LIPOIDS AND BLOOD PLATELETS WITH REFERENCE TO BLOOD COAGULATION AND THE HEMORRHAGIC DISEASES

by

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Im Tätsächlichen grösster Prägnanz,

im Hypothetischen äusserste Vorsicht. (Wöhlisch, 1923)

When theory, fancy free,
Bids "hither -- yon's the way",
Calm fact exhorteth thee:
"Consider, tyro! Stay!" (Orig.)

EDITORIAL NOTE

The author adopts the American spelling in the text.

Bibliography:

(1) References are given in text by elevated numerals, corresponding to alphabetized bibliography in Appendix III.

(2) Titles of journals are abbreviated in accordance with the widely-used "List of periodicals, abstracted by Chemical Abstracts", Amer. Chem. Soc. (Columbus, Ohio), 1951.

(3) Abstracts (abstr.) and 'personal communications' are indicated.

(4) Authors are listed alphabetically. In case of multiple authorship, the second and subsequent author's names follow in alphabetical sequence.

(5) Diphthongs are replaced by separate letters. Modified letters (accent, etc.), when not anglicized (e.g. ue, German ü) in the original reference, appear in the usual alphabet order, but include the modification.

(6) Hyphenated and compound names are listed in alphabetical order under initial letter of surname, including De, Le, Mac, Mc, Van, Von.

Author

A B S T R A C T

By specifically analyzing for the various active principles of plasma, platelets, tissues and their fractions, much new information has been obtained concerning the role of lipoids and platelets in blood coagulation and in the hemostatic mechanisms in health and disease. Analyzed components are studied in artificial clotting systems, especially a two-stage thrombin-forming system. Some 86 cases of bleeding disorders, 32 newborn normal infants and their mothers, and many normal adult bloods have been analyzed with respect to components of the clotting and hemostatic functions.

The detailed considerations embodied in the thesis are encompassed under the following heads:

1) the importance of certain lipoids, especially cephalin,

2) the normal need, in plasma clotting, for platelets,

3) the particular significance of a platelet component, which has many analogies to cephalin, in the thromboplastic system,

4) potentiation of the thromboplastic actions of cephalin, of platelets, and of tissue thromboplastin (to some extent) by a variety of experimental additives. Part of this may be explained as a 'thromboplastin generation' through co-participation of certain plasmatic components (antihemophilic globulin, PTC, etc.). Part, however, may be the result of certain proteolytic enzymes, particularly trypsin, 'disaggregating' lipoproteins and thus rendering their phospholipid (and sometimes calcium) available for participation in the clotting reactions,

5) possible Ca-containing and lipid-containing 'intermediates' in the thrombin-forming reactions,

6) myelin figure formation as an explanation of 'alterations' of platelets and certain other formed elements such as thrombocytes, megakaryocytes, and stromatolytic erythrocytes,

7) the multiplicity of factors which platelets may contribute to the blood clotting and hemostatic mechanisms,

8) the occurrence of many clinical disorders due to deficiency of platelet functions. Thrombocytopenias denote deficient numbers ('counts' and total bulk in body). Thrombocytopathias are deficiencies of specific platelet components, e.g. thromboplastic factor, accelerator, vasoconstrictor (5-hydroxytryptamine), or retractor factor. Such deficiencies can be clinically significant even when the platelet count is normal. Bleeding in leukemias, uremias, etc. may often be accounted for in these terms,

9) the nature and modes of action of heparin and other 'antithromboplastic' inhibitors, and of some antiproteases, in relation to the mechanisms discussed,

10) the 'cephalin availability theory' of the author, as a useful working hypothesis to explain the importance of the natural thromboplastic phospholipid. Lipid release from platelet, tissue, or possibly plasma sources may very well be the long-obscure 'trigger mechanism' which initiates blood coagulation.

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PART I. BACKGROUND

FOREWORD

In presenting a thesis embodying experimental work conducted over a 27-year period (1929-1956), it is necessary to review the knowledge of the field at the commencement of this period and its subsequent progress to the present time, in order to evaluate each contribution in the light of contemporary knowledge and theory. Several volumes, and possibly several authors, would be required to review critically and in detail all the extensive literature of the blood coagulation field. From the physiological approach alone, several thousand titles are referred to in the monumental reviews of P. Morawitz [346,347] and E. Wöhlisch [505,506] and in more recent articles, including those appearing in the Annual Reviews of Physiology, two of them by the present author [133,140]. The purposes of this thesis may best be served by a selection of those ideas and trends of thought which afford the background for the topics presented.

A thesis, more than a definitive scientific publication, reflects the interplay of ideas that influence the mind of its author and it is not inappropriate that it indicate, to some extent, the personal element in research. In the period covered, Science itself has undergone a change which is deserving of mention. This change was gradual in the beginning, but it has accelerated in the last few decades of the twentieth century. Characteristic of earlier periods was the eminence of individual scientists, whose discoveries opened up new fields, or special areas within those fields, often single-handed. Students and young investigators came to work in the laboratories of the distinguished authority and thus developed the various 'schools', justly adding to the reputation and prestige of the 'master'. A fine example in our field was the

Dorpat school, under the leadership of Alexander Schmidt (1860-1895).

The modern scientific era has seen much larger numbers of scientists working simultaneously in the same field. Through scientific gatherings of regional, national, and international scope, workers of kindred interest have frequent opportunities for exchange of ideas. Thus, all are kept fully informed of many lines of progress and of the definition of numerous problems in advance of their solution. A not infrequent consequence is that the same solution (or almost) is reached simultaneously by several independent groups of workers, perhaps widely scattered over the world. An illustration of this may be cited from a field peripheral to that of the present enquiry. In 1939, the Journal of the American Chemical Society simultaneously published four papers from different laboratories, all of which must share the credit for the definitive solution to the problem of the chemistry of vitamin(s) K. Much the same sort of thing is true of the recent discoveries of several new factors in the blood clotting mechanism. Here, another consequence is manifest, namely, some uncertainty because of the difficulty in exact identification of the factors in question, thus leading to a confusion in nomenclature. Such confusion is more troublesome to the casually interested scientist than to those actually working on these problems, however.

In the coagulation field, at least in America, perhaps the last of the old school authorities was W. H. Howell, of the Johns Hopkins School of Medicine in Baltimore. This eminent scientist and distinguished teacher was quietly continuing his critical experimentation in a modest laboratory, which he occupied as Emeritus Professor of Physiology, during the period of the 1930's when the present author had the privilege of paying him numerous visits. His friendly charm, advice and critical

discussions, and warm personal interest and encouragement were of inestimable value in guiding a new researcher into the complexities of the blood clotting field.

On the occasion of the annual meeting of the Federation of American Societies for Experimental Biology at Detroit, in 1935, a small group of interested coagulationists met together and agreed to maintain an exchange of ideas. By common consensus, this 'coagulationist' group has never become formally organized. It has, however, continued to meet at each annual meeting of the Federation, and the sustained interest has expanded with each succeeding year so that our mailing list now has over two hundred names. There are similar groups of workers in other specialized fields, and a very important result of their coordinated interests has been to secure inter-society sessions and symposia at such gatherings as the Federation and the International Physiological and Hematological Societies' meetings. Other significant contributions to the cross-fertilization of ideas among specialized scientific groups are the numerous Research Conferences, which are a distinctive feature of the modern scientific scene. Some examples, in which American coagulationists get together, are those promoted by the National Research Council, the U. S. Public Health Service, and the Josiah Macy Jr. Foundation, to mention just a few.

With this broad perspective, we may selectively summarize the early history of blood coagulation, in what we may call the personal era, and then proceed to a brief review of its modern status, in which due credit for individual investigators need not be denied by presenting this largely from the depersonalized point of view.

The topic of the blood platelets will be separately reviewed.

CHANGING CONCEPTS IN THE CLOTTING FIELD.

A. HISTORICAL HIGHLIGHTS

1. EARLY ORIGINS: HEWSON, DENIS, BUCHANAN.

The earliest significant efforts toward an understanding of the coagulation of the blood were made in the 18th century, and the Experimental Inquiries of young Wm. Hewson [218], associate of the famous brothers John and William Hunter, are particularly worthy of mention. These established the clotting phenomenon as essentially a physiological property of the 'coagulable lymph' (Hutt, 1760; cf. 'coagulated lymph' of de Senac, 1749), which we nowadays call plasma. Malpighi (1666) and Ruysch (1707) had already noted the 'fibrous' material obtainable from blood clots, and clinicians with wide experience of ubiquitous bloodletting talked of the 'cruor' or 'crassamentum' appearing after separation of the "serosity" or serum. Much was made of the 'buffing' of blood in disease. This we now interpret as an increased erythrocyte sedimentation rate with the consequent easier observation of clotting sequences starting in the supernatant plasma-rich layer. The term fibrin, which we owe to the French encyclopedists Chaptal [72] and de Fourcroy [99], was not really an advance, since they used the crudest methods of preparation of the clot material which they tried to characterize by appearance and elementary analysis. Mulder introduced the word protein in 1839, but it has been a long and difficult road, still with unexplored bypaths, to define individual proteins and, among them, fibrin and fibrinogen. The latter term we owe to Virchow [482].

The 1830-1859 publications of P.-S. Denis are of historical interest. Denis' [102] most significant contribution, all things considered, was the practical preparation of his 'plasmine'. This was a precipitate

obtained from plasma with strong solutions of sodium chloride and other neutral salts. Redissolved on addition of water, it subsequently underwent coagulation, indistinguishable from the appearance of natural fibrin clot.

The false impression that fibrin was the result of some 'spontaneous' change in a soluble precursor (fibrinogen) was first questioned by Buchanan [68,69], particularly in his observation that the washings from a blood clot could coagulate certain hydrocele fluids. He compared this with the rennet curdling of milk and also did much to try and prove a cellular origin of the blood coagulant. Buchanan's work was neglected until republished by Gamgee[183] in support of his confirmatory experiments. Gamgee's method of preparing thrombin (as it has come to be termed) from blood clots was further improved by W. H. Howell [229,232] at the beginning of our current era.

2. THE DORPAT SCHOOL

Alexander Schmidt, of Dorpat, established his famous school in the latter half of the 19th century. From the master and his pupils came a large body of work and publications, reviewed in Schmidt's two monographs [420,421] in 1891, 1895. It is easy, from our advanced modern viewpoint, to be extremely critical of the crudity of their experimentation and highly theoretical presentations of their ideas. It should be remembered, however, that the difficult biochemical problems of the coagulation field humble us even to this day. Then again, the 19th century discoveries, especially in the field of ferments (or enzymes), aroused such interest that contemporary enthusiasm sometimes got out of bounds. Analogies were suggested and their implications tacitly accepted, with little of the caution demanded of more recent experimental

science. Schmidt used a crude technic of storing blood serum under alcohol to obtain his 'fibrin ferment', which was a very weak and impure thrombin by modern standards. His numerous experiments endeavoured to search out all possible factors influencing the clotting mechanism. 'Proplastic', i.e. fibrinogen-containing, liquids such as hydrocele fluid, or plasma obtained from blood kept fluid with $MgSO_4$ and other neutral salts (cf. Hewson [218]), were clotted by Schmidt's fibrin ferment. He obtained good evidence for a precursor of the coagulant. The terms thrombin (for fibrin ferment) and prothrombin (for its precursor) were introduced in his later writing. Thus Schmidt has deservedly been called "the father of the thrombin theory". Many details of his particular theories and terminologies have long since been discarded and need not be reviewed. A few words may be said, however, about some of the experimental facts. In attempting to unravel the thrombin problem, Schmidt did much to implicate (he thought) both (1) a plasma protein, 'paraglobulin', and (2) tissue or cellular factors, which he called 'zymoplastic substances'. As to the first, it did seem rational at the time to look for a proteolytic 'split product' accompanying the postulated enzymatic breakdown of fibrinogen to fibrin.

Schmidt and his pupils did a great deal with the tissue materials noted under the second head, namely, 'zymoplastic substances'. These were obtained both in aqueous extracts and by means of fat solvents, hence chiefly lipoidal in character. Heat stability and many other properties were investigated. The most significant conclusion about these zymoplastic agents was that they were not thrombin but could participate in the activation of prothrombin.

The essential technique whereby Schmidt sought to characterize tissue

fractions was, first to obtain an alcoholic extract ('zymoplastic substance'), then a succeeding watery extract ('cytoglobulin') and its acetic acid precipitable fraction ('preglobulin'), and finally an insoluble residue ('cytin'). Of historical interest were the observations: (1) zymoplastic substance promoted clotting under a variety of test circumstances some of which led to the conclusion (above) that it could participate in the activation of prothrombin to thrombin; (2) the defatted cellular extractives, especially preglobulin, had considerable clot-inhibiting powers, e.g. for mixtures of $MgSO_4$ plasma and fibrin ferment (thrombin); (3) zymoplastic substance, however, was able to restore clotting in these inhibited mixtures.

Schmidt's final summing up of his views on coagulation [421] included the idea of something normally present in blood which could be regarded as an antithrombin. This term was actually introduced by Fano (1881) to designate the thermolabile (on boiling) agent which caused incoagulability of blood after peptone injections in the dog, as first shown in Carl Ludwig's laboratory by Schmidt-Mülheim (1880). Extracts from the medicinal leech contain a substance hirudin which Haykraft [217] showed to have similar 'antithrombic' powers.

