's plasma or serum (1:50, 1:100, 1:500 final concentrations in the incubation mixture). If plasma is being tested for an anticoagulant, the incubation mixture is made up with 0.15 ml. of 1:2.5 normal plasma plus 0.15 ml. of diluted, unknown plasma. If serum is being studied, the incubation mixture is made up with 0.15 ml. of 1:5 normal serum plus 0.15 ml. of diluted unknown serum. If generation of thromboplastin is good with the high dilutions and poor with the low dilutions, an anticoagulant is present.

FIG. 1

THROMBOPLASTIN DILUTION CURVE FOR THE TGT.

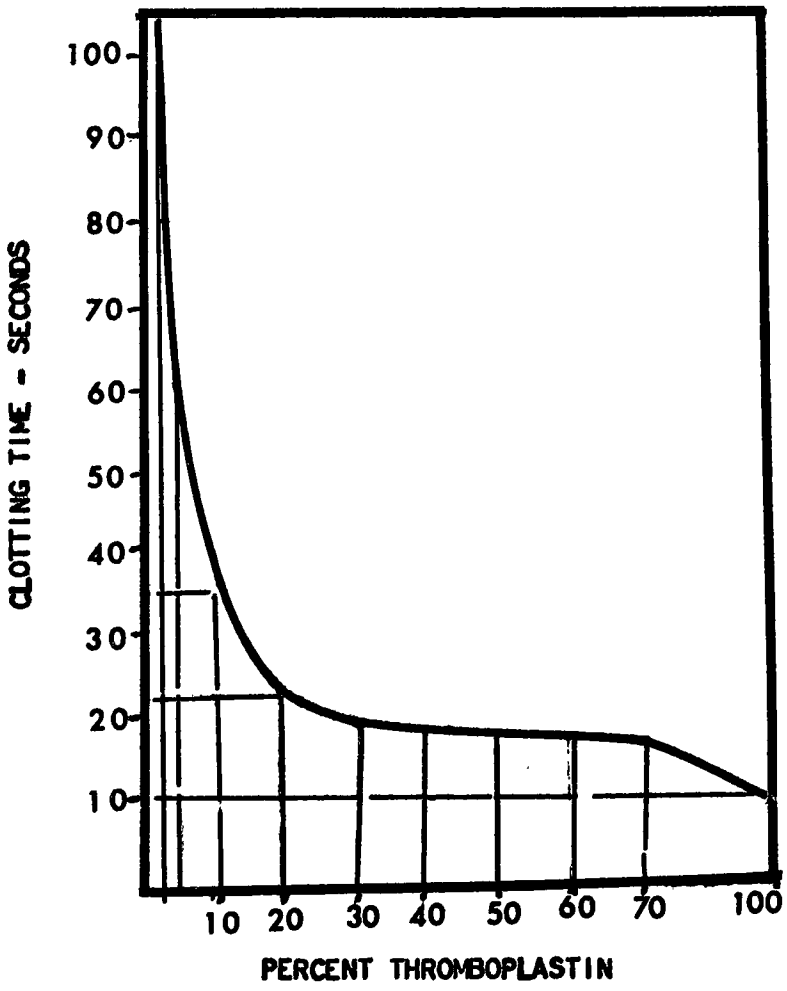
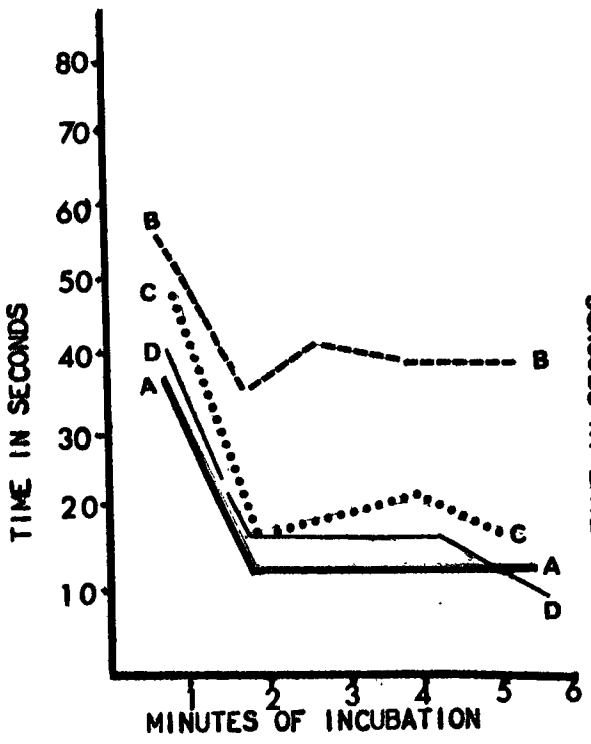


FIG. 2

THE TGT IN FACTOR VIII DEFICIENCY



A —— NORMAL CONTROL

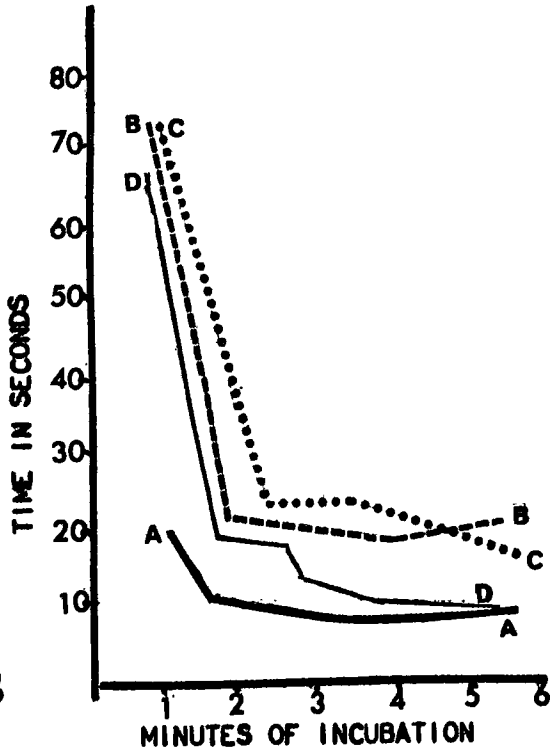
B ---- FACTOR VIII-DEFICIENT PLASMA

C ····· FACTOR VIII-DEFICIENT PLATELETS + NORMAL PLASMA

D —— NORMAL ABSORBED PLASMA SUBSTITUTED

FIG. 3

THE TGT IN FACTOR IX DEFICIENCY



A —— NORMAL CONTROL

B ---- FACTOR IX-DEFICIENT SERUM SUBSTITUTED

C ····· FACTOR IX-DEFICIENT PLATELETS + SERUM

D —— NORMAL SERUM + PATIENT'S PLATELETS

TABLE 2

REAGENTS PREPARED FROM NORMAL BLOOD

REAGENT	PHASE I AND 2	PHASE 3	PHASE 4
WHOLE PLASMA	FACTORS VIII IX XI X	FACTORS II V VII X	FACTOR I
ABSORBED PLASMA	FACTORS VIII (IX)	FACTOR V	FACTOR I
SERUM	FACTORS IX XI X	FACTORS VII X	

Results and Interpretation

(a) General Results

The results of the TGT in various coagulation factor deficiencies and other haemorrhagic states are listed in Table 2.

The range of normal values obtained in 120 normal persons is illustrated by Fig. 1. The average is 11.4 seconds, with an S.D. of ±3.0 seconds. The variations could not be due to technical factors, for repeated tests performed with the same reagents check within a few tenths of a second. A value of from 9 to 15 seconds, occurring during the six minute period of incubation, may be regarded as evidence of normal generation of thromboplastin. Normal maximum activity is usually reached within three to four minutes of incubation. At least one test or run using the three normal reagents is included in all instances in which an unknown blood is studied, as this gives the control value for 100 per cent generation.

The usefulness of the TGT is in the study and differentiation of deficiencies of factor VIII, IX, X, platelet activity and titration of abnormal antithromboplastins which act as circulating anticoagulants. Particular reference is made to platelet adsorptive activity contained in step 5, preparation of reagents, TGT, which is a modification of the technique as revised by the author.

The principle of the TGT is based on the fact that normal plasma treated with aluminum hydroxide or barium sulphate (which plasma now contains factors V, VIII, XI, and XII, Table 2), platelets and normal serum (which supplies factors IX and X, as well as XII and XI) react in the presence of calcium ions to form blood thromboplastin. It is apparent that factors VIII and V act as substrates for the reaction with the other factors XI, X and IX which serve as enzymes or co-enzymes in the reaction. A lack of any one of these seven factors will cause deficient thromboplastin formation and will be revealed by the test.

If thromboplastin generation is deficient when the patient's absorbed plasma, serum, platelet suspension and calcium chloride are incubated together, then the defect is contained in one of the cross tests.

If the TG is still deficient when the patient's absorbed plasma, normal serum and normal platelets are incubated, then factor V or factor VIII deficiency exists. The two possibilities are easily differentiated by the prothrombin activity test (Appendix A) which is prolonged in factor V deficiency but not in a deficiency of factor VIII substrate activity.

Similarly, of the deficiency persists when normal absorbed plasma, patient's serum and normal platelets are incubated, then a deficiency of factor X or IX is evident. These can, likewise, be differentiated by the prothrombin activity which is normal in deficiencies of factor IX but prolonged in factor X insufficiencies.

If the defect exists in neither of the above tests but only when patient's absorbed plasma, patient's serum and patient's or normal platelets are incubated, then the cause of defective thromboplastinogenesis may

be either a defect of factor XI or factor XII. These can be differentiated by further assays and cross checking using absorbed serum to identify factor XI activity.

If the defect is present when normal absorbed plasma, normal serum and the patient's platelets are incubated, then the patient has either a quantitative insufficiency or qualitative platelet defect. A normal platelet count might indicate that of thrombocytopathia or other qualitative abnormality. A correlation factor between platelet activity and vascular epithelium integrity can be ascertained by the procedure in step 5, preparation of reagents, which assess the adsorptive properties of the platelets.

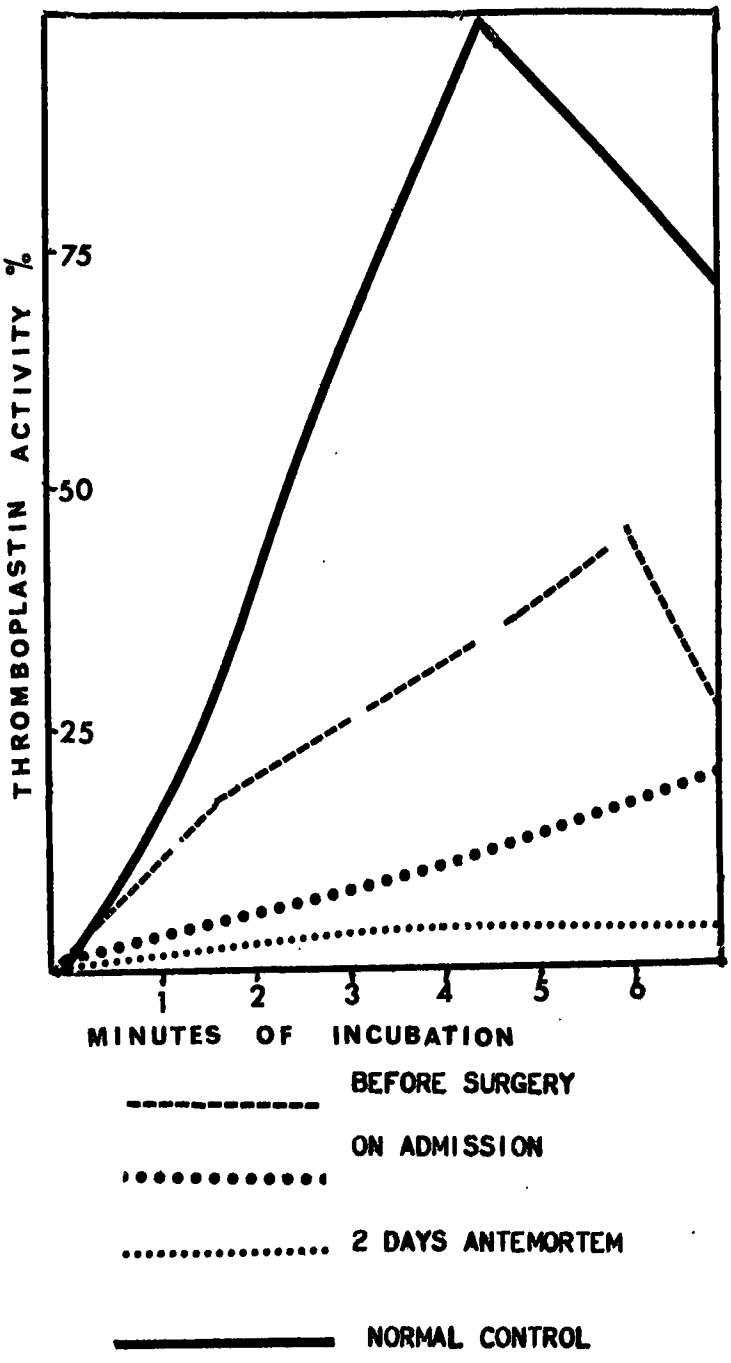
In a patient with either factor VIII or factor IX deficiency, the substitution of the former's plasma or the latter's serum yields an abnormal curve (Figs. 2 and 3). In studying the female siblings or the mothers of these male children, the TGT did not distinguish significantly the abnormalities of factor VIII or IX levels in carrier states or in cases of vascular abnormalities associated with such deficiencies. It was therefore necessary to revise and modify the TGT(82) so that an assay of factor VIII (or, with further modification, factor IX) could be based on the ability of the test plasma to correct thrombin generation of a constant amount of a factor-deficient plasma as compared to the correction obtained with a standard control plasma. Factor V assays were performed also by a modification of this method by substituting factor V-deficient plasma of known concentration in the place of the factor-VIII deficient plasma of the original method.