It was generally known in this era that: (a) the 'thrombic' action of fresh serum progressively decreased on aging even for a few hours; (b) thrombin clotted plasma less readily than a fibrinogen solution; (c) the weak thrombin preparations of that time had little, if any, capacity to produce coagulation (thrombosis) on intravenous injection into experimental animals.

3. THE WORK OF HAMMARSTEN AND WOOLDRIDGE.

Olof Hammarsten [205], in 1883, seemed to simplify Schmidt's theories

by suggesting that 'fibrinoplastic substance' ('fibrino-globulin') was not a component of the coagulant, but appeared as a split product of fibrinogen when acted upon by the fibrin ferment. However, this eminent biochemist went on to considerable success in the purification of both fibrinogen and thrombin. We owe to him the application of the 'salting-out' technique to precipitate fibrinogen from plasma, using ½ sat. NaCl or ⅓ sat. $(NH_4)_2SO_4$. Weyl, Heynsius, and others (ref. 371) were then developing the use of neutral salts, rather than the earlier acids and alkalies, etc., for the separation of all kinds of proteins. The distinction between albumins and globulins on the basis of solubilities in such salt solutions was made in this era. When oxalate or citrate (see later) replaced the older $MgSO_4$, etc. as anticoagulants used in separating (centrifugally) the plasma from the blood corpuscles, the preparation of fibrinogen and other clotting factors by the salting-out method was much improved. The technique of elementary organic analysis by combustion methods, dating back to Liebig (1840), was continued and, despite the relative crudity of the materials analysed, did give data on N, S, P, and minerals, e.g. Ca, which contributed some information of real value in these early days of protein- and other organic- chemistry. Hammarsten eventually purified fibrinogen until he was able to obtain up to 94 per cent of its protein in the fibrin clot which resulted from treating its solutions with thrombin. His thrombin was fractionated from fresh horse serum. The conclusion from these careful experiments was that the small quantity of non-coagulable protein unaccounted for was merely a minor contamination with what we would now term serum globulins. Hammarsten's work on the calcium factor will be reviewed on p. 9.

L. C. Wooldridge [508] had considerable justification for some of his

claims to priority, over the Dorpat school, in the investigation of phospholipids ('lecithins') in the blood clotting system. He obtained many interesting experimental facts, but his tissue fibrinogen theory and many aspects of his work were too unorthodox to gain him wide recognition. Even the publication of his obscure works by Horsely and Starling, in 1893, failed to shake the widespread acceptance of the thrombin theory.

4. THE CALCIUM FACTOR: Early work of Arthus, Pekelharing, Hammarsten, Sabbatini.

Alex. Schmidt was never convinced of the need for calcium in the thrombin-forming reaction. There had been previous suggestions [201,404] for a role of calcium in blood clotting, but the significant evidence was provided by Arthus and Pagès in 1890 [19]. They used oxalates or fluorides to prevent clotting, and restored it by simple re-addition of calcium salt.

Pekelharing [372,373] confirmed this and introduced citrates for the same purpose. This worker improved upon Schmidt's methods for obtaining prothrombin. Dilution and acidification of plasma with weak acetic acid separated this agent, by a principle which we would now characterize as isoelectric precipitation. Pekelharing, however, went to undue lengths to identify his material as a "nucleo-albumin" and suggested that it had a cellular, perhaps platelet, origin. These were not useful ideas and they somewhat lessen the value of his contributions. He did insist upon the importance of calcium salts in activating prothrombin to thrombin (fibrin ferment), but he loses us in his further speculations about its transfer of calcium to the fibrinogen in the act of clotting.

Hammarsten [206] provided more convincing evidence of the specific importance of calcium in prothrombin activation, while denying it any

really significant role in fibrin formation. By oxalation and 'iso-electric' precipitation of his fibrinogen and thrombin, he obtained reagents which interacted to give a fibrin analysing as little as 0.005 per cent calcium. This could reasonably be dismissed as a trace impurity (but see Howell's [238] comments).

The question raised by citrates, which are unlike oxalates in that they do not precipitate calcium, was settled at the turn of the century by Sabbatini [117], who showed that they do remove Ca^{++} ions, by forming inactive $CaCit^{-}$ anion complexes.

5. THE MORAWITZ-FULD THEORY.

Morawitz [345,346] and Fuld and Spiro [181] clarified ideas concerning the role of tissue factors in the clotting mechanism. Fuld's term 'cytozyme' was later adopted by a minority of workers, notably including Bordet [55], whereas most European investigators accepted Morawitz's term 'thrombokinase'. The argument was still by analogy with a tacitly accepted enzyme hypothesis and it may be remembered that Pavlov (1899) had just discovered enterokinase to explain the intestinal activation of pancreatic trypsinogen into active trypsin. The Morawitz-Fuld theory did clearly state that <u>both</u> calcium ions and tissue factor were needed to convert prothrombin into thrombin.

Morawitz [345] also gave us the term 'metathrombin' for the inactive form of thrombin in serum, believed due to some combination with anti-thrombin, which Morawitz, following earlier leads by A. Schmidt, thought he was able partly to reverse with an acid and alkali treatment.

6. THE HETERODOXY OF WOLF.

P. Wolf [351], in the later 1900's, restudied the phenomena of peptone shock in experimental animals, particularly in relation to the

liver. He not only confirmed earlier French workers (Contjean, Gley, Hedon and Delezenne) as to the hepatic origin of antithrombin (which Nolf preferred to call 'antithrombosin') but also gave good evidence that the liver produced fibrinogen and antifibrinolysin. Nolf was especially interested in the fibrinolytic phenomenon, pointing out the loss of fibrinogen due to appearance of an active proteolytic enzyme (fibrinolysin) in the blood, partly explaining the incoagulability encountered not only in peptone shock but also in liver poisoning with phosphorus (Corin and Anciaux, 1894) or with chloroform (Doyon, 1905). Even when the blood remained coagulable, the clots would soon undergo lysis. As little as 1/60 vol. of normal dog plasma could prevent this, indicating that normal plasma contains a powerful antifibrinolysin. This, however, is reduced or absent in the above cases involving failure of liver function. Nolf performed many noteworthy experiments, but his theories were heterodox. In brief, he tried to bring into a dual clotting-fibrinolytic scheme: (1) fibrinogen, (2) 'thrombogen', which is possibly equivalent to the orthodox prothrombin, although Nolf also indentified it with the antifibrinolytic agent, (3) 'thrombozyme', possibly equivalent to Schmidt's zymoplastic substance or Morawitz's thrombokinase, since Nolf asserted that it was "produced by certain white cells (thrombocytes and platelets) and by the endothelium of the capillaries". Nolf accepted thrombin only as a by-product of the reaction between the above three substances and not as the coagulant itself, as in the thrombin theory. He used the term '<u>thromboplastic</u>' for a heterogeneous group of agents, including wettable surfaces, e.g. ground glass, "calcium oxalate in colloidal suspension", chloroform and similar emulsification, which assist in the colloidal

reactions. ...."The thromboplastic agent intervenes solely to make the reaction possible between these (3 cited) precursors. It acts as a catalysator. And so also do the alcoholic or aqueous tissue extracts". It may be remembered that Freund [174] and Bordet and Gengou [58] retarded blood and plasma clotting by use of paraffined surfaces.

7. THE HOWELL THEORY

Around 1910, W. H. Howell and his pupils were re-investigating (a) the thrombin-fibrinogen reaction (Rettger, 1910; Howell, 1910), (b) the intravenous injection of thrombin (Davis, 1910); and (c) the clotting of peptone- and hirudin- plasmas, especially by glycerinized (Cecil, 1911) tissue extracts. In a 1911 paper, Howell [230] carefully reconsidered the role of 'thromboplastic' tissue extracts and, because of their ability to clot the above-mentioned inhibitor-containing plasmas, he concluded in favour of an alternative to Morawitz's theory, namely, that tissue extracts act only by neutralizing certain clot-inhibitors. Furthermore, he argued for a type of (natural) inhibitor which does not merely antagonize active thrombin but actually prevents the formation of thrombin from prothrombin. Hence the new term 'antiprothrombin' and the specific idea (Howell's 'thromboplastin') that tissue extracts, particularly the thermostable lipoidal fraction, act by neutralizing antiprothrombin.

In 1912, independent of Zak [514], Howell [231] identified the thromboplastic agent as cephalin in some form of protein combination. Lecithin, whether from tissues or egg-yolk, was inactive. Howell's cephalin preparation followed the method of Thudichum [464,465], pioneer in the field of brain chemistry. Diakonow had separated 'lecithin' from other brain lipoid fractions and shown that its hydrolytic products were

glycerophosphoric acid, choline, and fatty acids such as oleic and margaric (ref. [103]). Thudichum found that there were a variety of phosphorylated lipoids (phosphatides; phospholip(o)ids) differing especially in their nitrogenous basic groups. Retaining the name lecithin for those containing choline, the new term cephalin was given to those containing ethanolamine. In 1915, Howell set his pupil, Jay McLean, to test, in the blood clotting system, all phosphatides described in the literature to that time. An unexpected result will receive due consideration in a later paragraph (p. 14). The data for Howell's [239] last paper, on "The isolation of thromboplastin from lung tissue", were completed just before his death in 1945, but it was his daughter Dr. Janet Howell Clark, who wrote it up for publication, kindly submitting it for my appraisal before sending it to the publishers. This paper showed the progress Howell had made toward characterizing thromboplastin as a "protein compound of a phospholipid" from which "it was possible to remove the protein, leaving a residue with marked thromboplastic activity, the chemical nature of which has not been determined".

Howell [233] used a crude preparation of prothrombin, simply obtained by precipitation of plasma with acetone, washing with ether, drying on a Büchner funnel, and subsequently extracting with alkalinized water or saline. None of his prothrombins failed to activate with calcium alone. In fact, many of them activated to thrombin 'spontaneously' and this was enhanced by treatment with $CHCl_3$ and other lipid-solvents [71]. Howell [229,232] also prepared a thrombin by 8% NaCl extraction of washed blood clots (see p.5) and could purify this by shaking with Chloroform until it no longer gave any reaction to chemical tests for phosphorus. These data

caused Howell to reject the European thrombokinase idea and insist that calcium alone could activate prothrombin. Reviewing these matters in his 1916 Harvey Lecture, Howell [235] agreed that thermolabile (50° - 60°C) aqueous tissue extracts or relatively thermostable cephalin do assist in prothrombin activation, but he proposed an alternative explanation, namely, that "under normal conditions the prothrombin is protected from the activating influence of the calcium ions by a combination of some kind with an inhibitory agent or antisubstance, and that cephalin exerts its accelerating effect upon coagulation by neutralizing the influence of this inhibitory substance thus liberating the prothrombin so that calcium can convert it to thrombin". This is the essential Howell theory.

Howell's pupil, McLean [326], in the above-mentioned study of a variety of relatively crude phosphatides, found some that, instead of aiding, had a marked inhibitory effect upon coagulation. These included two preparations of 'cuorin' (method of Erlandsen, 1907) and a 'heparphosphatide' (method of Baskoff, 1908). In personal conversations with Dr. Jay McLean during visits to Columbus, Ohio, around 1940, he told me how elated he had been to "discover antiprothrombin". But he had to leave for active service in World War I and miss the follow-up work in which Howell and Holt [242] and Howell [237] established the more important aspects of this anticoagulant action, including need for a plasma co-factor, and identified the agent, not as a phosphatide, but as a sulfonated polysaccharide amine, which Howell called heparin. In his 1925 Pasteur Lecture [236] and 1935 Physiological Review article [238], Howell clearly identified his antiprothrombin with heparin. He did note that Mellanby [330] was not able to obtain any evidence that heparin

combines with prothrombin, finding that it did not prevent the activation of prothrombin in a mixture of prothrombin, calcium, and kinase (tissue extract). Howell's "opposite results" were thought to be due, possibly, to differences in the two prothrombin preparations or in the test substrate (purified fibrinogen vs. dialysed oxalated plasma). Howell failed to mention a much more significant point, namely, that Mellanby's 'kinase' was a crude aqueous tissue extract, whereas Howell's 'thromboplastin' was purified cephalin. Howell cites Fuch's [177] suggestion that the prothrombin complex might be the same as the 'proserozyme' postulated by Bordet and Delange [56]. No further comment is made in the review, but I know from personal conversations with Howell that he was unimpressed with this idea. Bordet's proserozyme was said to change to serozyme (prothrombin) on contact with wettable surfaces, but heparinized plasma or prothrombin solutions are unaffected by the surface of the container. Howell [238] did not bring the heparin cofactor into the antiprothrombic thesis, but only into the antithrombic action of heparin. Thus ... "unlike hirudin, ... heparin ... has no inhibitory effect upon the reaction between thrombin and fibrinogen" ... although ... "heparin added to plasma or serum ... causes the production in these liquids of a true antithrombin by a reaction with some unknown thermolabile constituent of the blood". Let us anticipate some of the modern era of our theme to record the reasons for the refutation of the Howell theory. This came about because of failure experimentally to verify the essential facts which the theory requires. Here are the answers to some key questions:

1) Are significant amounts of heparin to be found in the blood normally? The best answer is that of Jaques [249] who used a reliable

chemical assay method which failed to find more than 10 micrograms per 100 ml in normal human bloods. This is only about 1/10 the amount of the heparin needed to keep blood from clotting in vitro. Heparinemia can occur in peptone-shock and other anaphylactoid reactions [255].