By extrapolation of the straight line of the logarithmic calibration, conversion to percent activity was made on plasmas which had a shorter mean clotting activity than the standard controls. The values obtained by this method could be duplicated within the statistical mean of error allowed by Brinkhaus (83) in coagulation studies.

In the usual haemophilic patient who had received many transfusions of blood and plasma, the results are complicated by the frequent occurrence of antithromboplastin substances in his blood and it was necessary to do repeated studies of the TGT before and after treatment. When such serial determinations were made, there appeared to be very little improvement in the TGT or the vascular permeability after therapy. There was, furthermore, complete lack of agreement between the results of the venous coagulation time, prothrombin consumption time (Appendix A), and the TGT, and between these tests and the clinical course. Only in cases in which transfusion of fresh platelet-rich blood was followed by transfusion of factor VIII-rich plasma was there any marked improvement in the TGT activity.

One particular case, that of an eight-year old boy, was intensively studied. This haemophiliac had an original factor VIII titre of less than 3 percent activity. Surgery was necessary in this case to correct a severe anastomosis. Repeated transfusions of fresh concentrated plasma failed to raise to factor VIII level above thirty percent activity. Purified Cohn fraction (AHG) was given until the pre-operative level attained 45 percent activity. The child had an excellent recovery until he became sensitized to plasma protein and developed a potent antithromboplastin which resulted in profuse haemorrhage and death (Fig. 4).

FIG. 4


THROMBOPLASTIN ACTIVITY %
75
50
25
1
2
3
4
5
6
MINUTES OF INCUBATION
BEFORE SURGERY
ON ADMISSION
2 DAYS ANTEMORTEM
NORMAL CONTROL

Actual cases were studied which included a deficiency, major or minor, of at least one each of the coagulation factors. Other cases were evaluated by the TGT which showed not only quantitative deficiencies of one or more specific coagulation factors, but also qualitative derangements of the normal coagulation mechanism. These disturbances of the coagulation process not associated with specific deficiencies per se, acted as "circulating anticoagulants" against specific phases of the coagulation reaction and are, therefore, discussed as antithromboplastins, antithrombins or specific fibrinolysins.

The results of the experiments and the interpretation of the thromboplastin generation test of the blood of the above cases indicated that four distinct phases of the coagulation process could be assayed as to specific activity and the individual coagulation factors as well as platelet factors activity could be assayed and estimated in duplicate and triplicate samples within the statistical mean of error. All activity assays were compared to the activity of normal control specimens.

(b) Stage One - Initiator Reaction

1. Factor XII appeared to be the only factor which is directly activated by surfaces and the degree of its activation varied inversely with the particle size of suspensions. In siliconized tubes, prothrombin consumption is poor when platelet-rich plasma is clotted. Paraffin had a somewhat similar effect. Exposure of the plasma to contact with glass in the presence of platelets initiated the reaction effectively and the addition of

serum to platelet-rich plasma in silicone-coated or paraffin-coated tubes activated factor XII activity which paralleled the reaction which was obtained in contact with glass without the addition of serum. It was necessary to rigidly control the size of the test tubes used in the experiment and to insure that chemically clean test tubes were used throughout all of the determinations.

2. Incubation of serum containing a suspension of capric acid and serum containing arachidic acid were compared as to their activation activity of factor XII. The coagulation time obtained with the arachidic acid was almost twice as long as the coagulation time when capric acid was used in the experiment. The results of this experiment were qualitative rather than quantitative due to contamination by factor XI activity.

3. A one-stage assay for factor XI activity was used (86). This assay is based on the ability of the test plasma to shorten the long partial thromboplastin time of factor XI-deficient substrate plasma in the presence of an optimal activating surface. Kaolin was used as the surface or contact activator.* The factor XI-deficient substrate plasma and the test plasma were stored frozen. The levels of factor XI activity in 56 controls derived from the blood from normal adults were distributed in activity over the range between 65 and 135 percent of the standard reference plasma. These levels were consistent with the range of normal values found in the same control group for assays of factor VIII activity. De-

* Platelin, W-3906

Supplied by Warner Lambert Co., Morris Plains, N.J.

ficiencies of factor XI involved major bleeding potentials below 20 percent activity and minor complications when the activity level varied between 30 and 55 percent.

(c) Thromboplastinogenesis

4. Factors VIII (AHG) and V disappeared during the normal clotting process and it may be assumed that they serve as substrates in the TGT reaction.

When normal platelets, previously incubated in normal adsorbed plasma, were substituted in the TGT there was a partial correction of the factor VIII defect as compared to when the haemophiliac's own platelets and adsorbed plasma were substituted. The addition of factor VIII to the saline platelet washing fluid showed increased generation activity of the previously demonstrated factor VIII deficiency of platelet function of the haemophiliac's own platelet.

Similarly, in the case of three factor IX deficiencies, the addition of normal serum to the platelet washing saline slightly improved the generation of thromboplastin after six minutes incubation in the TGT. Incubation of the factor IX-deficient platelets with both adsorbed plasma and serum restored the platelet activity to a normal level. One case which was studied failed to show the latter correction and by serum electrophoresis the child, his sibling sister and his mother were proven to be cases of congenital hypogammaglobulinemia, whose platelets were unable to adsorb factor IX during a state of infection.

Two classical examples of induced factor V deficiency were studied. Case one was a 14 year old boy who had extensive liver surgery for the removal of cysts whereas case two had intensive involvement of liver tissue by histoplasmosis. The factor V deficiency in each case was not corrected in the TGT after six minutes incubation when the platelets of the individual patients were used in spite of the fact that the serum agent used in the test contained an optimum amount of factor V. However, when the same platelets were previously incubated in a factor V-rich media, TGT activity was restored to normal within four minutes of incubation.

5. Factors IX, X, XII and XI are not consumed in the TGT reaction and appear to act as specific enzymes and co-enzymes.

It has been reported that alkylating agents such as methylbromide and ethylene oxide specifically and reproducibly cause a factor X deficiency in mice (109).

A true induced deficiency of factor X was detected by the author as the cause of an haemorrhagic state associated with necrosis of the liver following an acute toxaemia of pregnancy.

6. Antithromboplastin activity was demonstrated in specific cases which behaved as a lipid inhibitor and appeared to bind either factor VIII or factor IX and make them totally or partially unavailable for thromboplastin generation. Excessive bleeding was evident in all cases and three cases showed a prolonged coagulation time in the presence of normal blood fibrinogen levels.

(d) Thrombinogenesis

7. The mechanism of prothrombin conversion depends on the type of thromboplastin substance supplied in the test. When tissue thromboplastin is used, factor VII is considered necessary for prothrombin conversion.

Hypoprothrombinemia which is characterized by a prolonged plasma prothrombin time is determined with the use of a tissue thromboplastin plus optimum calcium concentration. In conjunction with the TGT and prothrombin consumption test (Appendix A) hypoprothrombinemia is defined as a result of one or a combination of (1) diminished factor II activity, (2) factor V activity, (3) factor VII activity, (4) diminished factor X activity, or the presence of excessive antithrombin activity.

The induction of a clinical haemorrhagic state due to hypoprothrombinemia was evident when the concentration of factor II or factor V fell below forty percent of normal activity, whereas a similar effect due to factor VII deficiency was usually induced only when the level of this factor fell below five percent of normal activity. Therefore, it may be concluded that prothrombin (factor II), factor V and factor VII do not exert an equal effect in vivo on the coagulation process.

8. Russell's viper venom was used to distinguish hypoprothrombinemia due to factor VII deficiency from hypoprothrombinemia due to a deficiency of factor X. The venom is dependent on the presence of platelets and plasma lipids in the conversion reaction of prothrombin to thrombin and is not affected by any concentration of factor VII.

9. Incubation of factor V deficient platelets with trypsin destroyed factor V activity and did not affect platelet morphology. Platelets from patients who showed a factor V level of less than ten percent activity, were washed twice in saline and incubated in suspension in normal plasma. Factor VII was adsorbed in minimum quantities (less than 1.3%) and could not be removed by four saline washings.

(e) Stage 4 - Fibrin Formation

10. Pure thrombin was added to washed platelets suspended in siliconized containers. The platelets microscopically showed clumping and viscous metamorphosis. Two cases of afibrinogenemia of the blood were studied in which this process did not occur. Another case, which presented the diagnostic problem of normal activity of all coagulation factors yet was a classical case of purpura in the presence of a normal platelet, count showed failure of adsorption of fibrinogen by the platelets although the fibrinogen level of the citrated plasma exceeded 200 mgms. percent. No fibrinolysins were demonstrated in this latter case.

11. Eight cases of purpura occurring in the presence of renal disease with an elevated blood urea nitrogen were examined by coagulation studies. All of these particular cases, except one, demonstrated a qualitative platelet incompatability although quantitatively the platelet count exceeded 165,000 per cu. mm. With the addition of thrombin to the platelet suspension, viscous metamorphosis was incomplete and the fibrin network which was formed was very pliable and almost completely soluble when a few drops of one percent urea solution was added to the suspension. Fibrin polymerization, therefore, was incomplete in the presence of a criti-

cal level of urea concentration in the platelet or in plasma.

The above studies were repeated on two of the above cases when the urea nitrogen level of the blood exceeded 60 mgms. percent. Platelet agglutination or viscous metamorphosis could not be demonstrated at this level of renal failure.

12. All control standard plasmas were assessed for prothrombin activity. The serum from whole blood was tested for residual prothrombin activity exactly one hour from the clotting of the specimen and the amount of prothrombin consumed in the reaction was calculated. In all normal cases prothrombin is present in the blood in excess of that needed to complete the reaction in phase 3 and the conversion to thrombin. The most reproducible and reliable results were obtained when the prothrombin activity in the serum was measured using a standardized source of factor I in a one-stage modified assay (Appendix A). A graph was prepared from the various dilutions of normal prothrombin activity (Fig.15). The application of this method has inaugurated a simple procedure for the detection of potential bleeders prior to surgical operations.

Commercial preparations of factor I produced unpredictable variations in results and failed to properly predict bleeding tendencies which might result from qualitative platelet deficiencies.

All cases in which the prothrombin activity of the serum exceeded less than eighty percent of prothrombin consumed in phase 3, showed mild, moderate to severely prolonged tendency to haemorrhage spontaneously or after surgical procedures.

13. Antithrombins were demonstrated to be of four specific types:

1. Delay of conversion of thrombin to fibrin due to a deficiency of fibrinogen below the optimum level.
2. Heparinoid in activity and inhibiting the interaction of thrombin and fibrinogen. This antithrombin could be neutralized by the addition of 0.05 ml. of 1% protamine sulphate or toluidine blue to the incubation mixture.
3. An antithrombin was detected both in the plasma and serum. It was stable at 4°C and could be neutralized by the addition of 0.5 ml. of ether to the incubation mixture.
4. Antithrombin 4 was demonstrated in one case only and behaved at the interaction of heparin with a heparin cofactor dissimilar to that of antithrombin 2, as in this particular case the antithrombin was neutralized by toluidine blue but protamine sulphate rendered the plasma incoagulable in vitro.

Patients who developed antithrombins showed an anticline effect of the TGT curve. This finding is not the consistent observation in cases of specific coagulation factor defects.

14. When thrombin was added to washed platelets a coagulum was formed. Analysis of the residual fluid after centrifugation revealed the disappearance of fibrinogen which had been previously assayed in the mixture before the mixture was activated and the coagulum had formed. In the case referred to in item 10 no coagulum was found when thrombin was added to the washed platelets. The mixture revealed an absence of fibrinogen in the platelets.

15. Fibrinolysins were demonstrated in many cases due to extensive exposure to burns, to accidental shock and specifically in gynecological cases of placental retention. A coagulum was formed when thrombin was added to the washed platelets and fibrinogen activity could be detected in the washing fluid. This fact would indicate that the platelet has some protective action against anti-coagulation factors which might interfere with its physiologic function.

16. Twenty-five cases of increased vascular fragility or permeability were examined because the patients complained of excessive bleeding or bruising. Eleven of these cases showed a defect in the arteriolar end of the capillary loops of the nailbed which was examined by a dissecting microscope. Ten cases showed a combination of capillary abnormality and a decrease below the optimum level of factor VIII activity.

In contrast to the true sex-linked inherited deficiencies of factor VIII in which the titre remained constant, the cases associated with vascular fragility showed variation in the titre of factor VIII dependent upon whether the blood was examined after or during an infection, during or between menstrual periods or during periods of mental stress.

17. Assays of factor V, VII, XI and particularly factor XII were easily plotted by using arithmetical serial dilutions of standard plasmas or sera plotted on semilogarithmic graph paper. However, the sex-linked recessive deficiencies such as factor VIII and factor IX were only successfully assayed by plotting the dilutions by logarithmic means.

(f) Platelet Activity

18. Platelet activity as determined by step 5(c) TGT demonstrated that their activity was diminished by repeated washings but the loss of such activity could be restored by the addition of the original saline supernatant wash in which they had been previously incubated.

When washed platelets were added to whole blood they retained their effect on the coagulation time of the blood.

When saline substrate washings from qualitatively defective platelets were added to normal washed platelets the complete activity in the TGT was not restored (Figs. 10 and 11).

Washed platelets from cases of factor VIII or IX deficiencies as well as other factor deficient sera or plasmas appeared to be qualitatively defective when the patient's own platelets were used. However, they regained their normal activity in the TGT after incubation in normal media which contained the previously deficient coagulation factor or factors.

Optimum activity of the platelets was obtained only when buffered saline was used which consisted of one part of barbital buffer and nine parts of 0.76 percent saline, the solution having a physiological concentration of 0.80.

Microscopic examination of the platelets revealed that tiny spicules were still present as a part of the cell morphology and no cellular breakdown had occurred in the in vitro system during experimentation.

A case of neonatal thrombocytopenic purpura combined with thrombocytasthenia was studied intensively by the author. The infant was born with extensive areas of petechiae and a tendency to haemorrhage and trauma.

The examination of the platelets revealed a thrombocytopenia of 17,000 platelets per cu. mm., the platelets being grossly enlarged (from 5-8 micra) and showing anisocytosis. Further examination of stained suspensions of the infant's blood revealed platelet adherence to the erythrocyte surface.