2) Can heparin form a prothrombin-antithrombin type of complex? Because of its highly acidic groups heparin can combine with the basic groups of many proteins, etc. (Fischer [162]). Chargaff [75] obtained evidence of a stoichiometric combination between heparin and protamine (a basic split-product of salmon-roe protein). It can form a polyanion complex with fibrinogen, experimentally [42]. No one, however, has isolated anything like the postulated heparin-prothrombin compound. The inhibitory actions of heparin have proved multiple and complex. They are still insufficiently understood, but much that is now known can explain these actions without need for postulating Howell's complex [425].

3) Does thromboplastin neutralize heparin and release prothrombin? As to the first part of this question, let us for the moment identify thromboplastin with cephalin (thromboplastic phospholipid). Phosphorylated lipoids are acidic in much the same way as the sulfonated heparins. Hence a direct neutralization seems improbable chemically. There is much more reason and evidence (e.g. Chargaff [75]) to think of both the phosphatide and the heparin as competing for the basic groups of proteins. Cephalin combines stoichiometrically with protamine [74]. Heparin, the stronger acid, can 'deviate' cephalin from this combination. "When heparin is made to react with the thromboplastic lipoprotein from lungs [87], the phosphatides are split off the protein carrier and a heparin-protein compound results. This compound, in losing its

thromboplastic property, has acquired a new one: it exerts a markedly anticoagulant effect!" [83]. The protein moiety of the thromboplastic agent must obviously also be considered. Furthermore, the plasmatic cofactor (cf. [242]) has been shown [67,151a] to intervene in the antiprothrombic as well as the antithrombic actions of heparin and this requires further explanation. As to the second part of the question, real evidence is still lacking that prothrombin was bound in the first place.

4) Can prothrombin be activated by calcium salts alone? This essential corollary to the Howell theory is seriously questioned by more recent work, including data presented in this thesis, in which it will appear from experiments with both Howell-type and with newer types of prothrombins that cephalin or tissue thromboplastin play a direct role, along with calcium, in the conversion of prothrombin to thrombin. Furthermore, wholly new factors [363,364] have now become recognized as equally essential in the highly complex thrombin-forming reactions. In summary, then, the Howell theory was too simple and has not stood up to the inexorable pressure of more modern experimental facts.

8. J. MELLANBY, "STUDENT" AND LEADER IN THIS FIELD.

In 1909, J. Mellanby, working in the Physiological Laboratory at Cambridge (England) as George Henry Lewes "Student", published two [327] noteworthy papers in the field of blood coagulation and, in his maturity, he returned to this field with further investigations in the 1930's [328,329].

Mellanby first employed fowl plasma, as recommended by Delezenne (1897), to prepare fibrinogen by a dilution and acidification technic, in principle similar to the techniques of Alexander Schmidt [420,421]. He rejected Hammarsten's [205] salting-out method because of "baneful

influences of salts on proteins" and stated ..."it is clear that if fibrinogen is a globulin, the best method to prepare it is to dilute and neutralize any fluid containing it. This procedure will certainly precipitate all the globulins contained in the fluid used, but there has not yet been adduced a certain proof that any globulin other than fibrinogen exists in plasma.... As a matter of fact the majority of experimental results point to the conclusion that fibrinogen is the only globulin contained in plasma [327]." This error is not only confounded completely by more modern knowledge (ref. [114]), but was untenable even with the data on plasma and serum proteins available in Mellanby's time. Furthermore, it is inconsistent with many observations in his own work. Elsewhere, Mellanby cited Pekelharing [373] as identifying prothrombin with 'kinase'. We have noted (p. 9) that this authority did believe prothrombin to be a 'nucleo-albumin' of cellular origin, but he was very clear that it was a plasma component and quite distinct from the zymoplastic cellular substance of Alexander Schmidt.

Credit must be given to the attempts of the Cambridge "student" to further the knowledge of blood clotting, including his efforts to be quantitative in many experiments. Yet one finds all sorts of inconsistencies and contradictions. For example, compare (a) "every fibrinogen solution which has been tested has coagulated with calcium chloride alone", and the statement (b) ..."but a consideration of the properties of kinase and prothrombin and the constitution of plasma point to the conclusion that calcium alone will not clot fibrinogen solutions". As 'thrombokinase' Mellanby used diluted testicular extracts from the bled cockerel, after the manner of Rauschenbach (1882). With calcium, this readily clotted his 'fibrinogen' and he properly interpreted this as

evidence for prothrombin contamination in the substrate. In this early work [327], Mellanby did not separate prothrombin but merely deduced its presence and activation from crude experiments, especially varying the concentration of kinase.

Mellanby's 1909 thrombin (fibrin ferment) was simply "the fluid expressed after complete coagulation ... of a fibrinogen solution by kinase and calcium chloride". It was with such reagents that he attempted to quantitate the thrombin-fibrinogen reaction both as to clotting-times and fibrin yields. He varied (a) calcium and other alkaline earth salts, (b) neutral salt (NaCl) concentrations, (c) acid (HCl) or alkali (NaOH), and (d) oxalate, citrate, fluoride, etc.

In 1930 Mellanby [328] presented a purification technique for 'prothrombase', which was his term for prothrombin, based upon the prevailing enzyme idea. Starting with dilution and acidification, patterned after the earlier methods of A. Schmidt and Pekelharing and essentially anticipating what we would now call an effort toward isoelectric precipitation, he triumphed over many difficulties. Thus, ..."the precipitate obtained from the diluted plasma is a complex mixture. The greater portion of it consists of prothrombase, fibrinogen and serum globulin, but the pigments of serum, cholesterol, and thromboldinase (!) are always present in variable quantities. The separation of prothrombase from this complex precipitate has presented considerable difficulties". Nevertheless, by diluting with lime water and bubbling CO_2 through, until a pH of 6.8 - 7.0, and calcium bicarbonate thus formed about 0.0092 per cent, he claimed splitting of the prothrombase from the complex. The amount of calcium was said to be too small to activate the prothrombase in the time required for filtration and subsequent precipitation by

acetic acid, to pH: 5.3. The final product was dried with acetone (cf. Howell, see p. 13). Admissions of its frequent contamination with thrombin conflict with assertions of "no spontaneous activation", e.g. on dialysis. Mellanby's product did have considerable potency and, in some tests, was partly stable to boiling for 5 min. at pH 7-8.

" ...Addition of tissue extract alone (thrombokinase) to a solution of prothrombase always causes the generation of a corresponding quantity of thrombase in the course of a few hours. This fact holds good even when the prothrombase has been prepared from solutions containing no calcium salts, i.e. oxalate plasma and the replacement of calcium bicarbonate by sodium bicarbonate in the second stage of the process". However, ..."minute amounts of calcium ... have a marked effect .... on the velocity of activation". Hence Mellanby balked at any final conclusion, thus ..."no definite statement on the place of calcium salts in the reaction may be made until thrombokinase has been isolated in a pure condition".

Mellanby's 'thrombase' (thrombin) described in a later paper [329] was obtained by slightly modifying the prothrombase method so as to permit conditions favorable to 'spontaneous' activation.

We have cited the foregoing in some detail in order to emphasize the unsatisfactory state of experimentation with the blood coagulation problem, as late as the 1930's. Fibrinogen had come a long way with Hammarsten [205] and Howell [230], but prothrombin and 'kinase' and even Howell's [232] purified thrombin were far short of critical requirements. It is in fact all too evident that most workers preferred to avoid facing the issue of contaminant impurities. Most deductions were made as if these did not exist, even if at times this meant a considerable

twisting of the experimental results. It would not seem that it was asking too much, by 1930, to ensure that fibrinogen preparations were free from all traces of prothrombin. Yet nowhere can we find a product which failed to clot with calcium and tissue extract, except possibly in some of the earlier experiments of Howell [230]. Most of the time, however, Howell only used calcium salt in the control test, since, according to his theory, this is all that was needed to activate prothrombin.

9. MILLS: FRACTICE VS. PRECEPT.

C. A. Mills, of Cincinnati, was the other notable authority whose personal interest and exchange of ideas played a role in the present writer's entry into the blood clotting field at that time. Of his voluminous writings [334,337] little will be said in this review. We do recall, however, Mills' insistence on a critical control of fibrinogen preparations for freedom from prothrombin. Mills used cephalin as well as calcium in the preliminary test. Unfortunately, much of Mills' magnificent conceptual approaches ended in data which must be discredited because of the failure to realize in practice what he preached in theory. The critical control was inadequate when most needed to support the interpretation of his experiments. The fact was that Mills [338] obtained clotting in his 'prothrombin-free' fibrinogen on adding lung extracts or platelets in the presence of calcium. This led him back to the heterodoxy of a second type of clotting and even to the nomenclature of Wooldridge's [508] 'tissue fibrinogen'.

The refutation of Mill's experiments and conclusions was made in 1934 by the Iowa pathologists Smith, Warner and Brinkhous [442]. In searching for a suitable fibrinogen these careful workers finally

adopted the technic of adsorption with $Mg(OH)_2$ for removal of traces of prothrombin, as previously recommended by Fuchs [180]. Their second contribution was in the preparation of the lung extract itself. Being highly vascular, lung is ordinarily full of blood, the prothrombin from which can appear in crude aqueous extracts. By perfusing the lung with water or saline the Iowa workers were able to obtain a subsequent prothrombin-free extract. This did not clot the critically prepared fibrinogen even on adding calcium.

Prothrombin-adsorption techniques were not new. Pickering [375] reviewed a variety of reagents which had been tried for this purpose. We may list $Ca_3(PO_4)_2$ (Bordet and Delange, 1914), [55] $BaSO_4$ (Dale and Walpole, 1916) [98], $Mg(OH)_2$ (Fuchs, 1929) [176], $Al(OH)_3$ (Quick, 1935) [381]. Many including Alexander [6] and ourselves [147] have come to prefer $BaSO_4$. In their 1938 purification of prothrombin by CO_2 (under pressure) elution from the $Mg(OH)_2$ plasma adsorbate, and its subsequent transformation to thrombin by means of calcium and lung extract, the Iowa workers were assisted by W. H. Seegers [435,428]. After establishing his own laboratory at Wayne University, Detroit, Seegers and his colleagues further purified prothrombin [424,431,495] and thrombin [433], from bovine plasma, to give us the most potent and possibly the purest preparations to date. There still remain some questions of trace impurities and it must be remembered that both adsorbents and precipitants yield several other components of the clotting system the presence of which in the final product may call for some critical comment.

B. MODERN VIEWS ON BLOOD CLOTTING.

The following selective outline of present day (1956) knowledge is presented with slight personal bias but is essentially the concensus of the modern 'group thinking'. All workers agree that many details and perhaps still undiscovered factors await further exploration and that the present 'working hypothesis', useful as it is, especially in many clinical applications, yet fails to give an adequately detailed answer to the deceptively simple, but truly involved, questions: (1) why does blood not clot in the normal circulation, and (2) why does it clot (a) in thrombotic conditions and (b) when shed? To unravel the complexities we shall try to analyse the clotting mechanism step-by-step, bearing in mind, however, the inter-relatedness and frequent simultaneity or overlap of the several and successive reactions.

1. SKELETON SCHEME OF CLOTTING REACTION.

Figure 1 presents an intentionally over-simplified scheme. It merely indicates the two 'classical' phases of clotting. The essential and final (second) phase is the conversion of the plasma protein fibrinogen into fibrin clot through the physiological intervention of thrombin. From one point of view, this reaction is colloidal, namely, a change from 'sol' to 'gel' involving many physico-chemical considerations, including surface electrical charges depending upon polar groups of the reacting molecules and the 'ionic atmosphere' of the surrounding salt-rich medium. From another point of view, it is enzymatic, referring to a specific mode of action of thrombin.

Thrombin is not normally present in the circulating blood. Indeed there are antithrombic factors naturally present to inhibit or remove any thrombin which might possibly form in the blood. The plasma does

contain a precursor protein, prothrombin. The classical first phase of clotting, then, is concerned with the conversion of prothrombin to active thrombin. This is particularly complex and will require a searching analysis of what our scheme collectively calls 'activators'. Possibilities of inhibition of the activator mechanisms are indicated in the scheme and will also require investigation. It is questionable whether we can group such first phase inhibitors as 'antiprothrombic' without becoming involved in the old controversies of the Howell theory.