The phenomena of platelet metamorphosis was easily distinguished at all stages in the cells of this exceptional case.

The early stages of adherence showed a depression in the erythrocyte stroma in which the platelet was firmly fastened. The hyalomere of the platelet was unchanged and similar to the hyalomere of platelets individually suspended in the media. The mitochondrial granules of the chromomere showed no specific organizational pattern.

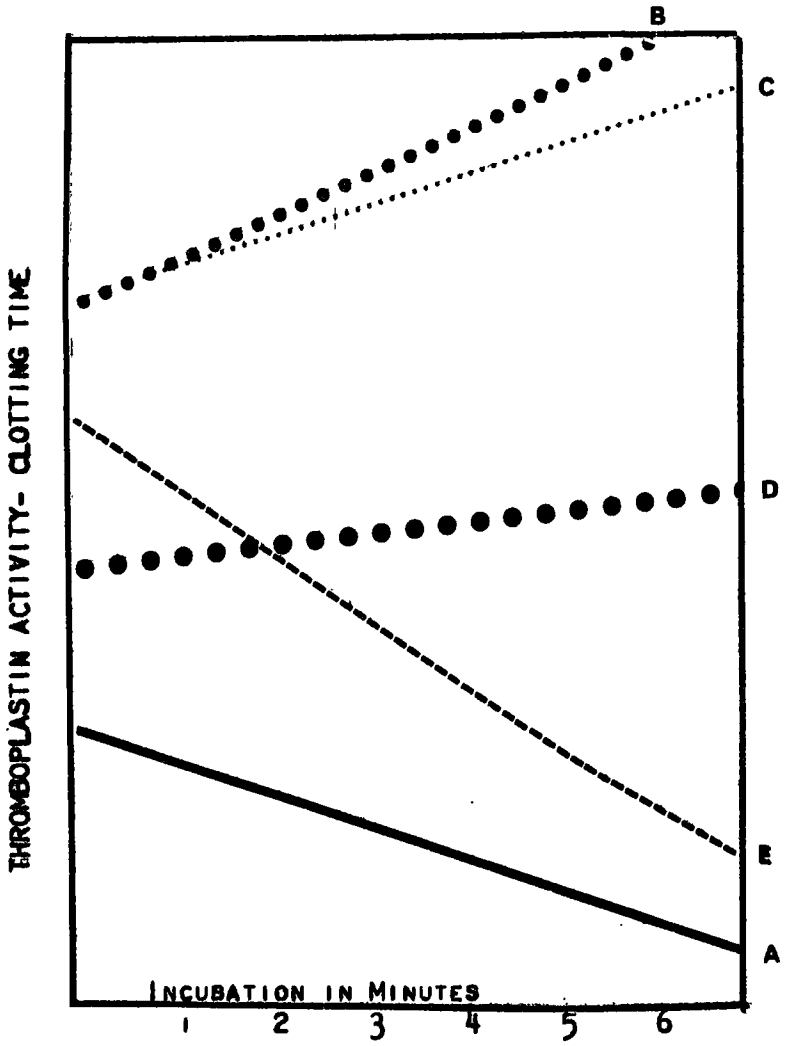
Progressive changes were noted in the behaviour of platelet structure dependent upon the time it was exposed to the erythrocyte surface. The hyalomere proceeded to enlarge and dendritic structures became evident which, on the surface of some erythrocytes, extended to embrace over one-half the circumference of the erythrocyte. The mitochondrial granules of the platelets underwent organization into a concentric ring close to the periphery of the hyalomere. Fibrillae-like structures were noted emanating from the condensed granules towards the center of the cell, each granule and its fibrillae giving a clothespin-effect with the heads all pointing towards the surface of the dendritic structures of the hyalomere.

The general arrangement of the fibres was reproducible and no cells were examined at the later stage which showed random arrangement of the fibrillae-like structures.

The final phase of platelet activity on the erythrocyte structure was evident as a progressive disintegration of the hyalomere substance, with the chromomere arrangement still intact on many of the erythrocytes.

Hyperprothrombinemia was evident with an excess of formed prothrombin as well as an excess of unconsumed prothrombin in the serum which on assay, showed only 70% consumed in the clotting process. Further incubation of the clotted blood for four hours showed an increased level of prothrombin activity in the serum, the level being double the value of the first assay. Assay of the platelets for factor V activity showed an increase in this factor twice the normal value per unit of plasma and this is assumed in vitro to account for the hyperacceleration of thromboplastin generation when the infant's platelets were substituted in the test.

FIG. 5



A ——— NORMAL CONTROL

B ●●●●● FACTOR VIII 3.0 % PLUS FACTOR XII DEFECT.

C ···· ···· B PLASMA TREATED WITH ETHER

D ● ● ● ● B PLASMA, ETHER + NORMAL PLASMA

E – – – – – MOTHER OF HAEMOPHILIAC, CARRIER STATE. AHG- 46%

FIG. 6

THE EFFECT OF INFECTION ON FACTOR IX ACTIVITY
IN A FAMILY WITH CONGENITAL HYPOGLOBULINEMIA

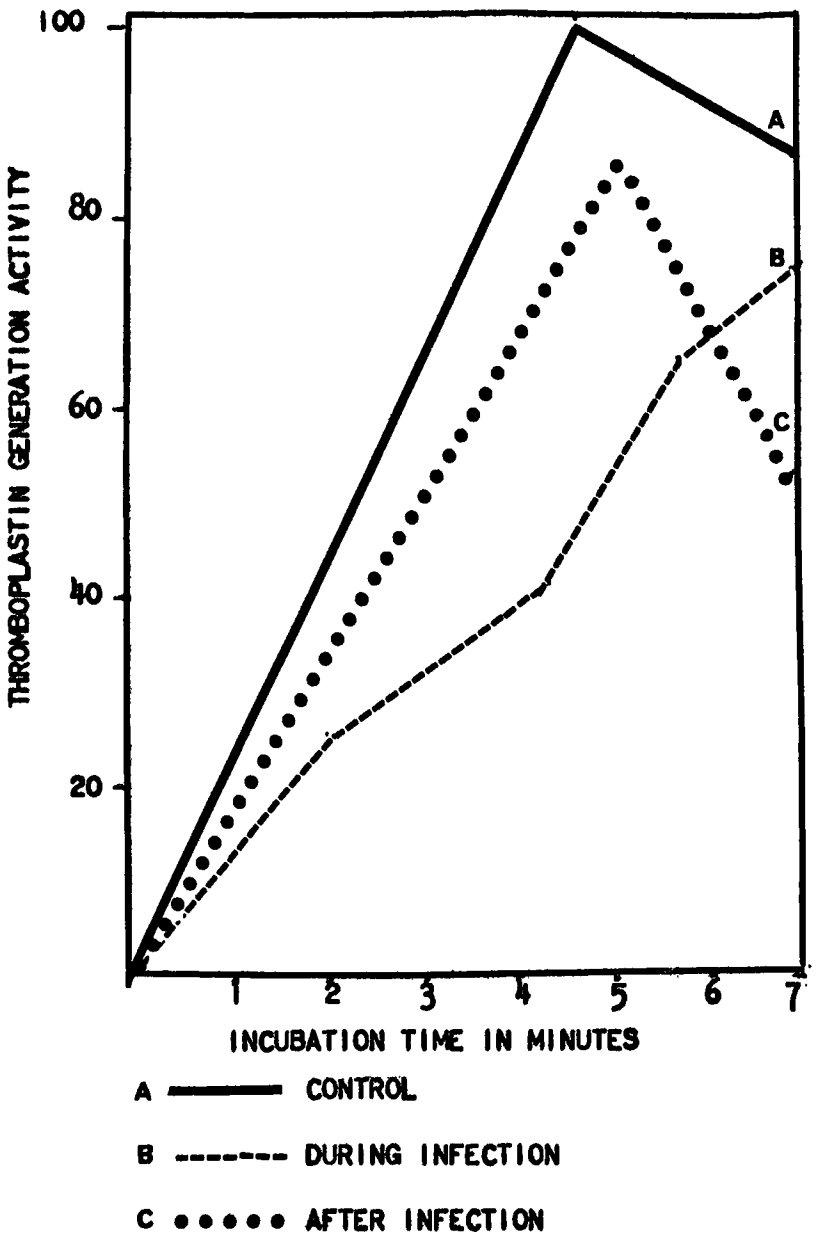


FIG. 7

THE EFFECTS OF NORMAL PLASMA, NORMAL DEPROTHROMBINIZED PLASMA AND NORMAL SERUM ON THE COAGULATION ABNORMALITIES IN DEFICIENCIES OF FACTORS VIII, IX AND XI.

REAGENT ADDED TO PATIENT'S PLASMA	DEFICIENCY		
	FACTOR VIII	FACTOR IX	FACTOR XI
NORMAL PLASMA	CORRECTS	CORRECTS	CORRECTS
NORMAL ABSORBED PLASMA	CORRECTS	NO CORRECTION	CORRECTS
NORMAL SERUM	NO CORRECTION	CORRECTS	CORRECTS

FIG. 8

DIFFERENTIATION OF PROTHROMBIN COMPLEX DEFICIENCIES

DEFICIENT FACTOR CAUSING PROLONGED PROTHROMBIN TIME	EFFECT OF ADDITION OF		
	NORMAL PLASMA	SERUM	ABSORBED PLASMA
FACTOR II	CORRECTS	NO CORRECTION	NO CORRECTION
FACTOR V	CORRECTS	NO CORRECTION	CORRECTS
FACTOR VII	CORRECTS	CORRECTS	NO CORRECTION
FACTOR X **	CORRECTS	CORRECTS	NO CORRECTION

** DIFFERENTIATED IN THE TGT AND BY VIPER VENOM.

FIG. 9

SCHEMATIC REPRESENTATION OF THE FOUR PHASES OF BLOOD COAGULATION BASED ON EXPERIMENTAL DATA AND ELECTROPHORESIS.

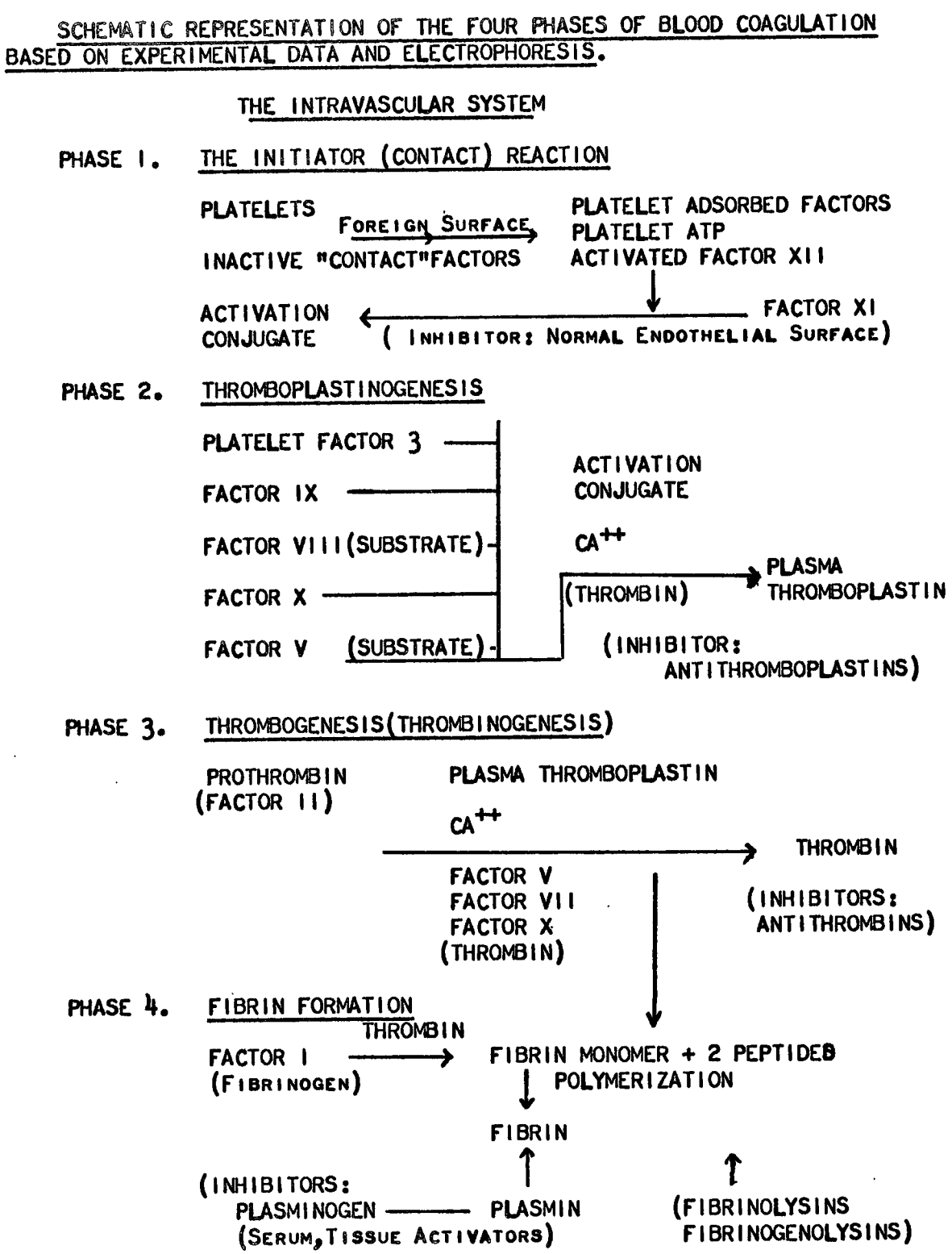
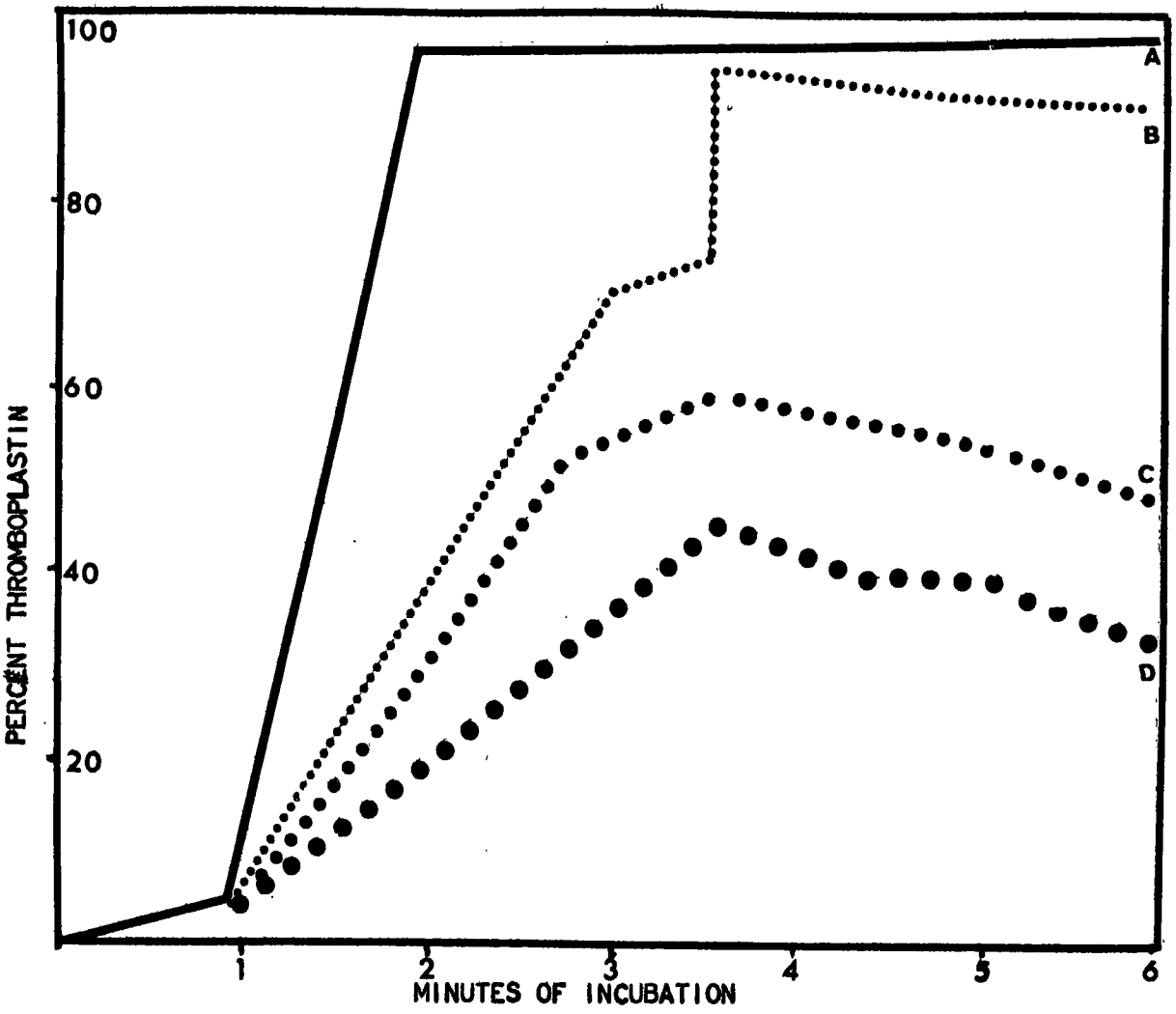


FIG. 10



A ——— PLATELETS(NORMAL)-280,000/CU.MM.

B ..... PLATELETS(NORMAL)-149,000/CU.MM.
INCUBATED 4 MINS. IN NORMAL PLASMA.

C •••• PLATELETS(THROMBOPATHIC)- 149,000/CU.MM.
INCUBATED 4 MINS. IN NORMAL PLASMA.

D ●●● PLATELETS(THROMBOPATHIC)- 149,000/CU.MM.
INCUBATED 4 MINS. IN PATIENT'S PLASMA.

FIG. 11

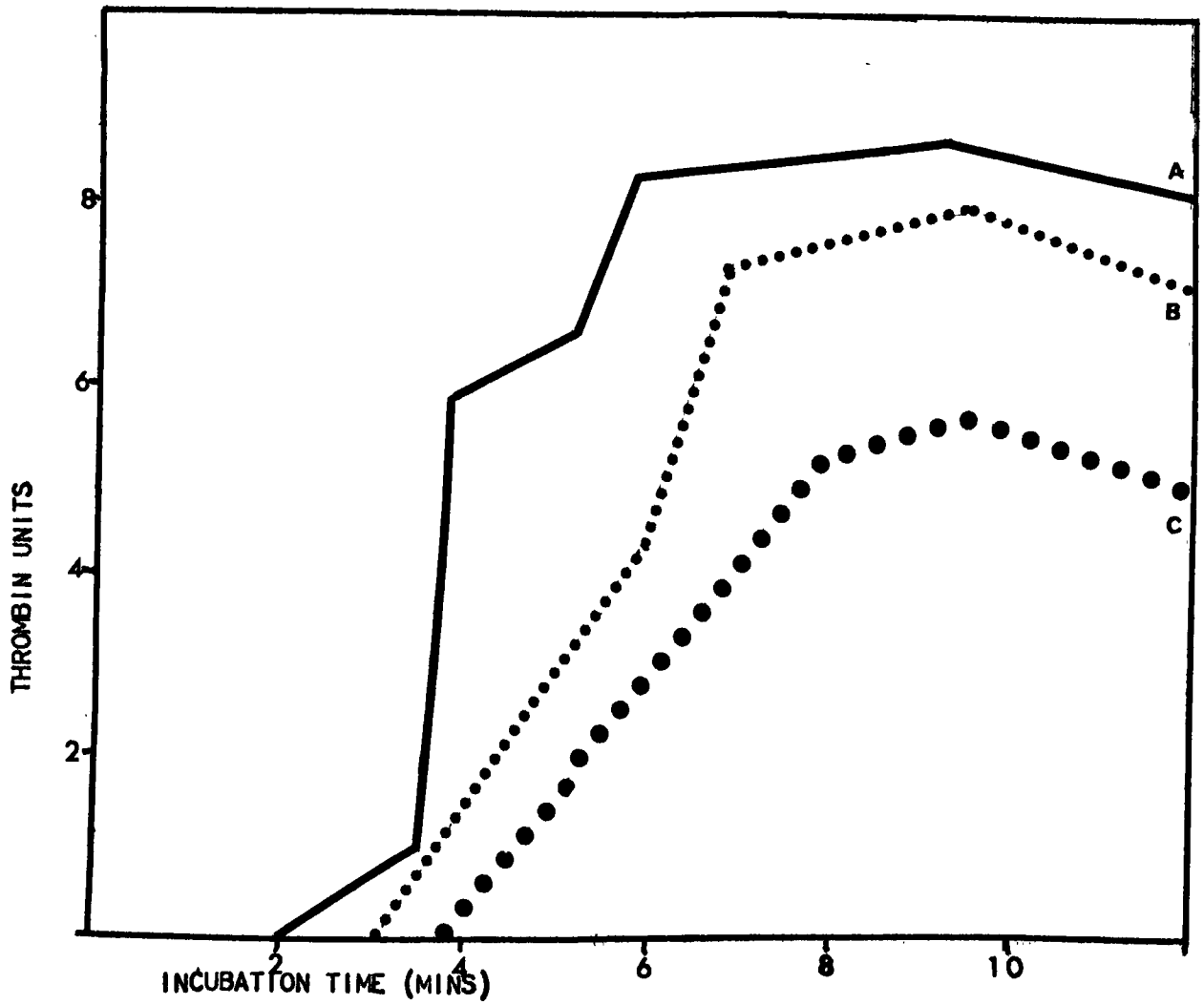

THROMBIN UNITS
2
4
6
8
A
B
C
2
4
6
8
10
INCUBATION TIME (MINS)
A ——— NORMAL PLATELETS__ 280,000 PER CU. MM.
B ••••••• NORMAL PLATELETS__ 149,000 PER CU. MM.
C ● ● ● ● THROMBOPATHIC PLATELETS __ 149,000 PER CU. MM.

FIG. 12

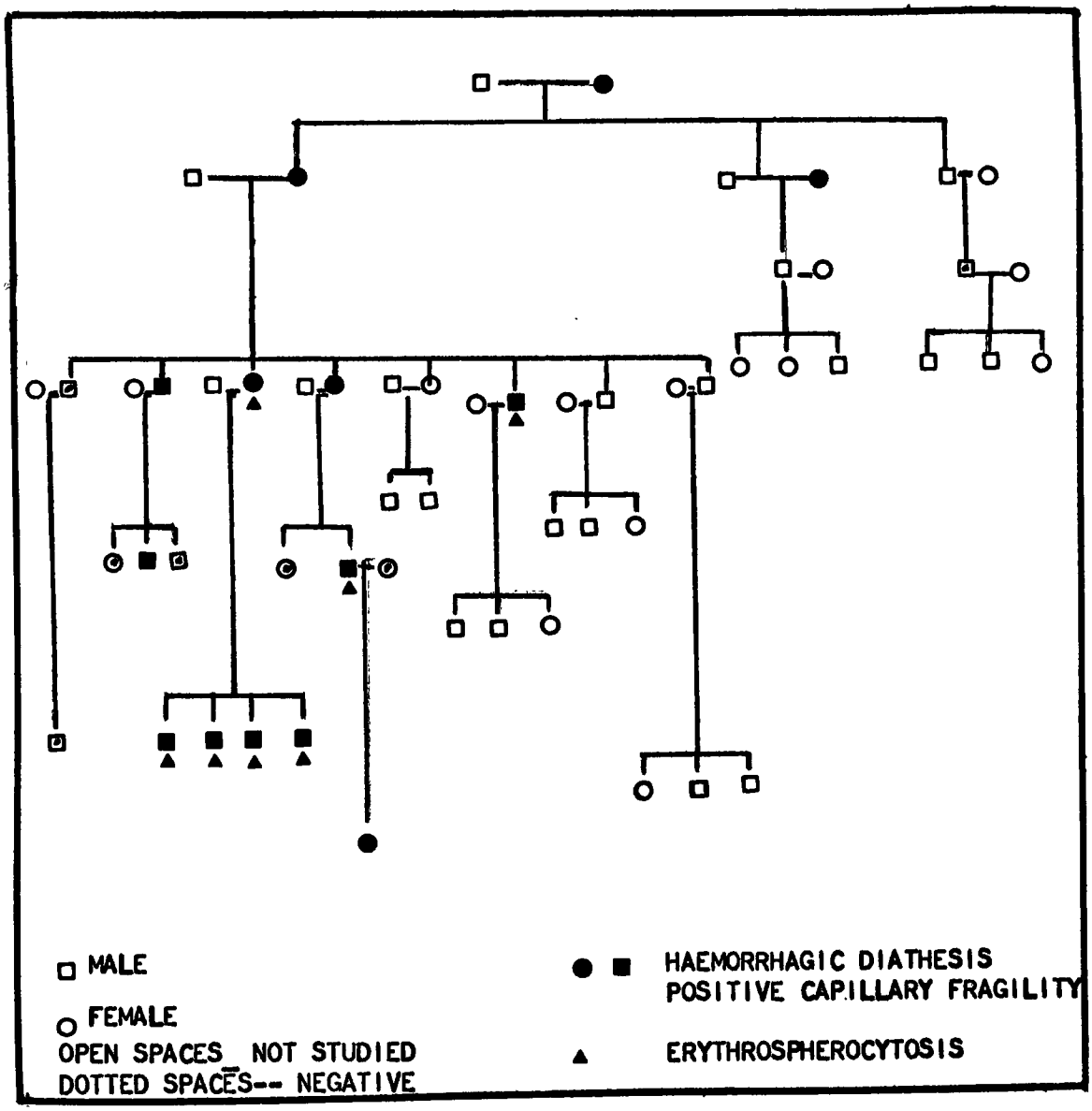
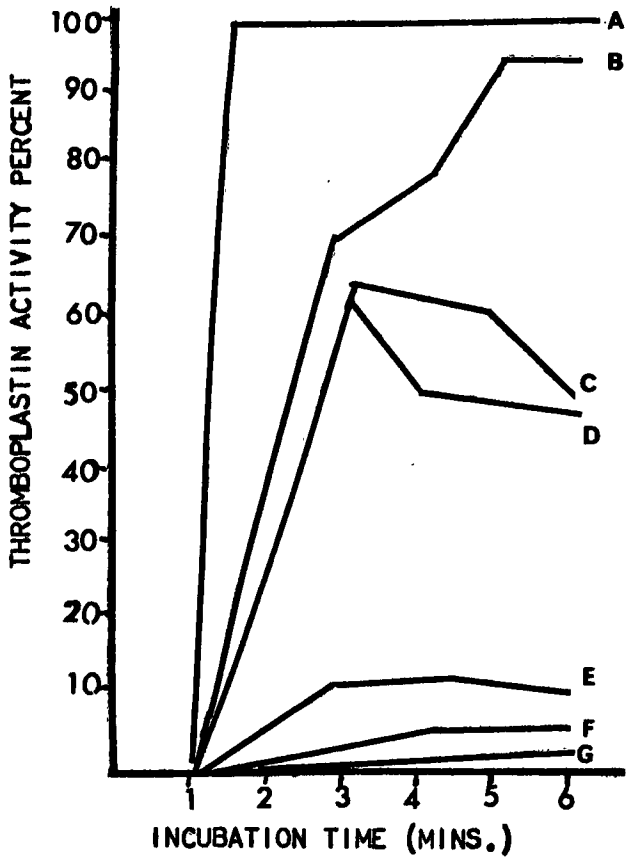

MALE
FEMALE
OPEN SPACES NOT STUDIED
DOTTED SPACES-- NEGATIVE
HAEMORRHAGIC DIATHESIS
POSITIVE CAPILLARY FRAGILITY
ERYTHROSPHEROCYTOSIS

FIG. 13

THE EFFECT OF PLATELET CONCENTRATION ON THE GENERATION OF PLASMA THROMBOPLASTIN



A – 330,000 PLATELETS / CU. MM.