2. EXTENDED SCHEME OF CLOTTING AND HEMOSTATIC MECHANISMS.

Figure 2 presents a more detailed scheme of the major factors in blood clotting and hemostasis, with some indication of inter-relationships. For clarity, all inhibitor factors are omitted and must be considered separately. Briefly summarized, the following ideas are covered:

1) Hemostasis is a physiological function involving:
   (a) vascular integrity and vasoconstrictor mechanisms,
   (b) platelets (and other formed elements) participating in the cell-thrombus,
   (c) fibrin clot.
2) Vascular factors will not be considered, except to mention:
   (a) vasoconstrictor role of serum serotonin derived (in part) from 5-hydroxy-tryptamine of platelets,
   (b) role of damage to vascular endothelium in (i) initiating platelet participation (adherence, clumping, breakdown) and (ii) affording site for fibrin deposition.
3) Platelet factors (incompletely reviewed) including:
   (a) the above vasoconstrictor (2a),

(b) a platelet component, which reacts with plasma factors in 'thromboplastin generation',

(c) a platelet 'accelerator',

(d) platelet retractor factor,

(e) a role of thrombin in the platelet mechanisms.

4) Plasmatic thromboplastic factors (AHF, PTC, etc.) are indicated as reacting with platelets (3b) in 'thromboplastin generation'.

5) Injured tissue sources of a 'complete' thromboplastin are indicated.

6) Prothrombin is the inactive precursor of thrombin, in plasma, normally activated by Ca^{++} and thromboplastin in the presence of (7) and (8), which are two essential plasma 'co-factors'.

7) Proconvertin, yields convertin in the presence of Ca and thromboplastin.

8) Proaccelerin, yields accelerin, under the influence of a trace of thrombin.

9) Active thrombin, formed by the indicated reactions,

10) Conversion of fibrinogen into fibrin. 'Serum factor' (p. 33) should be included.

11) Clot-retraction subsequently results, from action of a platelet factor (3d).

12) Fibrinolysis and clot-resolution result eventually, from activation of the proteolytic enzyme system, whose components are also indicated in the scheme.

13) Various inhibitors should be included in the concepts outlined above (see pp. 49-57).

3. THE THROMBIN-FIBRINOGEN REACTION.

Most of the early work in this area was poorly quantitative and

beset with uncertainties and errors caused by impurity of the reagents used. Modern materials are by no means completely free from all uncertainties, but have clearly proved their value in many exacting quantitative tests. Particularly significant are data which have been obtained with physical measurements, e.g. opacity, rigidity and tensile strength of clots, light-scattering (Stufenphotometry), streaming double refraction, diffusion, viscosity, electrophoresis, ultracentrifugation, and electron-microscopy. Solubility studies [89] are very valuable in preparative procedures, when worked out in relation to the five variables: (1) temperature, (2) pH, (3) ionic strength, (4) protein concentration, and (5) content of water-miscible organic solvent, e.g. ethanol, zinc-glycine, etc. Monumental contributions in these areas of our field have come from the late E. J. Cohn's Department of Physical Chemistry and Plasma Fractionation Laboratories at Harvard [114].

a) Fibrin yields. The modern chemist seeks to follow the course and kinetics of reactions, especially enzyme actions, by timing changes in the substrate, as well as by measuring amounts of the final products. This logical approach is beset with technical difficulties in the case of the thrombin-fibrinogen reaction. The extent to which these may be overcome is illustrated in the selected references [159,348,60]. Fibrin yields, as measured by the amount of coagulated protein recoverable in washed clots, is, of course, primarily dependent upon the initial amount of fibrinogen available. That the thrombin concentration determines only the rate of the clotting reaction but not the ultimate fibrin yield is clearly shown in Figures 3 and 4. These are from experiments with purified fibrinogen and thrombin made in the author's laboratory [60]. Figure 3 shows effects of varying the relative thrombin

concentration a thousandfold, but employing a constant amount of fibrinogen. Despite clotting-time (see below) variations between 18 sec. and 30 min., the final (10 day) fibrin yields are identical and all 100 per cent of the original (coagulable) protein in the fibrinogen. These findings are strong evidence for the enzymatic nature of the thrombic action. Figure 4 shows fibrin yields, with time, employing four different thrombin concentrations. The linearity of the logarithmic plots (of residual substrate concentrations), within small limits of experimental error, indicates a first order reaction [273] again consistent with an enzymatic activity of thrombin.

NOTE: The results do not rule out a pseudo-first order reaction in which preliminary phases might be slower than the action of thrombin. Other things which do not affect the fibrin yield, within reasonable limits, are (1) temperature, (2) pH, (3) salt concentration, e.g. NaCl, $CaCl_2$, (4) 'fibrinoplastic'* colloids, e.g. acacia (except for minor occlusion effects) (5) minor partial denaturation of fibrinogen ('profibrin' of Apitz, 1937). Things which may affect fibrin yield, include (a) extremely low fibrinogen concentrations (0.002 - 0.003 per cent) where 'soluble fibrin', i.e. intermediate polymers of insufficient degree of aggregation, forms a significant proportion of the end-product, according to Morrison [348]; (b) occluded proteins [348]; (c) reversible inhibitors of the type studied by J. D. Ferry, S. Shulman, et al. in the Wisconsin Laboratories [160,438,440], for the same reason; (d) fibrinolytic enzymes, if active, which are apt to be

FOOTNOTE: * Ferguson (1940) [134] proposed a revival of this old term of Alexander Schmidt's in order to indicate agents which shorten clotting-times through nonspecific effects best interpreted as adsorptive or similar ways of bringing fibrinogen and thrombin 'reactive groups' together and thus facilitating their interaction.

contaminants of thrombin and some fibrinogen preparations and can destroy fibrinogen and fibrin at approximately equal rates, according to experiments in our laboratory [291].

b) Clotting-times. The earliest efforts to obtain quantitative information about the clotting process were by measurement of the time required to form a visible or a solid clot. Because so many factors enter into the determination of any 'clotting-time', the whole concept would seem to be fraught with extreme empiricism. In fact, it is possible to advance the view that there is no such thing as clotting-time, since the blood does not normally clot in the circulation, so that we must merely be measuring the results of highly artificial conditions. In actual practice, however, these conditions can be defined sufficiently to give clotting-time determinations real value in following and interpreting the clotting phenomena. As a technical point, particularly referring to experimental systems of artificially isolated clotting agents, most workers prefer to time the first appearance of a definitely visible clot (fibrin strands), rather than a solid gel (tube invertible). Both end points are empirical and depend upon a certain amount of fibrin formation, by no means indicative of the ultimate fibrin yield. The appearance of successive crops of clots when a weak thrombin is used, is a well-known phenomenon. Factors known to influence clotting-time include:

(1) Concentration of thrombin: the stronger the thrombin, the shorter the clotting-time, other things being equal. This is a common feature of enzyme reactions. Figure 5 shows an experiment, from the author's laboratories, in which, over at least a 10-fold range of relative thrombin concentrations, the clotting-times obeyed an 'inverse law', an old idea

in the coagulation literature [163]. It is, perhaps unfortunately, very difficult to define the precise experimental conditions for obtaining this result. In most experiments, the deviation from a linear relationship is significant. It may still be possible, however, to translate clotting-times into relative thrombin concentrations ('units' of activity) by use of this type of reference curve (simple plot) obtained under closely similar experimental conditions [20].

(2) Concentration of fibrinogen: over a considerable range this has remarkably little effect on the clotting-time, which is not so strange, remembering that we time only a partial change in the substrate (see above). The quality of the clot varies, however, and may affect reading of the end point. Particularly with very weak fibrinogens, a poor clot and the probability of slower and less complete polymerization (referred to under fibrin yield), may cause longer clotting-times.

(3) Temperature: It has long been known that cooling retards the reaction, while warming accelerates it, up to temperatures where complications appear because of thermal denaturation of the fibrinogen. Thrombin is much more heat resistant, but this depends on pH, salt content, and other considerations. Some plasmas and impure fibrinogens may be completely clear when frozen and kept at -20°C, but show clots on subsequent thawing. This may indicate that traces of thrombin can act on fibrinogen slowly even in the frozen state, but it is just possible that the clotting occurs in the act of thawing.

(4) pH: There is an ill-defined pH optimum for the thrombin-fibrinogen reaction, with some dependence upon the experimental conditions. It is important that the normal pH of the plasma (about 7.4) is close to this optimum. Clotting-times are prolonged, but only slightly, until we

approach the extremes of (a) 5.3 in the acid range, or (b) 10, in the alkaline region. In (b) we run into the 'reversible inhibitor' problem (see (7)) of insufficient polymerization of the fibrinogen [466]. This may also be a factor in the acid region [405].

(5) Ionic strength: the most important action of sodium chloride and most common neutral salts is a delay in clotting-times with increasing ionic strength [115]. Numerous data on this go back all the way to William Hewson (1770). Polyvalent anions [191] e.g. ferrocyanides, ferricyanides, have especially marked inhibitory effects. Table I summarizes an experiment of the author's [145]. The experiment shows that the inhibitory action of strong neutral salt (4.5% NaCl), which can be removed by subsequent dilution, does not alter fibrinogen or thrombin and only prevents the appearance of visible fibrin in a mixture of these two agents. Confirmation of the fact, that clotting-times become progressively shorter, the longer the thrombin-fibrinogen mixture is held with the 4.5% NaCl before dilution, is regarded as evidence that the essential step in fibrin formation proceeds independently of the presence of the salt, which merely inhibits or retards the gel formation (precipitation). cf. Apitz[18].

(6) Specific ion effects: calcium is anomalous in that it definitely accelerates the clotting-time of thrombin-fibrinogen mixtures, in a narrow range of low concentrations e.g. 0.008 - 0.032 molar, in one of our [60] experiments, although this depends on total ionic strength [115,310]. By removal of the Ca-effect, EDTA ..."hinders the fibrinogen-fibrin transition" [409].

(7) Reversible inhibitors: in an extensive recent study by Shulman [438] 42 (out of 80) substances tested markedly delayed or inhibited clotting

in thrombin-fibrinogen mixtures, under this author's test conditions. This type of inhibition is reversible by dialysis. Important conclusions as to the 'polymerisation' process by which fibrinogen is transformed into fibrin, can be drawn from these and other experiments. We shall consider this topic in a later paragraph.

(8) 'Fibrinoplastic' colloids [60]. The shortening of clotting times by these agents has been mentioned in the preceding section on fibrin yields and the footnote on p. 27 suggests an explanation of their mode of action. Gum acacia is of some importance in that it is used "to stabilize the reactivity of fibrinogen" in the Iowa 'two-stage' prothrombin (and thrombin) bioassay [443]. Protamine has similar effects [134], over a wide pH range. We have also noted it with a highly purified lactoglobulin. Also see 'platlets' (p. 89).

(9) Partial denaturation of fibrinogen [60]. The present author's view is that the effect of this is due to the same general type of 'fibrinoplastic' action as noted under (8). That all of the denatured fibrinogen is included in the fibrin clot may be evidence of an adsorptive or occlusion phenomenon. The Wöhlisch [504] 'denaturation theory' of clotting is now largely discredited.

(10) Adsorptive removal of thrombin: compared with whole blood or plasma clotting (see later), the surface of the container has little effect on the thrombin-fibrinogen reaction. An exception is seen in the case of very weak thrombins, where adsorption on to glass, etc., removes part of it from solution and hence lengthens the clotting-time. This can be minimized by coating the tube with non-wettable silicone [60]. It is a common experience that poorly washed glassware, thus contaminated with a trace of thrombin, can clot fibrinogen solution stored therein

later.

c) Nature of Fibrin Formation. Progressing from the uncertain experiments of Apitz [18], Laki and his colleagues [278,274] produced evidence of a step-wise process in the formation of fibrin from fibrinogen under the influence of thrombin. The latest interpretation of these and other data was well brought out in the presentations and discussions at the 1951 Conference of the Josiah Macy Jr. Foundation [274]. J. T. Edsall (representing the Cohn laboratories), J. D. Ferry (formerly of the Cohn team, but now at Wisconsin), K. Laki (now at Bethesda, Md., N.I.H. Laboratories), D. F. Waugh (Massachusetts Institute of Technology) were the chief authorities at this meeting, with the present author contributing a minor role. The modern concept of fibrin formation is as follows:

Fibrinogen is a fibrillar protein whose long filamentous molecules approximate 38 Å in diameter and 700 Å in length (usually), with a molecular weight close to 500,000 (perhaps 330,000). In solution, these large molecules, moving with Brownian movement, exhibit considerable swirling and 'steric hindrance'. During clotting, however, they line up 'like logs in a stream' and unite end-to-end and also side-to-side, until the 'polymerization aggregates' are large enough and hydrophobic enough to separate from the watery solution as visible fibrin. Fibrin filaments or needles were seen to appear during clotting under the ordinary transilluminating miscroscope by Ranvier (1873) and Schimmelbusch, (1885). Stübel, in 1914, and Howell, in the same year, demonstrated them very clearly with the dark-field microscope. Howell [234] discussed the problem of the fibrin gel formation in 1916. Electron microsopy, first used for observing fibrin by Wolpers and Ruska [507] in 1939, has

been used to support the modern views, by Schmitt [422] by Hawn and Porter [212], Hall [204], Kaesberg and Shulman [264] and others. Even the electron microscope does not resolve the fibrinogen molecules and the simplest polymers or protofibrils, but it does show variations in meshwork density and the fibrillar composition, together with cross striations, of the fibrils which appear uniform under the ordinary (incl. dark-field) microscope. Consideration of clotting conditions (see above) in relation to electron microscopy and many physical measurements (see earlier) affords strong support for the modern concepts. Thus, under inhibitory conditions, much smaller aggregates are formed and resemble the normal early or lag phase, in which there is yet no visible clot, but viscosity, birefrigence, opacity and other data indicate some intermediate polymerization. Under some conditions, the fibrin polymer is reversible, undergoing disaggregation and re-solution, e.g. in urea, guanidine, lithium bromide (strong solutions), etc. [307]. Irreversible and urea-insoluble clots have recently been shown to depend upon two things, namely, (1) calcium [266] and (2) a special 'serum factor' Laki and Lóránd [277], Lorand [304,305,307], Shulman [439]. An earlier indication of this was found in 1944 by Robbins [405] in studying the somewhat similar problem of 'solution' of fibrin in acid (0.03% HCl) or alkali (0.5% Na_2CO_3). These data assist in explaining some of the peculiar actions of calcium when present in the thrombin-fibrinogen (+ serum factor) mixture (see above), the 'serum factor' being a common contaminant of 'purified' fibrinogen, but see p. 115.