B – 166,000 PLATELETS / CU. MM.

C – 83,000 PLATELETS / CU. MM.

D – 42,000 PLATELETS / CU. MM.

E – 22,000 PLATELETS/ CU. MM.

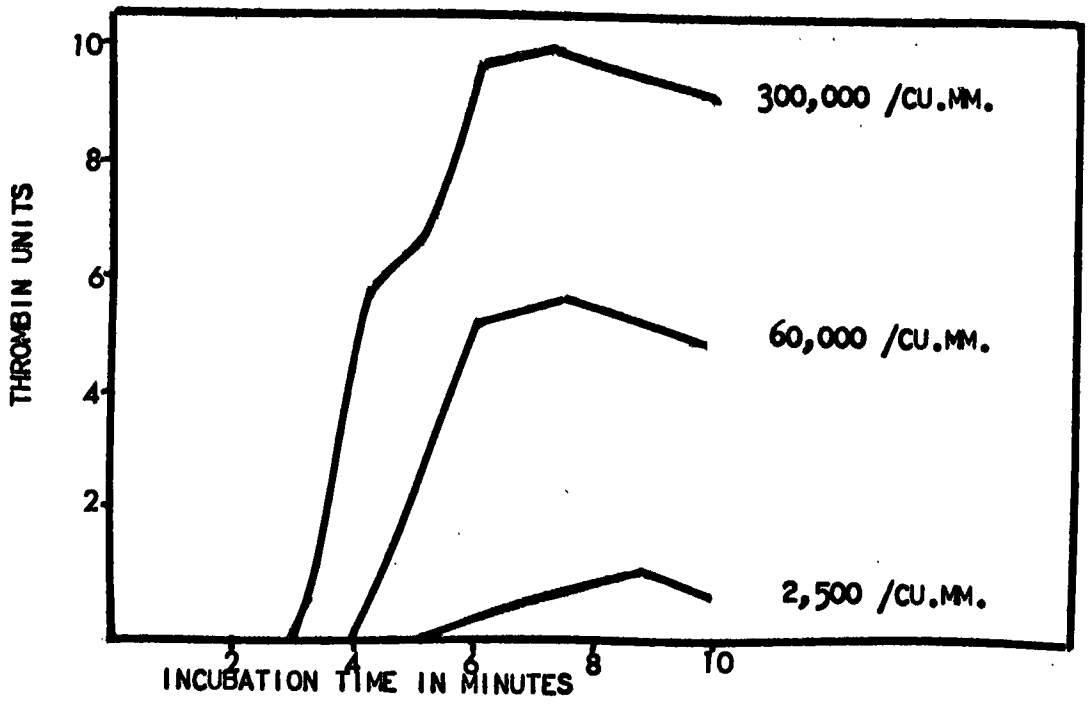
F – 5,000 PLATELETS / CU. MM.

G – 3,000 PLATELETS / CU. MM.

THE CONCENTRATIONS SHOWN ARE FOR PLATELETS PER CUBIC MILLIMETER OF FINAL INCUBATION MIXTURE.

FIG. 14

RELATIONSHIP BETWEEN PLATELET CONCENTRATION AND THROMBIN FORMATION



THE CONCENTRATION SHOWN IS THE NUMBER OF PLATELETS PER CUBIC MILLIMETER OF PLASMA USED TO GENERATE THROMBIN.

General Discussion

Mechanisms of Reactions. Reactions Involved.

Four phases of reactions are recognizable and can be proven by experimental evidence. The presence of an inherited disorder of a coagulation factor deficiency can be distinguished from an acquired inhibitor directed against a specific coagulation factor by the study of representative cases. The paradox of normal so-called 'screening' tests for bleeding and coagulation followed by excessive post-surgical or spontaneous capillary bleeding can be explained by careful analysis of the phases of the coagulation mechanism in relation to the integrity of capillary vascular epithelium.

1. Phase I - Initiator Reaction

Experimental evidence shows that a plasma factor becomes activated by contact with glass (Page 45) but in the absence of platelets this activation cannot be measured successfully because the action of the activated agent is on the platelets. In siliconized tubes, prothrombin consumption is poor when platelet-rich plasma is clotted. Freezing and thawing has no effect on increasing prothrombin consumption by platelet disintegration in silicones. In glass tubes, however, consumption is high.

In aged normal serum, the platelet factor is not supplied and prothrombin consumption is low. However, with the addition of serum to platelet-rich plasma, prothrombin consumption occurs in silicone. Therefore,

serum contains a factor which is activated by a contact surface such as glass which can then react with platelet factors. This is the so-called Hageman factor or factor XII.

The activity of factor XII is related not only to the surface area to which the material is exposed but also varies inversely with the particle size of suspensions. In the case of fatty acids the diameter of the micelles is proportional to the chain length. Hence the specific surface area of a suspension of capric acid (C_{10}) should be twice that of one of arachidic acid (C_{20}).

Two conditions must be fulfilled for a surface to be "active". Firstly, there must be an affinity for factor XII and this is apparently determined by the presence of electro-negative polar sites (84). Secondly, there must be sufficient surface area to permit the unfolding of the large molecule of factor XII. In vivo, intravascular thrombi must be the result of (1) free fatty acids, uncoated or diverted by blocking agents, come into contact with blood and (2) the blood must contain factor XII in some specific sequence of its peptide chain. Margolis (84) identifies the reaction to simulate the kallikrein-kallidin system. Other recent work indicates that factor XII is required for the formation of kallikrein for the release of polypeptides in blood vessels (85).

Assays for concentrations of factor XII are crude estimates of its activity as, in order to assess the concentration accurately, it must be completely activated prior to its measurement. This can be done by bringing the preparation into contact with glass, kaolin or some other effective

agent. Only in its activated form will factor XII correct the specific defect in plasma deficient in this factor or accelerate the coagulation of normal plasma. The only specific way to measure the concentration of factor XII is to test its effect upon a substrate of plasma obtained from a patient with factor XII deficiency trait. Such plasma, however, inhibits the activation of factor XII by surfaces. The use of chicken plasma, which has apparently been proven to be deficient or to not contain factor XII has not proven satisfactory in the experiments of the author. It is therefore essential that the activation of factor XII be complete before the addition of the substrate of factor XII-deficient plasma.

Contamination of the activated factor XII with factor XI activity rapidly shortens the clotting time of factor XII-deficient plasma. Factor XI (PTA), once activated in the presence of activated factor XII, shows a very rapid contact and interaction of the substrate bonds, thus producing a condition which favours initiation of a second phase enzyme substrate reaction involving factor IX, and specific platelet factor with factor VIII as a substrate.

Rapaport et al (86) by quantitative assay measured the activity of factor XI in all available members of the families of eight propositi with severe factor XI deficiency. From his data and from my study of four cases it is evident that factor XI deficiency exists only in two forms: major deficiency, characterized by the potential for serious post surgical bleeding with factor XI levels up to 20 percent of the activity of reference plasma, and minor factor XI deficiency with levels between

30 to 65 percent of the standard reference plasma and characterized by insignificant or easily-controlled bleeding.

It would appear that the gene for factor XI deficiency is an incompletely recessive autosomal gene which produces major factor XI deficiency in the homozygote and minor factor XI deficiency in the heterozygote.

Studies on the treatment of factor XI deficiency reveal that the defect may be corrected and controlled by the administration of stored plasma with the effect gradually disappearing over the period of eight days.

The levels of factor XI in 56 controls from normal adults were distributed over the range between 65 and 135 percent of a standard reference plasma. The thromboplastin agent used was the cephalin-kaolin reagent supplied by a commercial firm.*

Attempts to utilize absorbed functions of chicken blood (87) in the assay gave confusing results either combined with human thromboplastin or Russell's viper venom. However, further investigations and correlation of results might indicate alternative pathways or mechanisms of the coagulation factor enzymes.

* Platelin - W 3906

Warner Lambert Co., Morris Plains, N.J., U.S.A.

2. Phase II - Thromboplastinogenesis

Reactions Involved

There is evidence that factor VIII and factor IX are normally combined with a lipid inhibitor in the blood. When plasma thromboplastin is first formed, its activity is very powerful. As developed in the TGT, its activity equals the most potent of tissue thromboplastin preparations. The fact that it rather rapidly loses potency within an hour after incubation and generation appears to be due to neutralization by some antithromboplastic component in the blood. This can be confirmed by the deficient thromboplastin generation noted in the presence of excess production of blood antithromboplastins (88). A similar though less marked effect is product by an increase in antithrombin because thrombin accelerates the formation of thromboplastin.

Experiments on factor VIII-deficient plasmas by the author (Fig. 5) indicate that the factor-inhibitor conjugates can be dissociated artificially by ether extraction but that, when repeated transfusions of fresh previously-frozen plasma are given, the inhibitor conjugates are more firmly adapted to the inhibition of factor VIII activity and dissociation is incomplete or impossible to attain. This may further indicate the action of antibodies which completely adapt the inhibitor conjugates to factor VIII or factor IX thereby further arresting their activity in the generation of thromboplastin. The platelets from such cases also show inhibition of adsorptive ability as compared to normal platelets previously incubated in the factor-deficient plasma or serum from the same case.

The quantitative activity of factor VIII or factor IX in no way appears to change from its original estimate on assay after birth but qualitatively its activity may be changed. Studies by curtain electrophoresis (89) indicate that factors V, X and VII are formed in the albumen and prealbumen areas, factor VIII in the alpha-1 and alpha-2 globulin regions and to a minimal extent extend into the albumen area. Factor II and factor IX were located in the alpha-1, alpha-2 and albumen areas. Antifibrinolysin was present in the alpha-1 and albumen regions; antithrombin, in alpha-1 and alpha-2, whereas factor XI was found over a broad range in the middle mobilities. Fibrinogen (factor I) was largely present in the faster-moving gamma globulin and slower beta-globulin regions.

A case studied by the author was that of a male child, aged six, whose plasma on admission showed less than 3 percent factor VIII activity. Pre-operative therapy included the administration of freshly-thawed plasma and bovine extracts of concentrated factor VIII (Cohn fraction I) until the titre of factor VIII remained constant at a level of 30 percent activity. Similar therapy was maintained postoperatively for four weeks with minimal indications of bleeding. However, a point of deterioration in the patient's condition was reached where, in spite of transfusions of fresh blood, factor VIII-concentrated plasma and steroids, the thromboplastin generation test showed an increasingly abnormal curve (Fig. 4). The activity of factor VIII was completely inhibited as well as platelet adsorption properties and partial inhibition of factor IX activity. The generation of thromboplastin was only partially corrected by the addition of human thromboplastin extract at the time the blood was taken and before the plasma was separated.

This indicated a stimulated allergic response in a sensitized recipient and the production of an antithromboplastin substance specifically against factor VIII, factor IX and platelet activity. Electrophoresis indicated no change in the alpha-1 or alpha-2 proteins, but a broadening in the albumen and gamma globulin peaks.

After death, imprints of bone marrow, lymph nodes, spleen and liver revealed extensive plasma cell proliferation and formation in all of these organs.

The evidence that lymphatic tissue is directly involved in antibody formation is no longer disputed. A variety of experimental methods have indicated that cells of the plasmocytic series do contain, release and presumably manufacture antibody (90). Sensitization of bone marrow by specific antigens of certain diseases induce plasma cell response (91). Autoradiographic studies by means of DNA-labelling in plasmolytic cells (92) stimulated by multiple injections of antigen show the enormous proliferative capacity of the early cells of the plasmolytic series. The ability of the proplasmolytes to synthesize RNA is much increased over that of the mature cell in which protein synthesis is minimal. The bone marrow of the case which is described above indicated a similar reaction. This reaction occurred in all haemopoietic organs, was not organ-specific and indicated a response to a circulating antigen.

It may be further stated that hypogamma-globulinemia, consistent or transient may behave as a low production syndrome, while the consumption mechanisms operate normally at a rate which is directly proportional to the plasma concentration of the enzymes or coagulation factors involved in thromboplastinogenesis.

A male child, aged 7, was investigated for excessive post-tonsillectomy bleeding. The TGT of the child's blood demonstrated an abnormal curve of the clotting activity of the substrate plasma mixture which appeared to demonstrate a factor IX insufficiency but it was not evident when tested against a known factor IX-deficient serum. An electrophoretic pattern of this child's serum revealed a hypogamma globulinemia. Vascular fragility was increased and the adsorptive power of the platelets was insufficient. This child, his brother and his mother all had a history of more than average frequency of infection during their lifetime, with easy bruising and episodes of bleeding which succeeded even minor exposure to infection.

Investigation of the blood of the female sibling and maternal parent showed a similar response in the TGT (Fig. 6) after acute infection with almost complete correction to normal within two months later. The electrophoretic pattern still showed a slurred reduced albumen peak and hypogammaglobulinemia but no change in alpha-1.

Normally in the TGT, interaction of the substances demonstrate an equilibrium in production and consumption. In this case and that of the brother and mother, a state of hypogammaglobulinemia, influenced by a low-grade infection, was sufficient to incur abnormal generation in the thromboplastin generation complex without alteration of the normal concentrations (qualitative) of factors V, VIII, IX, X or XI. In the original case this resulted in excessive bleeding after tonsillectomy. Any antithrombic heparinoid effect was ruled out.

The antithromboplastin was species-specific acting more specifically against human thromboplastin extract and interfered with the selective adaptation of factor IX in particular in the formation of thromboplastin in the TGT. Two later cases investigated showed a similar reaction against factor VIII. The inability to form sufficient normal antibody due to hypogammaglobulinemia apparently accentuated inhibitor complexes and produced an abnormal curve of activity (Fig. 6) which identified the process responsible for excessive post-operative capillary bleeding.