It is suggested on p. 34 how a limited attack on the fibrinogen molecule activates polar groups which enable the altered substrate

molecules to aggregate in the polymerization processes. The electrical charge distribution on fibrinogen molecules has been studied and there is good evidence that Coulomb forces pay a role in the preliminary phases of aggregation. Salt inhibitors (see ionic strength) are to be interpreted (Ferry,[157]) as 'shielding' of the polar groups by the charged 'ionic atmosphere' contributed by the salt. Further stages involve van der Waal forces (e.g. adsorption, etc.) and perhaps specific chemical bonds (currently debatable)[158]. At all events, the final steps do not involve thrombin. Like other enzyme systems, thrombin is not truly a part of the fibrin end-product. It is a practical fact, however, that a considerable amount of thrombin is adsorbed on to the fibrin clot[434]. Indeed, upon this depended the success of early methods of obtaining thrombin by extraction from washed blood clots.

d) Thrombin. This is by no means the simple proteolytic enzyme suggested by the 19th century coagulationists. It is unlike trypsin[458]. Most of the alleged evidence for its proteolytic powers[219,380] can be dismissed as due to contaminant fibrinolysin[149,462] or possible as a certain degree of solubility of fibrin[247]. However, Sherry and his associates[437] have recently shown that fibrinolysin-free thrombin can hydrolyse certain bonds in some synthetic polypeptides, e.g. TAME (tosyl-arginide-methyl-ester).

Lorand[307] has contributed some provocative new ideas. He uses Seegers' citrate-thrombin (see p. 39) and (a) bovine fibrinogen or (b) human fibrinogen[308], to show:

1) non-protein N is liberated pari passu with the clotting action of thrombin;

2) the glutamic acid missing from the fibrinogen after the action of thrombin can be identified in a 'fibrino-peptide', which has been isolated and characterised [309,306];

3) in bovine fibrinogen, thrombin is thought to split arginine-glycine bonds [30]. (NOTE: it does not do so in the case of insulin, according to Middlebrook);

4) alanine replaces glutamic acid in the N-terminal amino-acid residues of human, as compared with bovine, fibrinogen, but the general process is the same;

5) electrophoretic studies show a characteristic pattern and the paper-strip method permits characterization of the split products. The pattern with thrombin is quite different from that produced by fibrinolysin.

Lorand concludes that:

a) the 'fibrino-peptide' is a by-product, which does not participate in the fibrin polymerization, but does account for the small quantity (about 2%) of nitrogen not recoverable in fibrin, even in optimal clotting of the highly purified fibrinogen now available.

b) the fibrinogen molecule activated by this action of thrombin now contains some 20-30 acidic groups, which can very well account for the first phases of polymerization to fibrin;

c) with prolonged action of strong thrombin, e.g. 3-4 mg. thrombin acting on 150 mg. fibrin for 24 hrs. at 37° C, complete fibrinolysis can occur (Guest and Ware [203]), associated with further small N.P.N. increase [307]. Hence

d) "fibrin is just a transitory stage in the continuous degradation

process of fibrinogen by thrombin".

In this special way, therefore, we may now return to a proteolytic concept of the action of thrombin on fibrinogen.

e) Other Coagulants, etc. Properly purified fibrinogen solutions are quite stable and are never 'spontaneously' coagulable. Whereas thrombin is the coagulant always required under physiological circumstances, some other means of coagulating fibrinogen are available experimentally. Examples are:

1) crystalline papain [112], a proteolytic enzyme from the paw-paw (papaya) fruit. Unlike thrombin (usually), papain readily goes on to digest the clot.
2) staphylocoagulase [186], a product of the reaction of a prostaphylocoagulase, from broth cultures of certain micrococci, with a plasma factor [187] which is apparently unrelated to prothrombin.
3) certain snake venoms, e.g. 3 species of Bothrops and 3 or 4 of Crotalus, according to Eagle's studies [111].

True fibrin clotting should be distinguished from a variety of fibrinogen precipitations (which are reversible) and denaturations (most of which are not). The pseudo-clot with ninhydrin [82] is a case in point. Some of the author's [155] dark-field observations of (a) thrombin-, (b) papain-, and (c) ninhydrin- clots are shown in the accompanying photomicrographs (Figure 6): (III) and (IV) show the nondescript appearance of ninhydrin clots, compared with the fibrillar gels formed with (I) thrombin and (II) papain. Laki [275] has recently confirmed the last.

4. THE CONVERSION OF PROTHROMBIN TO THROMBIN.

It is with this area of the clotting field that the major investigations of this thesis are concerned. The best modern prothrombins do not change into thrombin 'spontaneously' (but cf. [425]). Among the activators mentioned in the schemes of Figures 1 and 2 are: (1) calcium, (2) thromboplastic factors, (3) 'accelerator' factors, (4) 'convertor' factors. Non-commital terms are used in approaching these problems with an open mind.

a) Purity of prothrombin preparations. Prothrombin is difficult to purify. The early preparations of Schmidt, Pekelharing, Hammarsten, Mellanby, Howell, Eagle [110], and others, were undoubtedly too crude, when reviewed in the light of modern advances [425,276,491]. Nevertheless, they gave us many of the basic ideas concerning the activation of prothrombin e.g. by calcium ions and thromboplastic (thrombokinase) factors, with possibilities of inhibitors, which have merely been confirmed with the modern 'purified' prothrombins. Contamination with (a) traces of activators, (b) traces of inhibitors, (c) fibrinolytic enzyme (or precursor, etc.), (d) perhaps other factors, may still need critical consideration, even in the best modern preparations.

b) Nature of prothrombin [425]. Prothrombin is a specific protein accompanying the globulin fraction(s) of the plasma proteins. Its quantity in the blood (15 mg/100 ml) [425] is very much less than that of fibrinogen (280 mg/100 ml). In fact, unlike the latter, it does not show an independent peak in plasma electrophoresis [491]. Seegers and his colleagues have prepared the most potent, hence presumably the purest prothrombin, to date, and their work provides the most reliable data on its characterization [425]. Seegers believes that most of the

difficulties in obtaining a biochemically pure prothrombin come from "some activation" and other "marked alterations in the molecule" during the purification, storage, and experimental procedures. The purest preparation examined by Lamy and Waugh [280] was monodispersed in the ultracentrifuge, but with some boundary spreading. Its physical constants led to computation of the molecular weight at 62,700, and the shape of an ellipsoid molecule of 119 x 34 Å. Chemical analysis yields a trace of S (cystine and methionine), some 4.3 per cent polysaccharide carbohydrate [431], and 3.75 per cent ash. Perhaps because of 'derivatives' (Seegers), prothrombin solutions show more than one electrophoretic component, but the major component constitutes 70-90% of the total [432]. The thrombin yield may depend upon changes during storage in the dry (lyophilized) state, but generally 'averages' 23,000 units (two-stage assay) per mg tyrosine or 1400 units/mg dry weight.

c) Biophysics of thrombin formation [280].

(1) 'Biothrombin': is a convenient term [280] for the thrombin obtained from purified prothrombin in the conventional manner, i.e. addition of calcium salts, tissue thromboplastin, accelerator globulin, (pro)convertin, etc. Maximal transformation to thrombin is obtained only when the prothrombin concentration is relatively low. For physical studies, concentrations of prothrombin of the order of 0.5 per cent must be used (about 10-100 times as concentrated as used to obtain maximal activation). Under these conditions not more than 50% of the 'maximal' thrombin yield can be secured. With the (lung) thromboplastin allegedly removed by high speed centrifugation, subsequent ultracentrifugation shows a single symmetrical peak, having an average sedimentation constant, S~4.8, corresponding to that of the original prothrombin. Some boundary

spreading suggests polydispersity however. The specific activity of the material at the peak was 30,000 (thrombin) units/mg tyrosine.

(2) 'Citrate thrombin': distinguishes a new product which Seegers et al.[432] obtained in 1948, simply by incubating purified prothrombin in 25 per cent sod. citrate. Several other salts (but not all) could be used and the reaction could be modified by certain diphenyl sulfones[432]. It is extremely difficult to evaluate the biological significance of this very artificial method of thrombin formation. However, it must have biochemical significance. Refractive index, ultracentrifugation, and electrophoretic studies on 'citrate thrombin' give evidence of an initial splitting of prothrombin into two similar (molec. wt.: 34,000) fractions, but on dilution there appear 'aggregates' and 'disaggregates' and continuing changes over a 24 hr. period[280].

(3) 'Trypsin thrombin': is also discussed in the cited reference[280] and will be mentioned again later (p. 42).

These modern data are difficult to analyse, but do seem to indicate that prothrombin can split up into a number of molecular fragments, some of which can re-aggregate, probably into new agents. Autoprothrombin I has been identified with proconvertin[491,425,13] while autoprothrombin II has properties resembling PTC[429]. In the formation both of 'biothrombin' and 'citrate thrombin', small but significant amounts of CCl_3COOH-soluble carbohydrate and tyrosine compounds are liberated, confirming the idea that thrombin forms from prothrombin as the result of some 'splitting' process[427]. These very latest ideas cannot yet be integrated with the older data on mechanisms of prothrombin activation, from the viewpoints which are presented in this thesis.

d) 'Prothrombinogen' (?). In 1949, Quick and Stefanini [396] claimed to distinguish between 'free' prothrombin and a precursor form for which they suggested the name 'prothrombinogen'. They compared their experiments with older data of Nolf [351] and Bordet [55] and concluded that their prothrombinogen differed from Bordet's 'proserozyme' in only a few particulars. Only a part of the plasma prothrombin is believed by Quick to be in the precursor form, and Quick and Hussey [389] (cf. [154]) have recently attempted to explain the normal 'prothrombin time' of infants (see our Table LII) on the basis of a lack of prothrombinogen. Few contemporary coagulation's consider that Dr. Quick has enough factual evidence to support his shrewd ideas (see Macy Foundation Conferences), but Ware [491] is now inclining toward the prothrombinogen idea. The present author[154] strongly opposes it.

e) Calcium in relation to the conversion of prothrombin into (bio)thrombin. Older plasma clotting experiments by Nordbö [352] and McLean and Hastings [325] extended the earlier idea of Sabbatini [417], that clotting depends upon calcium ions. There is a definite optimum of calcium required, approximately that available in the blood under physiological conditions. Not merely the calcium ion concentration, but the actual ion 'activity' (in terms of the Debye-Hückel theory) require consideration. Nordbö particularly studied this and further pointed out that the 'available' calcium ions in a mixture of plasma, oxalate (or citrate), and added calcium salt depend upon a time-consuming process of attaining an equilibrium of the different forms (free Ca^{++}, inactive $(Ca\ Cit)^{-}$ anion, and protein-bound Ca, etc.). It is because of this time factor that a several-fold excess of citrate or oxalate must be added to prevent clotting and, conversely, that much less than the stoichiometric

equivalent of calcium to anticoagulant need be added to restore clotting cited [125].