The substitution of normal platelet suspension may partially correct the defect of haemophiliac or factor VIII or factor IX-deficient plasma or serum in the TGT(93). Substitution of the platelets from factor VIII or factor IX plasma or serum shows a definite lack of the globulin fraction. The addition of factor VIII or factor IX to the washing fluid shows increased generation activity of platelet function which previously could be demonstrated not to have any specific factor VIII or IX activity. This suggests retention of the specific factor on the platelet surface. Cases, such as mentioned, which demonstrated antithromboplastin activity, showed inhibited adsorption on the platelet surface of either factor VIII or IX.

Insufficient attention has been paid to the probability that the severity of haemophilia is due more to increased vascular fragility and permeability than to the delayed coagulation of the blood. Twelve clinically severe cases, studied by the author showed a prolonged bleeding time, two had a positive test for capillary fragility whereas four showed increased vascular permeability. Once minimal trauma with capillary bleeding

is induced, it continues as though no vascular mechanisms were active to control it. The contrast between factor VIII or factor IX deficiency and a deficiency of factor I (fibrinogen) is striking. In both conditions the blood coagulation time may be extremely prolonged and yet persons with factor I deficiency are free from spontaneous haemorrhage. This indicates that vascular permeability or capillary haemostasis may be more dependent upon the adsorption of platelets of factor VIII or IX than upon the actual normality of adequate quantities of these factors in the prevention of capillary bleeding. The inability of the platelet to supply the factors for adequate catalysis of thrombin at the capillary epithelium surface would explain the inadequate thrombin formation thus resulting in easy capillary permeability.

Antithromboplastin Activity

Inhibition of Stage 2 activity

Antithromboplastin is normally present in blood, and can be demonstrated by adsorption techniques as a form of lipid inhibitor which binds either factor VIII or IX, making these unavailable for thromboplastin generation (88). Dissociation of factor-inhibitor conjugate must take place before thromboplastin can be formed. This dissociation occurs in vivo when platelet factor is made available in the catalytic system. Dissociation can be induced spontaneously on a glass surface or by dilution of serum or plasma.

1. The refactory state in deficiencies of factor VIII or IX have already been discussed.

2. Excessive antithromboplastin activity can be demonstrated in haemorrhage disease occurring in women and associated with childbirth. These cases show a prolonged coagulation time and can be differentiated from haemorrhage due to deficiency of factor I (fibrinogen). There is a possibility, which is difficult to disprove, that an antibody develops as a result of fetal:maternal immunization by placenta fragments.

3. Exposure to ionizing radiation can induce excessive antithromboplastic activity. Individuals subjected to C_{60} total body radiation have shown an elevation of antithromboplastin activity in excess of 200 percent of normal on the twelth to twentieth day after exposure. In combination with thrombocytopenia, antithrombins and vascular damage, the antithromboplastin appears to act specifically against factor VIII.

Several isolated cases in the series were studied in which excess thromboplastic activity developed without reference to the causative factors previously stated. These cases were individuals who developed sensitivity to specific drugs used in the treatment of skin diseases where surface adsorption is high.

Heparin, a highly active anticoagulant, is extracted from liver but it is present in most of the tissues of the body and found in the granules of most cells. The distribution of these cells among the smallest blood vessels and capillaries may indicate that heparin is held strategically in such locations where it may be used to prevent local thrombosis.

The main effect of heparin has been thought to be antithrombic, showing the rate of interaction of thrombin and fibrinogen and increasing the adsorption of thrombin of fibrin (99). Heparin inhibits, however, the activity of plasma thromboplastin and by way of its antithrombic effect interferes with the formation of the thrombin-factor IX complex necessary to induce factor IX activity. Protamine, a strongly basic protein, counteracts the effect of heparin, possibly by virtue of its electric charge, which neutralizes the negative charges of heparin; but protamine may also behave as an anticoagulant capable of also interfering with factor IX activity (100).

One case of toxaemia of pregnancy with subsequent necrosis of the liver was studied by the author. The blood of this patient showed a critical deficiency of factor X, which contributed to severe haemorrhage and increased vascular permeability. Protamine sulphate, when added in minimal quantities to the substrate, behaved as a powerful anticoagulant (Page 50).

3. Phase III - Thrombogenesis

Mechanisms of Reaction

The purpose of the reactions in first two phases is to produce the plasma thromboplastin needed for the conversion of prothrombin to thrombin. When this is made available, some of the prothrombin in the blood is converted to thrombin. Thrombin not only completes the final step, the clotting of fibrinogen, but acts also as an effective catalyzer of other reactions, notably the activation of more platelets and thus providing more energy source for the reaction to proceed.

Three substances are the prerequisite for the conversion of prothrombin to thrombon; (1) plasma thromboplastin, factor V and calcium. Factor V acts as an accelerator normally found in plasma. Once formed, thrombin itself acts to convert factor V to a more active accelerator which actually activates the prothrombin reaction.

When any tissue thromboplastin is used, the reaction involves an additional factor, factor VII, which has no activity in the thromboplastin system. Tissue thromboplastin used in the experiments was derived from rabbit lung* or human brain and reacted with factor VII and calcium first to form an intermediate product which in turn reacted with factor V to form the definitive prothrombin activator.

A third type of reaction occurs when Russell's viper venom** is used. Venom requires platelet activator or cephalin phospholipid plus calcium ions. In this reaction factor VII is not involved but maximum prothrombin conversion requires factors V and X (Figs. 7 and 8).

The mechanism of prothrombin conversion depends, therefore, on the type of thromboplastin substance supplied. Factor VII is considered necessary for prothrombin conversion when tissue thromboplastin is used.

Hypoprothrombinaemia which is characterized by a prolonged plasma prothrombin time, as determined by tissue thromboplastin plus optimum calcium concentration, can be a result of one of or a combination of (1) diminished factor II (prothrombin), (2) diminished factor V, (3) diminished factor VII, (4) diminished factor X, or (5) excessive antithrombin activity.

* Simplastin - Warner Chilcott, Morris Plains, N.J.

**

Tissue thromboplastin and calcium are added in optimum concentrations only and therefore, these two variables are eliminated. If no factor I (fibrinogen) is present in the plasma test, no clot will form. Therefore, the one-stage prothrombin time measures the total activity in phase 3 of the coagulation mechanism.

The three chief determinants of the prothrombin time activity - prothrombin (II), factor V and factor VII do not exert an equal effect. The concentration of the first two affect the result more than that of factor VII. In thousands of patients who had been treated with an anticoagulant drug, Dicumerol, a haemorrhagic state was induced only when factor VII fell below the level of 5 percent activity, whereas in acquired deficiencies of factor II or factor V such as by parenchymatous liver disease, the activity of these substances can be less than 40% to induce haemorrhage. Therefore, a higher concentration of factor V can compensate for a lower concentration of factor VII. To be effective in an optimum manner, therefore, anticoagulant drugs should be standardized to meet this criteria.

The assays performed to formulate these criteria were done on the individual factors and accelerators by depleting the substrate to be examined of one of the factors and utilizing optimum concentrations of the remaining factors in phase 3.

The conversion of prothrombin to thrombin by Russell's viper venom differs from the conversion by tissue thromboplastin in that venom is dependent on the presence of platelets and plasma lipids and is not affected by the concentration of factor VII. If plasma is made platelet-poor and

lipid-poor by centrifugation, clotting by snake venom is prolonged but can be restored to normal by adding either platelets or crude lecithin to the reaction. Hjort et al (94) showed that about 6 percent of all factor V activity is adsorbed on the platelet and cannot be recovered by washing. This specific globulin is activated tenfold by thrombin. Platelets were examined by this author from two cases of congenital factor V deficiency and several cases of acquired factor V deficiency including one case of histoplasmosis. Normal clotting time was not obtained when these patient's platelets were examined as above for factor V activity. However, when the platelets were incubated for at least four minutes and rewashed three times in saline, normal clotting activity was restored.

It has been further proven that incubation of factor V-deficient platelets with trypsin, which destroys factor V activity does not alter platelet morphology (94). Platelet surfaces from patient's deficient in factor V, washed twice in saline, adsorb at least thirty percent of the total factor V activity of the normal plasma by volume whereas only about two percent of factor VII can be demonstrated to be present on the platelet surface from which it cannot be removed by two saline washings.

Prothrombin is normally present in the blood in a concentration in excess of that needed to complete the reaction in phase 3. Immediately after clotting occurs, the residual activity of prothrombin in the serum can be measured by using the modified one-stage test with a standardized source of factor I added (Appendix A). A comparison of the initial prothrombin activity in the plasma and that of the serum can be used to measure the efficiency with which prothrombin is utilized or converted. High

prothrombin activity in the serum is indicative of a failure to convert prothrombin, a failure which is usually due to deficiency in phase 1 or phase 2 (Fig. 9).

Autothrombins

Immediately after coagulation of the blood, an excess amount of thrombin is formed which rapidly becomes neutralized by at least four demonstrable mechanisms:

Autothrombin 1. which is the absorption of thrombin by fibrinogen which becomes an important variable in the in vitro systems where the concentration of fibrinogen (factor I) varies.

Autothrombin 2. which results from the interaction of heparin with a heparin cofactor thus inhibiting the interaction of thrombin and fibrinogen.

Autothrombin 3. is not dependent on heparin. It is found in plasma and serum, is stable at refrigerator temperature and may be neutralized by ether extraction. The extract can then be assayed for autothrombin 4 by conversion of prothrombin to thrombin in a system freed of antithrombin 3 activity.

Autothrombin 4. is neutralized by protomine sulphate or toluidine blue.

Of twenty-three cases of factor V deficiencies which were studied by the author, nine were acquired due to parenchymatous liver damage. Three cases showed the presence of an antithrombin 2 after gallbladder operations and in three other cases, antithrombin 3 was demonstrated. The TGT curve showed an abnormal upswing in the curve after four minutes incubation. Such an anticline effect is not the usual finding in uncomplicated cases of acquired factor V defect.

4. Phase IV - Fibrin Formation

Phase 4 activity is simple and direct. The complexity of the previous reactions show that previous precursors of coagulation have only one goal, namely, the formation of the substance thrombin which alone is responsible for the sol-gel change of fibrinogen (factor I) to fibrin.

Action of Thrombin

The action of thrombin is one of a hydrolytic enzyme which breaks down the fibrinogen molecule into one molecule of fibrin and one soluble fibrinopeptide. Molecules of fibrin monomer proceed to form progressively larger molecules of fibrin polymer by successive steps in which first the single molecules and then the polymer molecules come together in an end-to-end and side-to-side fashion. The final fibrin polymer then forms a gel or clot.

Thrombin, once formed, acts as an enzyme merely to initiate the reaction, after which polymerization proceeds independently. The final gel consists of large aggregates of fibrin which have a molecular weight exceeding 4.4 million (88). The presence of large molecules of globulin cause a gelatinous clot to form but there is no retraction of the clot. The properties of the clot, therefore depend on the pH and ionic concentration of the medium; if these conditions are not optimum, ultimate polymerization is inadequate, and the intermediate polymer molecules will remain in solution without forming a gel.

Several cases of extensive renal damage have been studied by the author in which the fibrin clot was totally or wholly soluble in urea indicating inadequate polymerization which was no doubt influenced by an excessive concentration of urea nitrogen which interfered with the formation of the S-S bonds between molecules of fibrin polymer units. Physiologic fibrin normally found in vitro in the blood is not soluble in urea.

The formation in vitro of fibrin polymer molecules of intermediate size suggests that in vivo there exists a dynamic equilibrium between fibrinogen and fibrin which is characterized by the formation of intermediates of varying molecular weight. Fibrinogen, therefore, does not appear to exist only in a pure monomolecular form but it is being constantly polymerized and depolymerized.

The addition of pure thrombin to washed platelets leads to platelet clumping and vicous metamorphosis which is followed by the formation of a coagulum qualitatively similar to fibrin. However, in two cases of a fibrinogenemia which were studied, the coagulum did form and, therefore, cannot be analytically similar to fibrin. Logically, therefore, we can assume that the platelets play a role in this reaction by surface adsorption of very small amounts of fibrinogen, too insignificant to be detected by electrophoretic or chemical methods.

The fibrinogen in platelet extracts has been demonstrated by means of immunoelectrophoresis and agar diffusion cells using fibrinogen antibody (94). The addition of thrombin to washed platelets caused the formation of a coagulum-like material and simultaneously caused the disappearance of identifiable fibrinogen. This fact would justify the assumption

that fibrinogen is adsorbed onto the platelet surface rather than retained inside the cell because, by accepted techniques, it can be demonstrated that platelet antibodies have antifibrinogen activity. In three cases of thrombopathic purpura there was defective or absence of fibrinogen demonstrated.

Johnson et al (95) found that the coagulum formed by the addition of thrombin to washed platelets was resistant to the action of fibrinolysin. They were able to show that this resistance was not due to the absence of fibrin, but to high levels of antifibrinolysin in or on the platelets. These findings were later confirmed (96) and it was shown that antifibrinolytic activity could be washed off the intact platelet and, furthermore, that some was given off by the platelet during clotting. This favours the theory that antifibrinolysin is adsorbed onto the platelet surface.

Cases which were studied in which fibrinolysins or antithrombins were demonstrated in platelet depleted plasma showed no concrete evidence of adsorption by the platelets of these substances either by the patient's platelets or normal control platelets. It may be therefore assumed that experimentally the platelet is selective in adsorption of these factors which assist but rejects those factors which interfere with coagulation or the haemostatic mechanisms. High selectively (up to 75 percent) of adsorption capacity by platelets can be demonstrated with epinephrine or norepeneplicine which further demonstrates selective adsorption. Therefore, selective adsorption must play a role in the vasoconstrictor function which occurs at the site of platelet clumping.

Fibrinolysis

Normal and pathologic depolymerization of fibrin have been proven to depend on the activity of a fibrinolytic system in the blood. This system may be considered to be the last of several haemostatic mechanisms which either correct or prevent catastrophic intravascular coagulation.

We have observed that autocatalytic reactions are controlled by natural inhibitors which restrict intravascular clotting. When a thrombus once forms its natural potential is dissolution. Dissolution of the thrombus is the function in vivo of the fibrinolytic system of plasminogen and plasmin.

Cell-free extracts of beta-haemolytic streptococci, when added to clotted plasma, dissolve the clot. The work of Christenson (96) defined the reaction by showing that the streptococcal enzyme (streptokinase) was an activator of profibrinolysin (plasminogen) in human blood or serum; plasminogen was activated to a potent fibrinolytic substance, namely, plasmin.

The fibrinolytic system of the blood is shown in Fig. 9. The tissue activator (cytofibrinolysokinase) appears to be the only one which is active in vivo: under normal conditions the other activators are not involved.

5. Fibrinolysin Activators

Streptokinase is a product of actively-growing haemolytic streptococci. Most preparations contain streptodornase which is a deoxyribonuclease. Staphylococci produce a staphlokinase which reacts more slowly than streptokinase.

Tissue fragments and extracts have the ability to activate plasminogen. The active substance appears to be firmly bound to tissue proteins but can be extracted with potassium thiocyanate (97).

The presence of a proteolytic enzyme in urine is capable of activating plasminogen. This enzyme, urokinase is proteolytic and capable of hydrolysing lysine ethyl ester as well as tosylarginine ester. A related enzyme, but not similar, is present in human milk and saliva. Trypsin also may be used as a plasminogen activator.

It was necessary for duplication of results within the statistical mean of error to perform any test for fibrinolytic activity within thirty minutes after the blood had been collected. It was noted that plasma devoid of platelets retained any fibrinolytic activity longer than platelet-rich plasma if separation was immediate after collection of the blood. Therefore, we may assume that the platelets may contribute some degree of inactivation of fibrinolysin by antifibrinolytic properties or substances adsorbed on their surface.

Two cases investigated by the author had shown excessive bleeding after cytoscopic examination. Both cases indicated by examination of data the presence of fibrinolysin activity with a fibrinogen level of less than 75 mgs. percent. Cytologic and haematologic examination revealed anaemia and many syncytia of tumour cells replacing normal haemoprietic elements in the bone marrow including platelet production. The initial tests for fibrinolysin were performed on the plasma of these patients after intervals of 5, 15, 30 minutes, one, two and three hours after removal of the blood.

Positive activity in one case was noted up to one hour in the patient's own platelet-rich plasma. When normal platelets were substituted the fibrinolytic activity was progressively reduced with each succession of normal platelet suspension substitution.

Inhibitors of Fibrinolysis

Human blood contains one or more inhibitors of fibrinolysis. The chief fibrinolysin migrates with the alpha-2 globulins. Human albumen (fraction V) shows antifibrinolytic activity and it may be assumed that this albumen fraction may contain a form of activity derived from factors V and X. Recently, it has been shown that epsilonaminocaproic acid, an analogue of lysine, blocks the activation of plasminogen and neutralizes the action of plasmin (98).

6. The role of calcium (Factor IV)

The behaviour of calcium ions does not appear to be catalytic but rather one of affinity for plasma antithromboplastins so that, when calcium ions are removed or partially reduced by oxalates, citrates or ion exchange, the liberated inhibitor makes the blood incoaguable. Increase of citrate ions to 25 percent saturation can spontaneously activate prothrombin to thrombin in the absence of calcium.

Washed platelets clump when exposed to thromboplastin. This clumping ability is lost when the platelets are incubated in citrated or oxalated plasma. A similar loss of clumping ability occurs when platelets are incubated in saline. However, if calcium is added to the saline the clumping ability is restored, but not with the addition of either oxalated or

citrated plasma. Using radioactive calcium Odell (101) demonstrated that the level of platelet calcium activity rose and fell in parallel with the changing level of plasma calcium radioactivity.

7. Haemorrhagic Disorders Due to Vascular Defects

The disorders characterized by bleeding or bruising because of a defective structure or function of vessel walls are difficult to classify accurately. There is apparently a close relation between the coagulation factors and vascular function.

Twenty-five cases of demonstrated increased vascular fragility or permeability were examined by the author. Eleven of these showed a pronounced defect in the arteriolar end of the capillary loops of the nailbed when examined under the dissecting microscope. The integrity of the capillary wall is known to depend in part on the presence of a "cement substance" between individual endothelial cells and in part on the juxtacapillary "ground substance". Both have been demonstrated to contain hyaluronic acid. Experimentally one of the above cases responded to treatment with ascorbic acid which is one of the substances required for the synthesis of hyaluronic acid, but administration of this vitamin had no effect on the capillary permeability of the other cases examined. This one case was confirmed as a true vitamin C deficiency and would explain the nature of the defective cement and ground substance.

Ten other cases examined (Fig. 12) showed a decrease of factor VIII activity and increased capillary fragility, the latter being more pronounced following infection, during the menstrual period or at menopause. Administration of fresh blood followed by a small quantity of factor VIII-rich

plasma corrected the abnormal bleeding and coagulation times but administration of factor VIII alone corrected the bleeding time but did not restore the factor VIII concentration to normal limits.

Twenty-three individuals, including four generations were examined who had a history of bleeding due to so-called 'vascular haemophilia". On the basis of the genetic pattern and expressivity of the protein dyscrasia, it was evident that these cases fell into the category which had previously been described as to represent a double heterozygosity for a dominant autosomal gene affecting the synthesis of factor VIII and another autosome affecting the bleeding time. This hypothesis is in contrast to the proven recessive sex-linked inheritance of a deficiency of the protein chain involved in factor VIII or factor IX insufficiency. These factors have been shown by this author to remain constant throughout the individual's lifetime. Fluctuations of activity have been demonstrated to be due to antithromboplastins, physiologically induced by antigenic stimuli or immunological response thus altering the molecular protein chain by blocking its activity. No motility or shifting of the intrinsic factor is noted on the electrophoretic analysis of either of these factors.

Congenital deficiencies of factors V, VII, XI and particularly factor XII show autosomal dominance in their inheritance pattern. The behaviour of these factors in the coagulation mechanism indicates that these fractions originate from modifying genes which produce a genetic control in the specific catalytic synthesis of thromboplastinogenesis and consequent autocatalytic action. The moiety of haemorrhagic manifestations

expressed in vascular haemophilia differs when factor VIII activity is delayed as compared to when factor IX activity does not proceed at the optimum rate.

Cases investigated in which autosomal control of co-enzyme production with resultant deficiencies in either enzyme content or enzyme release show a greater tendency to form blocking substances due to immunologic response to pathological conditions such as streptococcal or staphlococcal infections, certain types of carcinoma and specific drugs.

8. Platelet Morphology and Functional Considerations

Phase contrast microscopy, together with cinematography has demonstrated the existence of contractile vacuoles in the platelets of man and of the guinea pig (102). These vacuoles are also found in the hyaloplasm of frog thrombocytes (103). They usually occur in the center of the cell, sometimes in the region occupied by the granules of the chromomere, and they are particularly well seen when the cell has spontaneously spread out on the surface of the slide.

Prolonged observation shows that these vacuoles can contract and disappear. The time taken for their development is from 20 to 30 minutes at room temperature. Their contraction, on the other hand, is rapid and occurs in about 15 seconds. This contraction is sometimes accompanied by a clear zone, fusiform in shape, in an adjacent part of the cytoplasm, as if the contents of the vacuoles has been transferred to another region of the cytoplasm. Vital stains do not accumulate in the vacuoles which appear, after fixation, as circular bodies without peripheral or internal structures. Vital staining with neutral red shows fine granules in the

hyaloplasmic membrane of the cell. Some granules, specifically stained with janus green are noted among the granules of the chromomere and lends credence to the presence of mitochondria. It is therefore plausible that the mitochondria provide the cell with usable energy which plays a role in the energy-yielding reactions of oxidation or stored in the form of high-energy phosphate bonds to be used as adenosine triphosphate which becomes the principal energy-transfer system of the cell and its part in the platelet-vascular endothelium reaction to prevent increased capillary haemorrhage.

Born et al (104) have shown that the ability of the platelet to absorb epinephrine, norepinephrine, and serotomin is directly related to the concentration of platelet ATP. However, Born contends that energy release by the platelet is synonomous with platelet breakdown (105) which is contrary to the experimental evidence as submitted by this author.

A very recent work by Johnson et al (106) supports the finding that the mechanism of the endothelial supporting function is due to intact platelets. Johnson examined ultrathin sections of dermal capillaries from skin biopsies in patients with thrombocytopenia-complicating disease after massive platelet transfusions. Patients were selected with reduced vascular integrity in the hope that the platelets supplied in the transfusion would be attracted to endothelial cytoplasm lining the capillaries immediately on entry to the circulatory system. The actual post transfusion level of the platelets was only 51 percent of the calculated level. Many platelets were in the process of incorporation into the endothelial cytoplasm

In addition to the energy-rich activity of adenosine triphosphate (APT) in the platelet, adenosine diphosphate (ADP) had been identified as a product released by the platelets of rabbits which induces platelet aggregation in the presence of saline extract of tendon (107). Therefore, we may assume the platelet plays a role in the creation of energy-rich bonds which may couple with other energy-requiring processes of some of the coagulation factors and vascular surface factors and such a product no doubt plays a great role in initiating further synthetic reactions in thromboplastinogenesis.

On the basis of this concept it may be possible that the mechanism is self-perpetuating, increasing the stability and size of the platelet plug and that this function exists as a part of platelet physiological mechanisms. The functional abnormality of the platelets in thrombasthenia (Fig. 10) may be identified with a deficiency of platelet ATP. Zucker and Bareli (108) isolated a connective tissue factor which caused clumping of platelets from patients with factor VIII or factor IX deficiencies but not with the platelets from cases of thrombasthenia.

In spite of the apparent selectivity of platelet adsorption, it can be demonstrated in the TGT that non-specific substances which coat the platelet can interfere with generation of energy activity. Interference of platelet function by alteration of the isoelectric point of platelets by the dextran molecule, can induce inhibition of activity, prolonged prothrombin consumption and impaired vascular integrity. Similarly, platelets from patients with macroglobulinemia showed inhibited function in the TGT.

It may be further stated that the mortality rate from spontaneous haemorrhage due to anticoagulation, thrombocytopenia, specific coagulation factor deficiency or heparinization alone is very low but a combination of two or three of these mechanisms is usually fatal.

REPLACEMENT THERAPY OF THE COAGULATION DISORDERS

1. Fresh whole blood or plasma.

Whole blood or plate-rich plasma collected in siliconized or plastic equipment

-- Thrombocytopenic Purpura

Factor V Deficiency

Factor VIII Deficiency

Von Willebrand's Disease — Vascular Defect only

Vascular Defect with Factor VIII Deficiency.

2. Fresh or Stored Whole Blood or Plasma.

Factor II Deficiency

Factor IX Deficiency

Factor XI Deficiency

Factor VII Deficiency

Factor X Deficiency

PLASMA REQUIREMENTS IN DEFICIENCIES OF COAGULATION FACTORS

STABLE when stored

Factor IX

Factor XI

Factor 11

Factor 1

Factor VII

LABILE when stored

Factor VIII

Factor V

CIRCULATION SURVIVAL TIMES OF CLOTTING FACTORS

Factor VIII	-	12-24 hours
Factor IX	-	48-72 "
Factor XI	-	24 "
Factor II	-	36-42 days
Factor I	-	4 days
Factor V	-	24-48 hours
Factor VII	-	24-36 hours

Summary

The discussion and conclusions are primarily based on the results of the evidence which was obtained from the data of standard and modified techniques which were employed in the investigation of a total of 598 cases. Each case did present some clinical evidence for an abnormal haemorrhagic state or a capillary defect.

The following data includes the number of individual cases in which defects were found in any one of a combination of coagulation factors, the platelets or the capillaries. The cases which are listed as negative include haemorrhagic states which were due primarily to trauma or mechanical injuries and in which cases no actual haemostatic defect could be demonstrated.

Deficiency	Number of Cases
Negative	222
Factor I	23
Factor II	40
Factor V hereditary	2
acquired	13
Factor VII hereditary	5
acquired	41
Factor VIII hereditary	23
acquired (transient)	5

Factor IX hereditary	17
acquired (transient)	3
Factor X hereditary and acquired	3
Factor XI hereditary	4
Factor XII hereditary	2
Platelets, idiopathic, quantitative	35
thrombasthenic, qualitative	30
thrombopathic	18
Capillary Defects with deficiency of Factor VIII or IX	34
Antithromboplastins, species-specific	16
Antithrombins	23
Fibrinolysins	28
All other induced defects	11

Enzymes exhibit catalytic activity at very significant dilutions, rigidly controlled temperatures and hydrogen ion concentration and are present in the blood in relatively low but optimum concentrations. Therefore, it is easier to measure the accelerated rate of an enzymatically catalysed reaction than to determine the quantity of enzyme protein in a given volume of solution. Quantitative enzyme analysis is difficult due to the uncertainty that the product of the reaction is free from other enzymes but an attempt was made to assess activity by deletion of a known coagulation enzyme from the substrate using every precaution to avoid risk of denaturation or inactivation of the remainder of the substrate components.

In the modifications of the method employed to determine the role of specific enzymes in the thromboplastin generation test it is possible to assess the generation process by three phases of reaction involving at least three enzyme-substrate reactions which proceed by interaction according to their selective adaptability. This, as stated, can be done by deleting a specific enzyme or a specific protein fraction from the substrate at one of the phases of interaction which generate thromboplastin.

The method for the termination of enzymatic activity required the study of reaction rates involving the measurement either of the rate of disappearance of the substrate or the rate of appearance of the product either of which were plotted against the time factor which is of crucial importance.

The specificity of the enzymes or coenzymes described as the coagulation factors differs from the specificity of the simpler organic catalysts in that they are restricted and can be isolated in their activity. For this reason, the coagulation factors have been classified on the basis of their specificity. This particularly applies to those coagulation factors which act as hydrolytic enzymes specifically at peptide bonds (peptidases) or at ester linkages (esterases). Furthermore, certain of the hydrolytic factors have been demonstrated to catalyse bimolecular reactions in which the substrate reacts not with water, but with an organic alcohol or amine. It may be assumed that each coagulation enzyme molecule has an active catalytic site or center of precisely defined chemical structure and the combination with the substrate occurs at this particular center.