Protein-bound calcium is present in the blood plasma normally and in prothrombin and other materials prepared from it, unless very modern technics of complete decalcification (e.g. by ion-exchange resins) are used to obviate this. A number of workers (cited [125]) have continued to suggest that (protein)-'bound' calcium can play a role in clotting. Even Quick [383,394] still retains the idea that prothrombin is a 'calcium compound'. There is little point in arguing about this, since the weight of evidence supports the conclusion that ionized calcium must participate at some point in the conversion of prothrombin to thrombin. A possible reconciliation of the two viewpoints will be suggested from data presented in this thesis. Under all ordinary conditions of activating purified or even crude prothrombins, omission of ionized calcium salt or the presence of sufficient excess of citrate or oxalate, fails to result in thrombin formation by thromboplastin (and adequate accelerator and convertor factors). The special conditions under which thrombin may be formed, apparently in the absence of ionized calcium, include (1) Seegers' [432] 'citrate thrombin' formation (see above), which we shall not attempt to explain; (2) experimental mixtures, such as those of Milstone [339] (see p. 83), and of recent workers [40] with the thromboplastin generation test, see p. 84, which are best explained as "intermediates" containing bound Ca (which, however, was ionized in an earlier phase).

f) Thromboplastin and the activation of prothrombin. This topic will be explored in much detail in the body of this thesis. Aqueous (saline) tissue extracts, of many kinds, are powerful activators of

purified (and crude) prothrombins, in the presence of calcium salts and adequate amounts of 'accelerator' and 'convertor' factors. Purified cephalin seems to have thromboplastic actions, but much weaker, especially with Seegers' and similar prothrombins. It is now generally regarded as an 'incomplete' thromboplastin. Platelet suspensions also act as an 'incomplete' thromboplastin. So does a red cell lipid fraction [105]. However, when platelets or cephalin are incubated with several other plasma factors (including AHF, PTC, etc. - see later), a 'complete' thromboplastin is "generated". Modern workers are still trying to find full explanations for these experimental facts (see p. 82).

g) 'Thromboplastic enzyme'. The coagulant property of weak solutions of pancreatic trypsin, which some earlier observers erroneously concluded to be a 'thrombin-like' action, was clearly shown by Eagle and Harris [112] to be the result of some role in the activation of prothrombin, in experimental test systems. Some of the author's experiments with crystalline trypsin will be mentioned in this thesis. More recently, Schultze and Schwick [423] have examined extensively the conditions under which trypsin transformation of prothrombin to thrombin can be carried out. Lamy and Waugh [280] report some ultracentrifuge studies (see 'trypsin thrombin', p. 39).

The author [141] spent several years trying to establish a similar role for the natural plasma 'tryptase' (fibrinolysin, or plasmine) and possibly to give it an important role in the initiation of ordinary blood clotting, [136]. However, the final outcome [476] was evidence that trypsin-inhibitors could prevent the proteolytic actions of fibrinolysin without removing the 'thromboplastic' effect, — quite contrary to the findings with trypsin. Pending possible re-opening of this line of

investigation, it must be concluded that any thromboplastic effects of fibrinolysin preparations must be due to some unidentified impurity which they contain. Ratnoff, Hartmann, and Conley [402] also investigated the relationship between proteolytic activity of plasma and blood coagulation and, in a later paper Ratnoff and Colopy [401] suggest that they may have been dealing with "Hageman factor" (see later, under Hemophilia).

h) 'Accelerator' factors in prothrombin conversion. Many modern workers were fully aware that calcium and thromboplastin did not provide a sufficient answer to the question of prothrombin conversion. The most significant step, however, came from the very extensive studies of P. A. Owren [361], on a human patient suffering from a peculiar bleeding disorder, which he named 'parahemophilia' [362]. The new factor, which this patient was finally shown to lack, was called 'factor V' and later 'proaccelerin' by Owren [364]. Quick [384] had earlier evidence for a 'labile factor'. Another lead was the 'thromboplastin cofactor' of Fantl and Nance [119]. Owren [361] also provided evidence for conversion of his 'factor V' (proaccelerin) into a 'factor VI' (accelerin). Ware and Seegers [493] prepared the new agent under the name 'accelerator globulin', by which it is now widely known, or 'AcG' for short. They also found differences between 'plasma AcG' (proaccelerin or factor V) and 'serum AcG' (accelerin or factor VI), the latter being formed from the former by a trace of thrombin. Stefanini and Dameshek (p. 20 of ref. [453]) list other possible synonyms.

The present author and his colleague, Dr. Jessica M. Lewis, have studied (1) cases of congenital hypoproaccelerinemia [297]; (2) differences between proaccelerin and accelerin in the activation of

prothrombin (cf. [492]); (3) destruction of AcG by fibrinolysin [299]; and (4) its role in performance of the two-stage prothrombin assay and in determining prothrombin consumption in clinical cases [153]. In summary:

(a) (pro)accelerin is essential for the conversion of prothrombin to thrombin by calcium and tissue thromboplastin;

(b) a trace of thrombin converts the precursor form (proaccelerin) into the active accelerin;

(c) AcG has more effect upon the thrombin yield than upon the rate of activation, which is somewhat delayed when AcG is very inadequate, however;

(d) satisfactory assay methods are now available both by a 'one-stage' (specific) technique [395] and a 'two-stage' [493], including modifications (see pp.101,103) developed in the author's laboratory for application to clinical cases of hemorrhagic disorder. Plasma, serum, and platelets may be tested by these techniques;

(e) serum, except from the ox, cat, or rabbit [349] is apt to be devoid of this factor because of lability and due to the fact that it is used up during clotting;

(f) AcG levels fall after hepatectomy [322] or liver injury [461].

i) 'Convertor' factors in prothrombin activation. The discovery of this factor also owes much to the first clearly identified clinical case of B. Alexander and colleagues [9]. They named the factor SPCA (serum prothrombin-conversion accelerator) and obtained evidence for a precursor (pro-SPCA) [11,8]. Owren was on the track of this additional factor [360,366], but it was Koller (et al. [271]) who correctly re-interpreted Owren's data and named it "Factor VII". It is probably the same factor, which

(a) Warner, Brinkhous and Smith [497] suggested as a prothrombin 'convertibility' factor and (b) Mann et al. [319] called "co-thromboplastin". We prefer to adopt the names proconvertin (for the precursor) and convertin (for the activated form), as suggested by Owren [364]. There are other suggested synonyms (p. 20 of ref.) [453].

Graham and Hougie [198] report that mixture of the bloods of Alexander's original case, R. [9] and ours R. S. [298] results in mutual correction of the coagulation defects. Together with other experimental findings, this suggests that there may be more than one factor involved under the terms SPCA, proconvertin, factor VII, stable factor, etc. Calcium and thromboplastin convert proconvertin to convertin and abolish the lag phase in prothrombin activation to thrombin [295]. Serum often contains much unaltered proconvertin as well as some convertin.

In deficiency of proconvertin, prothrombin conversion is very slow and inadequate. Restoration by partly purified preparations from human [9] or bovine plasma (our laboratories) completely restores both rate and yield of thrombin formation. Dr. Lewis and the author [298] reported two cases of severe congenital hypoproconvertinemia, in whom the prothrombin consumption was reduced. This had not been observed in earlier case reports [9], due, we believe to their being a milder degree of this defect. Supporting this, we have two more recent cases (unpublished) in whom the prothrombin consumption was within normal limits. Our congenital hypoproconvertinemics did not respond to vitamin K_1, unlike the milder acquired hypoproconvertinemias, accompanying hypoprothrombinemias, in (a) vitamin K deficiency of infants, (b) liver disorders, or (c) after the drugs, dicumarol, tromexan, phenylindanedione, etc. (author's data), confirmed by Naeye [350]. The one-stage [101] assay

method is satisfactory for clinical use [298]. We [298] have also had some success with a two-stage technic which enabled us to compute a proconvertin index (see p. 89).

j) Plasma factors in thromboplastin generation. While tissue thromboplastin may very well play a significant role when blood is shed over injured tissues, it cannot explain the good clotting which occurs in blood which has been obtained directly from a blood vessel, e.g., by venepuncture, with every precaution to avoid tissue contamination. The necessary thromboplastin is generated in the blood itself by a complexity of reactions involving (1) platelets, (2) several plasma factors, which are present normally, but deficient in hemophilia and some recently indentified 'hemophilia-like' conditions, (3) some of the other prothrombin activators. The platelet factors and the thromboplastin generation test will be discussed in a later section.

(1) Hemophilia and related problems. Only a few key points will be reviewed from the extensive recent literature in this field and most of the older work will be excluded.

(a) Hemophilia A. Notwithstanding efforts to account for the hemophiliac's clotting difficulty on the basis of some inhibitor, e.g. the 'antithromboplastin' idea of Tocantins et al. [470,474,475], the major accumulation of evidence points to a specific clotting factor, present in the plasma of normal persons, but deficient in the hemophiliac. Patek and Stetson [368] and, later, other workers in F. H. L. Taylor's group at the Thorndike Memorial Laboratory of the Boston City Hospital, contributed convincing evidence for this antihemophilic factor (AHF), which they called 'antihemophilic' globulin (AHG). Koller [270] lists it as 'factor VIII'. AHG accompanies fibrinogen, etc. in the Harvard

Laboratories' plasma 'Fraction I'. It is somewhat unstable and difficult to isolate and purify. Bidwell [46], in England, claims a good bovine plasma fractionation, but the problem needs further solution [48].

AHF is utilized or consumed in the clotting process. With other factors, it participates in thromboplastin generation from platelets (or cephalin) (p. 80). In the natural clotting of hemophilic blood or recalcified hemophilic plasma, the 'prothrombin consumption' is very defective. Following this lead of Dr. Brinkhous's, we have also developed a method, depending upon the prothrombin consumption (1 hr. at 37°C) in recalcified mixtures of 'substrate' (known severe hemophilic) and normal (vs. test) plasmas, for quantitative estimation of plasma AHF levels. These tests can also be modified to assay anti-AHF [152]. By changing the substrate (known cases), they also assay PTC and anti-PTC, etc.

We shall say nothing about the important familial aspects of hemophilia, except to mention the very interesting work on canine hemophilia [197].

(b) PTC-deficiency (Hemophilia B). In February 1952, Dr. Lewis and the author [294] were investigating a 'bleeder' who seemed to be made worse instead of better by blood transfusions. Our tests immediately showed that he had a circulating anticoagulant (see later), but by preparing plasma fractions free from this inhibitor, an underlying clotting defect was disclosed, which was definitely not hemophilia. By withholding transfusions, the anticoagulant gradually diminished, as shown by the tests illustrated in Fig. 7, clearly revealing evidence (the long recalcification clotting times in the last tests) of the basic clotting defect. In April 1952, Aggeler and colleagues [5], in California, published the first clearly identified case of the new 'hemophilia-like' disease, which they named PTC-deficiency (plasma

thromboplastin component). Using their techniques, we quickly confirmed that our case was the identical disorder, but with a new type of inhibitor complication [294]. A few months later, Biggs, Douglas, Macfarlane and colleagues [51] published an English case, and from the name of the patient, coined the term 'Christmas Disease'. Koller's name for PTC is 'Factor IX' [270].

An earlier lead was provided by a number of observers, particularly the Argentinian, Pavlovsky [370], namely, that the mixing of two 'hemophilic' bloods, from certain cases, resulted in correction of the clotting-times, both in vitro and in vivo. Very interestingly, some of the classical 'hemophiliac' families (e.g. Von Tenna) extensively studied in Europe have turned out to be, not what has recently been accepted as hemophilia, but the similar PTC-deficiency (or Christmas disease). To avoid the confusion which has resulted, Dr. Koller [270], of Switzerland has proposed a revision in nomenclature, namely, (1) Hemophilia A (the usual type); (2) Hemophilia B (PTC-deficiency); and (3) others (see below). Another interesting recall is the obtaining of an 'antihemophilic globulin' from serum (rather than plasma) by the Dutch workers, Bendien and Van Creveld [39], a year or two ahead of the Boston (Thorndike) group [368]. Unlike AHG, PTC persists in the serum, since it is not completely consumed in clotting. The present author suggests, therefore, that the Dutch workers were really dealing with hemophilia B and that their 'globulin' was a PTC preparation.

(c) PTA-deficiency (Hemophilia C). R. L. Rosenthal and co-workers [413,412,414] have uncovered cases allegedly lacking a third plasmatic thromboplastic factor (PTF), which they call plasma thromboplastin antecedent (PTA). We have confirmed the defective prothrombin

consumption in one of their cases.

(d) 'Fourth-factor' - deficiency (Hemophilia D). Spaet, Aggeler, and Kinsell [446] encountered another type of case with "a possible fourth plasma component".

(2) 'Hageman Factor'. Ratnoff and Colopy [401] observed three patients with prolonged bleeding times but without significant hemorrhagic symptoms. Their studies suggest yet another factor, the chief action of which is failure, compared with normal plasma, to accelerate the clotting of platelet-poor plasma, obtained from normal blood, centrifuged at high speed without anticoagulant. They gave it the name 'Hageman factor', after the first patient, and obtained correction of the defect with a fraction prepared from normal $BaSO_4$-adsorbed heated serum.

(3) 'Factor X'. Duckert, Koller and colleagues [108], investigating sera, from various pathological conditions, in the thromboplastin generation test, concluded that still another factor must be recognized ('Factor X'). It is said to be deficient particularly in sera from cases with hepatitis, or persons under treatment with a dicumarol derivative (Marcoumar).

5. INHIBITOR PROBLEMS

a) Inactivators of thrombin. Quoting Seegers [425], the following mechanisms participate in the removal or neutralisation of thrombin (the comments are those of the present reviewer):

"1) Thrombin may be adsorbed on fibrin." Fibrinolysin can release it [268].