Competitive and non-competitive inhibition of enzyme reactions by means of substrate analogues have been proven to exist or be produced and occupy active sites for inactivation of specific coagulation factors. In most of the cases, the action was non competitive and the activation of the enzyme depended solely on the concentration of the inhibitor. The maximal velocity attained of the reaction was less than that found when the inhibitor was removed. In these cases the inhibitor combined with the enzyme-substrate complex but not the specific free enzyme factor.

By virtue of their specificity, the individual coagulation factors are able to preferentially direct the sequences of chemical reactions in optimum controlled conditions of pH and temperature.

Similar specific capabilities are present when the platelets are treated and coagulation factors are adsorbed on their surface. The criteria for adequate adsorption and duplication of results is the viability of the platelet which was maintained throughout the experiments.

Much of the energy produced in the oxidation process for the maintenance of capillary endothelial integrity is transferred by the viable platelet, no doubt in the form of energy-rich phosphate bonds. The proof that this mobile cell is able to produce adenosine triphosphate and, in a zone of tissue activity, can activate the formation of adenosine diphosphate suggests a practical physiological role of this cell in haemostasis.

Furthermore, the evidence suggests that many of the platelet's factors are selectively adsorbed onto its surface from the plasma and this ability becomes one of its basic, active functions in the dynamic equilibrium of coagulation and haemostasis. It is, therefore, quite conceivable that the main function of the viable platelet is to transport coagulation factors and perhaps vascular factors to maintain the epithelial supporting mechanism of capillary vessels and to provide the means of energy for this action.

APPENDIX A

1. PROTHROMBIN CONSUMPTION TEST

When the generation of thromboplastin is normal, prothrombin is normally converted to thrombin, and only small amounts of prothrombin remain in the serum. When thromboplastogenesis is defective, prothrombin conversion is deficient, and large amounts of the prothrombin factors remain in the serum. The residual prothrombin can be measured by a one-stage test, using barium sulphate-absorbed plasma as a source of factor 1 (fibrinogen).

The adsorbed plasma was prepared from pooled normal plasma and was devoid of prothrombin activity. The fibrinogen level was between 300 to 400 mgms percent of factor 1 activity.

Method:

1. Allow blood to clot without tilting or agitation of the tube. Serum is used from blood obtained in a serological tube, only one cubic centimeter of blood should be used.

2. After exactly one hour from the clotting of the blood, centrifuge the tube of clotted blood for one minute at 3000 rpm. Aspirate the serum and transfer to a clean tube, preferably siliconized.

3. To a serological test tube in a 37° waterbath add in rapid succession, 0.1 ml. of absorbed control plasma and 0.2 ml. of Simplastin*. Blow in 0.1 ml. of serum and start a stopwatch. Record the time required for the clot to form. Normal sera are used as controls in every determination.

Results:

1. Normally the serum prothrombin time is longer than 21 seconds which when plotted shows at least 75% of prothrombin consumed. Shorter serum prothrombin time indicates a decrease in prothrombin consumed and indicated an actual or potential tendency to post surgical bleeding, usually due to hypoprothrombinemia or abnormal thromboplastinogenesis.

2. Percent prothrombin consumed is calculated by reference to a standard table.

3. The data applies only when the one-stage prothrombin time is normal. When the prothrombin time of plasma is prolonged, this is taken as the reference point for calculating the prothrombin consumed. The prolonged plasma prothrombin time must be used as a 100% reference.

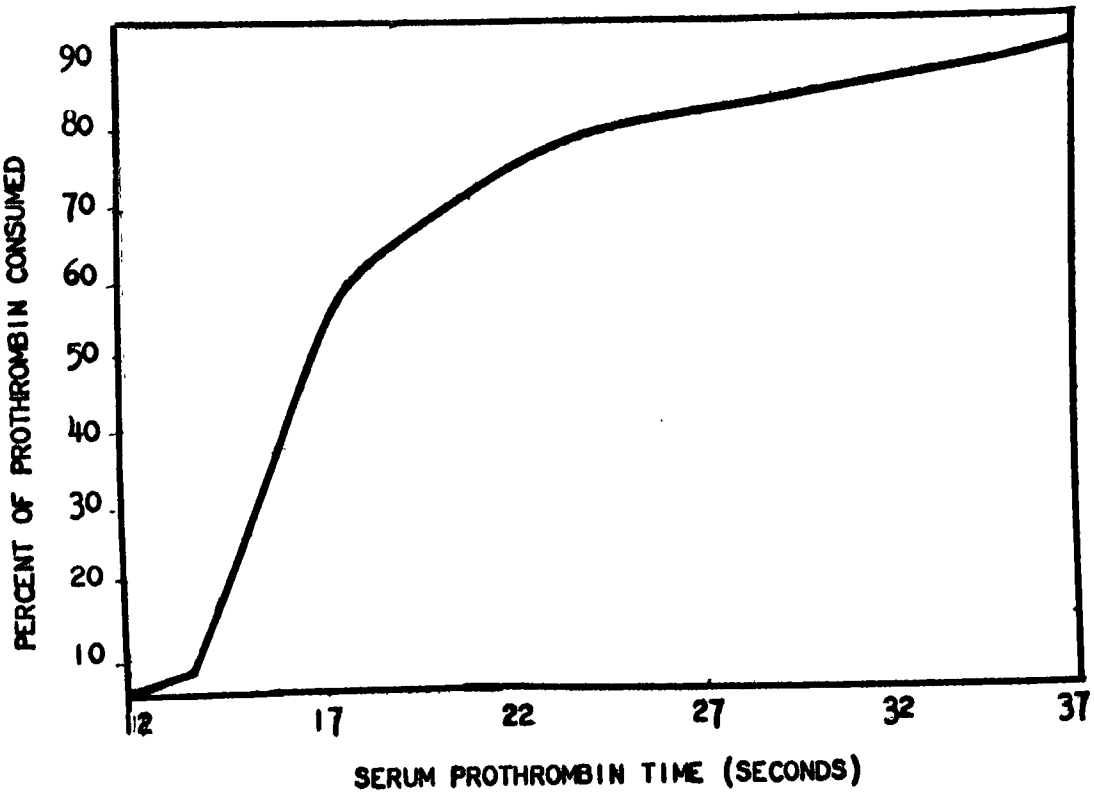
REPRESENTATIVE DATA

SERUM PROTHROMBIN TIME Seconds	PERCENT PROTHROMBIN CONSUMED
13 or less	0.0%
13.5	10.0%
14.15	20.0%
14.7	30.0%
15.3	40.0%
16.05	50.0%
17.5	60.0%
20.0	70.0%
22.2	75.0%
24.0	80.0%
35.0	90.0%

* Simplastin, Warner Chilcott Co., Morris Plains, N.J.

FIG. 15

RELATIONSHIP BETWEEN SERUM PROTHROMBIN ACTIVITY AND PERCENT OF PROTHROMBIN CONSUMED



APPENDIX A

2. ASSAY OF FACTOR VIII (MODIFIED METHOD OF JUNG)

All necessary reacting coagulation factors in the test system are kept constant except factor VIII. The resulting clotting time is then dependent only on the levelof this factor in the test plasma.

Reagents:

1. Aged plasma, 9 volumes of normal blood are mixed with 1 volume of 0.1 m. sodium oxalate and centrifuged for 10 minutes at 1700 rpm. Aspirate plasma and store unstoppered at room temperature until the one-stage prothrombin time is at least 60 seconds. The procedure may be hastened by placing the plasma in a 37° C. water bath. After the "aging" process is completed, the plasma is stored in small aliquots at 20°C. One thawed the plasma is stable from 24 to 48 hours at 4°C.

2. Bacto-Ac-Globulin* dissolve one vial in 2 ml. of distilled water. Dilute with 18 ml. of 0.85% saline solution to give a 1:10 dilution. Distribute in small aliquots and store at 20°C. One thawed do not refreeze.

3. Partial thromboplastin (Thrombofax** or Platelin***).

4. Calcium chloride 0.025 m.

5. Test plasma. To 0.5 ml. of 3.8% sodium citrate in a 12 ml. centrifuge tube (siliconized) was added 4.5 ml. of patient blood. If test could not be done immediately, the plasma was placed in a stoppered test tube and kept at 40°C. Test was done within a period of 4 hours after withdrawal of blood.

Method:

1. Place the tube containing 0.025 m. $CaCl_2$ in a 37°C. water bath.

2. To a 12 x 75 mm. test tube add the following:

   a) 0.2 ml. of Thrombofax of Platelin

   b) 0.1 ml. of Ac Globulin

   c) 0.1 ml. of Aged Plasma

   d) 0.1 ml. of Test Plasma

3. Place in water bath and allow to incubate 2 to 3 minutes.

4. Blow in 0.2 $CaCl_2$ while simultaneously starting a stop watch.

5. Gently mix and leave tube indisturbed in the waterbath for 45 seconds.

6. Remove tube from water bath and observe for clotting by tilting on a concave mirror with a lighted background. First formation of fibrin threads is the end point.

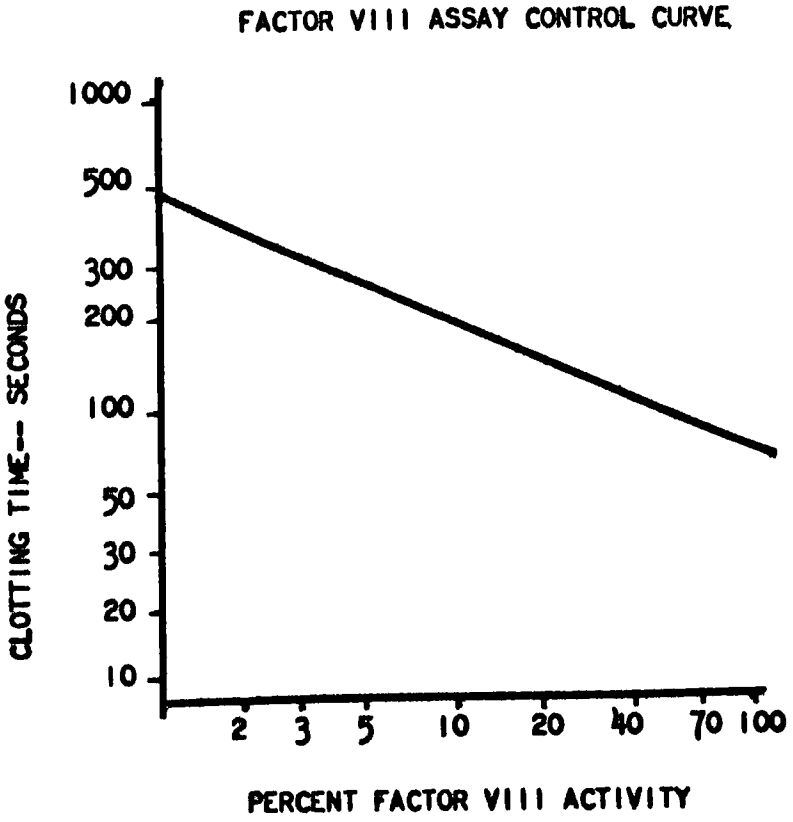
7. Resulting clotting time represents the factor VIII activity of the test plasma. To convert to percent, a dilution curve is made from fresh normal dilute plasma. The clotting times of undiluted (100%), 50 and 10% dilution of normal plasma are plotted on double logarithmic paper, with seconds on the ordinate against percent factor VIII activity on the abscissa.

The content of factor VIII of fro en plasma is stable for about 6 months, but upon thawing there occurs a variable loss of factor VIII. Plasmas assayed after being frozen were plotted against a control curve using fresh frozen normal plasma.

* Difco Laboratories, Detroit, Michigan.
** Ortho Pharmaceutical Corp., Raritan, N.J.
*** Warner Lambert Co., Morris Plains, N.J.

Reference: Jung, E.G.: A Rapid Quantitative Assay of Factor VIII (AHG) without the use of Hemophelia A Plasma. Thromb. Diath. Haemorrhage. 4:323, 1960.

FIG. 16


FACTOR VIII ASSAY CONTROL CURVE
CLOTTING TIME-- SECONDS
1000
500
300
200
100
50
30
20
10
2
3
5
10
20
40
70
100
PERCENT FACTOR VIII ACTIVITY

APPENDIX A

3. EVALUATION OF A ONE-STAGE PROLONGED PROTHROMBIN TIME

Reagents:

1. The same reagents and equipment used for the standard one-stage prothrombin time.

2. The blood specimens are collected in the same manner as for the one-stage prothrombin time.

3. Fresh normal $BaSO_4$-absorbed oxalate plasma is prepared as outlined in the thromboplastin generation test. Prothrombin (factor II) and factor VII are absorbed, but factor V is not.

PROLONGED ONE-STAGE PROTHROMBIN TIME CORRECTED BY:

REAGENT	DEFICIENCY
STORED SERUM	Factor VII
Fresh $BaSO_4$-absorbed PLASMA	Factor V
STORED OXALATED PLASMA	Factor II
* RUSSELL'S VIPER VENOM 1:10,000 in place of Simplastin**	To differentiate Factor X deficiency

4. Normal stored serum. Normal blood is allowed to clot, and incubated at 37°C for at least 2 hours to ensure maximum prothrombin conversion. Store serum at 4°C for about 24 hours.

5. Normal oxalated plasma is stored for two weeks at 4°C or 24 hours at 37°C. The prothrombin time of the stored plasma should exceed 28 seconds.

Method:

0.9 ml. of the abnormal plasma is added to 0.1 ml. of each of the above reagents in turn and a one-stage prothrombin time is done on each mixture.

* STYPVEN
Burroughs Wellcome and Co., Inc., Tuckahoe, N.Y.

** SIMPLASTIN
Warner-Chilcott Laboratories,
Morris Plains, N.J.

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