"2) Large amounts of thrombin are neutralized by antithrombin. This antithrombin does not require heparin for its action". This is

the classical antithrombin of serum or plasma [505,506]. It acts over a period of time in a progressive [190] manner and is limited in degree, i.e. a given amount of antithrombin can neutralize up to a definite limit of thrombin. Morawitz [344] used the term 'metathrombin' for the inactive product which thrombin forms with the antithrombin of serum. His claim for partial re-activation by treating serum with acid and alkali was confirmed by Gasser [184]. Astrup and Darling developed a method of assaying antithrombin [21], which they later [22] distinguished from the 'thrombin inhibitor' which heparin forms with some labile plasma component (co-inhibitor). They state [22] ..."while normal antithrombin seems to combine with thrombin to form an undissociable compound, the compound between thrombin inhibitor and thrombin, as well as the compound between heparin and thrombin coinhibitor, seems to be highly dissociable." That fat solvents can inactivate antithrombin was noted in the early literature, chloroform especially being used for this purpose [351], although it has the disadvantage of also destroying thrombin [476]. Seegers et al.. [433a] have done many experiments with ether, especially to remove this antithrombin from defibrinated plasma. Sternberger [456] used alcohol and has obtained some very provocative results [457]. Fibrinolysin will not work [476].

"3) Heparin co-factor, together with heparin, interferes with the reaction of thrombin and fibrinogen". This will be discussed further in a subsequent paragraph (p. 52).

"4) Antithrombin-accelerator activity arises during the clotting process and also neutralizes thrombin". Seegers' [425] new agent

(AA = "antithrombin-accelerator") refers to the antithrombin demonstrable after ether extraction of defibrinated plasma. To it is attributed the disappearance of thrombin formed therein after activation of the prothrombin by calcium, (lung) thromboplastin, and (platelet-) AcG, the latter reagents not being the source of the alleged AA factor. Further (Seegers) [425]: "There is an inhibitor of the antithrombin-accelerator action which we call antithrombin-accelerator inhibitor". AA-inhibitor is removed by $BaCO_3$ adsorption and is recoverable in the citrate eluate. These new ideas of Seegers are highly provocative, particularly in regard to his suggestion that AA-inhibitor is proconvertin or "associated with proconvertin in partially purified materials".

b) Heparin [242,382,121,151,22]. It is now generally agreed that little if any heparin is present in the circulating blood normally [249,61]. The protamine titration method [250] of measuring 'heparin-like' activity in plasma is subject to questions of interpretation, but does agree with anticoagulant tests, e.g. prolongation of clotting-times of whole blood [15] or recalcified plasma, routine in author's laboratory, p.107, when heparin is known to be present. This is true both in vitro and in vivo. In vivo, however, heparin may be removed from the circulation by (a) renal excretion [254], (b) metabolic alteration, or (c) a heparinase enzyme [248,252]. Chemical extraction and assay of the metachromatic color reaction of heparin with azur dyes are the most convincing methods [342]. They have recently been used in conjunction with paper chromatography [36]. Wilander's work [501] concerning the origin of heparin from the tissue basophils or Ehrlich mast cells is now generally accepted. These mast cells appear to break down and release

significant amounts of heparin (proved conclusively by chemical extraction) in certain pathological conditions, particularly those involving an anaphylactoid type of reaction [255]. They also yield histamine [195], which probably forms a complex with heparin [403]. Rocha e Silva [407] implicates a fibrinolytic enzyme activation and finds histamine release to be an accompaniment. Hyperheparinemia can cause bleeding disorders [448].

c) Heparin co-factor (heparin-complement) [242]. Fitzgerald and Waugh [164] have recently fractionated co-factor from Cohn's plasma 'Fraction I'. Their tests extend the work of Astrup and Darling [22] and do not support Klein and Seegers' [268] idea of "interference with the thrombin-fibrinogen interaction" (also Glasko and Ferguson's [190] 'immediate' antithrombic action) but rather point to a definitely time-consuming reaction. They conclude that heparin plus cofactor inactivates clotting by removing thrombin by direct combination. Thus, the reaction is 'progressive' and 'limited'. Further, "complications arise since reaction velocity, specific capacity of cofactor (units T/mg. C) and extent of reversibility (using protamine) are functions of cofactor concentration". These authors [164] suggest that cofactor does not occur, as such, in plasma — "but that heparin cofactors in effect represent altered normal antithrombins".

Some of the author's work with heparin and 'cofactor', etc., will be presented in this thesis. We shall not review the chemistry of the heparins nor the modern work on paritol, treburon, thrombocide, sulfonated dextrans, and other synthetic heparin-like substances, of current interest, particularly in the search for a cheap heparin-substitute.

d) Specific Inhibitors of individual clotting factors. These factors being protein in nature for the most part, 'antibodies' to individual clotting factors, considered a priori, are a possibility, which recent investigations are beginning to support with experimental facts. Such inhibitors are particularly significant when they can be demonstrated and shown to contribute to the bleeding tendency in clinical cases [453].

(1) Antiplatelet factors, causing thrombocytopenia and interfering with clotting and hemostatic functions, are discussed on pp.63-64 . Normal and pathological factors, which specifically inhibit one or other of the various components of the thrombin-forming system, are beginning to receive serious consideration, as follows:

(2) Anti-AHF and anti-PTC. The present author and associates [152] presented a paper at the September 1955 Meeting of the American Physiological Society and a definitive publication will appear shortly. By use of the prothrombin consumption test on specifically deficient substrates (lacking AHF or PTC, respectively) to which mixtures of normal and patient plasmas were added, we assayed the anti-AHF and anti-PTC inhibitors in 8 cases with hemorrhagic disorders. The routine tests on these cases showed prolonged clotting-times, but normal prothrombin time (Quick test). In 5 cases with primary hemophilia, the anticoagulant could not be demonstrated by simple clot delay on mixing with normal blood or recalcified plasma, but required the specific anti-AHF test. One hemophiliac bled to death in 18 days following a tooth extraction, despite a record 400 transfusions with whole blood, plasma, and antihemophilic globulin. We could demonstrate removal, by the potent inhibitor, of the transfused AHF faster than it could be supplied. In all five hemophiliacs the acquired inhibitor was specifically anti-AHF, whereas in the two PTC-deficients, it was anti-PTC. The eighth case was 'idiopathic', in a female,

and showed a very high titer of anti-AHF, as well as an apparently high titer of anti-PTC. The last might be questioned on technical considerations.

Inhibitors of the above type have been reported in a very small minority of cases of hemophilia; PTC-deficiency; certain allergies; some systemic collagen diseases [175,453]; and questionably associated with pregnancy. History of previous transfusion therapy can usually be elicited. In one of our PTC cases the evidence was very clear that transfusions led to the recurrence of high titers of the inhibitor. In this case, and in the 'idiopathic' female, electrophoretic analysis revealed an abnormal increase in serum gamma globulin. This is further evidence that the inhibitor is acquired by some unusual type of 'immunity reaction'. However, the result, in causing transfusions to become harmful instead of helpful, complicates the clinical control of the bleeding problem.

(3) Anti(pro)convertin (Factor-VII inhibitor). Wagner et al. [487] have obtained certain fractions from normal dog plasma which specifically inhibit active convertin. Jürgens [259] has prepared a similar agent from normal human serum and suggests that it has certain properties in common with plasma, and serum, antithrombin.

(4) Anti(pro)accelerin (Factor-V inhibitor), has been reported by Hörder [223] in a case with a bleeding disorder. It is said to be 'lipoid-like' and to cause the factor V deficiency which explains the bleeding syndrome in the case cited.

(5) Antithromboplastin, (A), in the sense of a circulating inhibitor, of added tissue thromboplastin, in cases with a bleeding tendency, is suggested in several accounts referred to on p. 209 of the new text by Stefanini and Dameshek [453]. The present author suggests that the positive

identification of a genuine antithromboplastin must fulfil certain criteria:

(a) it must specifically inhibit added tissue , i.e. 'complete' thromboplastin, e.g. in the one-stage prothrombin time (Quick) test,

(b) there should be no alternative explanation possible on the basis of inhibition or lack of some other factor in the thrombin-forming system.

Hougie [226] has already pointed out some of the interpretive difficulties. for instance, if the inhibition is demonstrable only at high dilutions of the added thromboplastin, it might be due merely to lack or inhibition of the plasmatic thromboplastic components (AHF, PTC, etc.). In fact, the test might be no more, in essence, than the performing of the partial thromboplastin test of Langdell et al. [282]. These authors, indeed, use it to assay the lack of AHF. When Fantl and Nance [120] investigated an Australian woman and reported inhibition of human brain thromboplastin, but not that of other species, they might simply have been observing the influence of dilution on two different reagents which did not have true equivalency of thromboplastic potency in the first place.

Among the several cases cited [453], the patient tested by Chargaff and West [81] and later restudied by Conley et al. [92] might possibly fulfil the above-mentioned criteria.

Antithromboplastin, (B), in the sense of Tocantins et al. [474], refers to an alleged plasma factor for which the following claims are made:

1) it is a constituent of normal (platelet-free) plasma,

2) it inhibits added cephalin or tissue thromboplastin, but requires a special series of minimal plasma dilutions in order to demonstrate and assay its effects,

3) it is less effective in the presence of wettable surfaces or after the blood has clotted,

4) it is somewhat species specific and does not affect the thromboplastic action of Russell's viper venom.

5) it can be concentrated in certain plasma fractions and in lipid-rich tissue extracts. Both these points were confirmed by Overman [358,359].

6) it is increased in cases of hemophilia [475],

7) it is also increased in post-irradiation hemorrhage [471].

Dr. Tocantins is a very able and careful worker, but there is now considerable doubt as to the significance of the data under discussion. One group [196] raises many objections, including questions as to the control of ionic strengths in the limited-dilution experiments.

e) Summary of the inhibitor problems and their clinical significance.

From the foregoing brief review it is evident that the inhibitors are a little known and difficult area in the coagulation field. More knowledge is needed because of their importance in occasional clinical cases. Valid information has begun to accumulate as the result of the devising of specific testing techniques. In some cases, the diagnostic question is settled. The therapeutic goal is elusive, however. Adrenal cortical hormones failed in an extensive trial on two of our clinical cases. The relationship of inhibitor appearance to transfusions necessitates a searching review of this form of therapy. There is need for more rapid advance in the difficult field of fractionation of the

individual clotting factors, from the blood in practical preparations, which can be used for rational replacement therapy.

BLOOD PLATELETS

A. HISTORICAL

Several careful microscopists in the early nineteenth century must be credited with the discovery of the blood platelets, although they were ignorant of their true nature and functions. Most of these workers are cited in a 1934 review of "the history of haematology" by Sir. Humphrey Rolleston [408].

Alexander Donné (1842) [106] termed them 'globulins' (a term still used in the French literature), but probably confused them with fatty particles of the chyle. Addison (1841), possibly Wagner (1842), and certainly Andral (1843) [17] observed them in commencing clotting of the blood. Andral thought them to be 'fibrin molecules', as did Fr. Simon. G. Zimmermann (1848-60) [516,517] also cited Gerber's 'free nuclei' and Fr. Arnold's 'Elementärkorperchen', as well as Simon's term 'Kügelchen'. Zimmermann used Simon's technic of receiving blood into potassium ferrocyanide and delayed clotting enough to permit investigation of "the supernatant, still more or less turbid, fluid". He also could "receive the blood into neutral or carbonic acid salts". In slowly clotting bloods, he clearly noted the 'little bodies' (kleine Körperchen) before fibrin appeared and described the latter as forming homogeneous masses or threads, often radiating from the 'little bodies' or, in some cases, from leukocytes. He concluded in favor of the idea that "...these little bodies already exist in the circulating blood". As they resisted ether and warming, Zimmermann showed they were not fatty globules, but an organic (vesicular) formation ("organische Bildungen

mit Inhalt gefüllte kleine Bläschen"). Zimmermann also studied birds and amphibia and described the nucleated cells (distinguishable from leukocytes, but confused with erythrocytes), which were termed 'thrombocytes' by later writers [100]. He discussed their formation in terms of Schwann's "cell theory" and suggested a lymphatic origin.

In 1865, Max Schultze again noted them in human bloods, as did Vulpian (1872) and Riess (1872), who thought they were remains of disintegrated leukocytes. In 1874, Wm. Osler [357] gave another classical description, which distinguished them from bacterial micro-organisms. In 1877 Hayem [213,214] called them 'hématoblasts' or the 'third' (formed)- element of the blood, and insisted that they were precursors of the erythrocytes, a view which he maintained as late as 1923 [216]. While much of the confusion of platelets with the other formed elements may be blamed upon poorly controlled observation in vitro, two more recently identified phenomena may be suggested as sources of error: (1) living leukocytes can lose fragments during observation (dark-field) on glass slides in a shed blood drop [122]; (2) erythroplastid [116] formation can occasionally be observed, under similar conditions from certain erythrocytes or their precursors.

Figure 8 shows one of a series of 14 photomicrographs, taken by a former colleague (Dr. P. H. Ralph) over a 2 hr. period in an observation of an erythroplastid forming from a bone marrow erythroblast, in a case of Hodgkin's disease.

Bizzozero's [54] work was most significant. He described platelets in the circulation in the mesenteric vessels of rabbits and guinea pigs, and later in the intact bat's wing. He demonstrated their adhesive quality, their participation in thrombi, and their role in the coagulation of the blood.

Löwit [311] tried hard to prove platelets to be mere artefacts, e.g. leukocyte fragments, but Bizzozero's data settled the controversy. Eberth and Schimmelbusch (1886) gave a classical description [113] of experimental thrombosis, including the role of the platelets and of fibrin, in the transilluminated mesentery.

J. H. Wright [510] identified the origin of the mammalian blood platelet from cytoplasmic fragments of the megakaryocytes of the bone-marrow. He also showed the phylogenetic relationship to the thrombocyte of the lower animals, which serves similar physiological functions in these orders. Dr. W. H. Howell showed me his beautiful tissue preparations obtained for a 1937 paper [241], in which he stated: "platelets represent a solid secretion from a unicellular gland, the megakaryocyte". This is a good way to emphasize the conception of a dual nature of platelets, namely, partly like a cellular element, and partly as 'amorphous' material contributing to the chemistry of clotting, etc. [469].

1. PLATELETS IN THE BLOOD UNDER NORMAL AND THROMBOTIC CONDITIONS.

Many workers have confirmed and extended the observations of Bizzozero [54] and Eberth and Schimmelbusch [113]. Several laboratories have shown fine cinematograph films of the circulation in small vessels, including platelet observations, e.g. those of (1) M. Knisely [269] (formerly of Chicago, now at Charleston, S. C.); (2) the research team at the Hoffman-La Roche Swiss laboratories; (3) the Boston University group under C. P. Fulton and B. R. Lutz [41].

In vivo, platelets are tiny little discs, which appear lenticular or "batônnet-like"* when viewed edgewise. Endothelial injury causes

Footnote: * Few workers have bothered to check the term "batônnet" as originally used by Aynaud [24]. H. Rosenthal in H. Downey's "Handbook of Hematology" [111] says "baton"-like and the latest note of the Boston workers [41] uses "bayonet"-like. Actually, Aynaud compared this platelet appearance to the little stick with tapered ends which is used in the childish game that the English call "tip-cat", the Americans "mumbly peg", and the South Africans "kennetjie".

leukocytes and platelets to 'marginate' from the axial stream to the 'skimmed' plasma layer closest to the blood vessel wall. Platelet plugs can be seen to form and seal off microscopic holes in an injured vessel, particularly in venules. The platelets adhere individually and to other platelets. They also break away one by one or in clumps of varying size. These may be arrested, temporarily or more permanently, further along the course of the circulation.

In observations on the formation of cellular thrombi in the frog mesentery, on pricking the capillary endothelium with a microneedle (Kite-Chambers micromanipulator), the author [127] noted the significance of rate of blood flow. In slower rates of flow, all the corpuscular elements enter into the thrombus formation and the red cells, being the most numerous, largely preponderate. In more rapid rates of flow, it is the stickiest elements which have the best chance of remaining adherent. Hence the distinction between 'white' (thrombocyte or platelet) thrombi and 'red' or 'mixed' thrombi, long recognized by pathologists. Hayem's (1889) term 'clou hémostatique' and the German 'Blutstillenden Keil' are highly descriptive (p. 82 of Quick) [383]. Wharton Jones (1851), Mantegazza (1869) and especially Zahn (1872) [513] preceded Bizzozero [54] in demonstrating the importance of white thrombi for controlling hemorrhage after vessel injury.

2. VISCOUS METAMORPHOSIS AND PLATELET ADHESIVENESS.

The thrombotic platelet mass later evinces a hyaline or granular appearance, for which the term "viscous metamorphosis" was coined by Eberth and Schimmelbusch [113], and later used by J. H. Wright and G. R. Minot [511]. At one of the Macy Conferences [509] Helen P. Wright reviewed this topic and described her quantitative approach, based upon rotating the platelet-containing fluids in glass tubes for definite time periods and comparing the platelet count (hemocytometry) with a vaselined-tube control. She reduced 'platelet adhesiveness' by heparin and chlorazole dyes, in vitro, and by dicumarol in vivo. Conversely, adhesiveness was increased after operations or parturition, at periods when the platelet count was also elevated and many young forms were circulating. Similar findings were reported for the thrombocytosis following injections of adrenalin or of pyridine. Splenectomy gave similar results, but now there was little further effect from pyridine. Moolten and Vroman [343], with an improved technique, failed to confirm Helen Wright's suggestion of an increased platelet adhesiveness in hemophilia. Her idea, in this disease, is also contrary to observations of many other workers. Dr. Wright's interpretations included the following (with the present reviewer's parentheses): ..."the factors which appear to maintain the various (formed) elements in suspension are first, their surface charges (p. 174 of Tocantins) [469] which are of the same (electrical) sign; and second, their constant motion, both as a mass and relative to one another". ..."It is believed, moreover, that the surfaces of the formed blood elements carry variable amounts of adsorbed water and that this helps to render them stable in suspension as well as augmenting the viscosity of the blood" ... "their surface reactions may be in part dependent on

some of the metabolic activities of the cell". The platelet surface may be modified by substances normally (fibrinogen, globulin) or abnormally (immune globulins of anti-platelet sera) present in the blood. Adhesiveness must also be related to alteration in the wettability or stickiness of injured vascular endothelium [419]. It may be recalled that Roskam [415] collected many data leading to the suggestion that platelets are surrounded by a firmly adsorbed protein film, to which many of their properties may be due. That this is not necessarily fibrin or fibrinogen, however, is indicated by the normal platelet adhesiveness, etc. in a case of congenital afibrinogenemia studied by Pinniger and Prunty [376], and confirmatory observations by Alexander et al. [10] and others [296], cited [7].

3. PLATELET 'AGGLUTINATION' (NORMAL).

According to Aynaud [24,25,26,27] platelets suspended in plasma may be agglutinated by a wide variety of substances, e.g. gelatine, egg albumin, peptone, gums, lecithin, heavy metals, dyes, and surface-tension lowering agents such as ricin, sod. taurocholate, and saponin. The peptone plasma experiments were particularly interesting as showing that platelet agglutination could be independent of clotting. Tocantins [469] reviewed many of the older papers dealing with platelet agglutination under various experimental conditions and in numerous clinical disorders. A number of modern workers have restudied some of these problems on well-washed (? plasma-free) platelets, especially aided by the silicone technique (see p. 120).

Copley and Robb [93] observed that <u>washed</u> platelets could be agglutinated by adding plasma or serum, especially the latter, and also by 'purified' preparations of globulin, prothrombin, thrombin, placental

tissue juice, and plasma with a high concentration of heparin (1). This last finding was unexpected and in conflict with the data of C. H. Best and colleagues [45]. These Toronto workers inserted a glass chamber between an artery and vein in an experimental animal and observed platelet agglutination and adherence to the flat-surfaced glass chamber, microscopically. The cinematograph film of these observations was re-exhibited at the Macy conferences (1949). Heparin caused a great reduction or even absence of observable platelet agglutination. Dr. Best, and others [161], believe that some heparin preparations contain impurities which could explain the findings of Copley et al.

4. PLATELET ANTISERA, PLATELET GROUPS, AND THE THROMBOCYTOPENIA PROBLEM.

Following the 1905 suggestion of Marino, Bedson [33,34,35] injected rabbit platelets into guinea pigs and obtained a platelet antiserum which caused thrombocytopenia and endothelial (capillary) damage when injected into 'non-sensitized' animals. Ackroyd [1] has recently reviewed the problem of "platelet agglutinins and lysins in the pathogenesis of thrombocytopenic purpura". He includes:

a) the 'abnormal splenic secretion' theory of Frank (1925);

b) the efforts of Troland and Lee (1938) and others to obtain spleen extracts ('thrombocytopen') allegedly capable of reproducing the human thrombocytopenic condition in experimental animals;

c) the demonstration by Evans, Duane, et al. (1949-51) of a 'thrombocyte-agglutinating factor' in the sera of some ITP (idiopathic thrombocytopenic) patients.

d) several reports that transfused platelets survive for shorter periods in ITP cases;

e) Harrington's [207] convincing experiments in which, by injecting

himself with plasma from an ITP case, he lowered his platelet count to dangerously low levels and was fortunate to recover;

f) confirmatory repetition of this, extremely carefully conducted, by Stefanini et al. [454];

g) Tullis' [478] finding that 9 out of 18 sera from ITP cases caused agglutination and lysis of normal platelets in the presence of complement.

h) demonstration, by both Harrington's [208] and Stefanini's [455] groups, that platelet agglutinins could occur in some patients after pregnancy or transfusions; or

i) even in some individuals who have never been pregnant or transfused [455], and

j) the confusion due to demonstrations [451,502] of fall in platelet count following transfusions of normal blood, etc.

This whole problem is beset with technical and interpretative difficulties, but the following conclusions represent current thinking:

1) antiplatelet agglutinins and lysins occur in humans, sometimes without, but usually with, thrombocytopenic purpura;

2) there are platelet 'groups' or 'types', analogous to those encountered with red cells;

3) 'acquired' cases of thrombocytopenia often have a demonstrable cause of agglutinin production. Ackroyd [1] has made some very clear-cut studies of drug-induced (sedormid; quinidine) thrombocytopenias, showing that both drug and specific antibody are needed for the platelet-destroying action;

4) complement is involved in the platelet lysis, as it is in hemolytic and bacteriolytic immunity reactions.

5. IN VITRO 'ALTERATION' OF BLOOD PLATELETS.

In 1873, Ranvier [397] observed the microscopic changes of platelets and formation of fibrin in a drop of shed blood. He described alterations in the shape of the platelets and in "the refraction of their granulations". However, his most significant observations were: (1) fibrin needles are often seen first at the edges of platelets or platelet debris; (2) platelet 'granulations' occur at the intersecting nodes of the fibrin network.

Aynaud (see p. 62) made a classical series of studies of platelet alterations in vitro, which have largely been neglected by more recent workers in this field. However, they formed the chief background for the present author's observations [124], which are embodied in this thesis. With extraordinarily skillful use simply of paraffined equipment, the centrifuge, and the microscope, Aynaud described the sequence of alterations from the original disc or batônnet (v. p. 60) form to the spiculated 'stellate', swollen and granular (disintegration) forms. The controlling factors studied by this worker include (1) surface, (2) temperature, (3) dilution of plasma (with citrate-saline), (4) the decalcifying (citrate, etc.) anticoagulants, and (5) many experimental additives (see previously).

Fonio and Schwendener [171] used $MgSO_4$ plasma to observe various platelet alterations, some of them bizarre.

Zucker [518], confirming unpublished observations in the present author's laboratory, agrees that even siliconed surfaces, which are our best non-wettable surface to date, fail to prevent, but merely retard, morphological changes in the platelets held in citrated plasma. The decalcifying ion-exchange resins, e.g. Amberlite or Dowex '50' do not

help. In fact, most recent workers find these unsuitable for platelet recovery and prefer the strong Ca-binding 'chelating' agent, EDTA (Sodium ethylene-diamine-tetra-acetate, also called Sequestrene or Versene), ref. Tullis [479].

$CaCl_2$ was not found to have much influence on the swelling, etc. shown by washed (x3) platelets in buffered saline (Milstone [340]). Whether or not thrombin can produce platelet alterations, as claimed by Zatti [515] and Fonio [169], is still an unsettled question (Zucker [518]), but is probably true and this may account for some new facts, such as low serotonin assays in severe clotting disorders (Dr. J. H. Lewis, the author, and M. B. Zucker, unpublished data).

B. PLATELET FUNCTIONS:

For the purposes of this thesis it is not necessary to review some platelet functions (e.g. metabolism; 'platelet loading'; etc. [469]). Included are those concerned with hemostasis and allied functions.

1. RELATION OF PLATELETS TO HEMOSTASIS, ETC.

As noted previously, platelets play a key role in thrombosis and embolism, besides acting as a defensive mechanism against blood loss. Pure platelet or 'white' thrombi are relatively rare and the usual thrombus is 'red' or 'mixed', with the bulk and numbers of erythrocytes preponderating. Moreover, clotting is initiated about the time (Erkelens' thesis) [118] that platelets adhere and begin to disintegrate. Hence a thrombus is also rich in fibrin, which forms a supportive meshwork and buttressing that adds greatly to the hemostatic effectiveness. In the clinical 'bleeding time test' (e.g. methods of Duke [109] or Ivy [244]), defects of platelet or vascular function, rather than of clotting function, are causative of prolongation of the bleeding.

The bleeding time is not infrequently normal, or nearly so, in cases of hemophilia, for instance. Conversely, the whole blood 'clotting time' test (e.g. Lee-White method [286]) is often normal in thrombocytopenics and primary vascular diseases.

Since hemorrhagic disorders [453] can accompany (1) vascular abnormalities, (2) platelet deficiencies, quantitative (thrombocytopenic) or qualitative (thrombocytopathic or thrombasthenic), or (3) coagulation inadequacies, it is evident that all three normally share in the defensive hemostatic mechanisms.

Vascular anomalies are beyond the scope of the present thesis. Just how important the contributions of the first two factors may be, is emphasized by known cases of afibrinogenemia [7], who lack the essential clotting mechanism. These cases do have bleeding problems but often manage much better than, say, a severe hemophiliac. May it not be that certain factor(s) of the thrombin-forming mechanisms are also necessary for some platelet functions?

Serotonin. Marjorie B. Zucker [518] performed experiments on the formation of the hemostatic plug which appears after cutting a (small) blood vessel in the rat. Noting a frequent vasoconstrictor effect at the time of platelet deposition, Dr. Zucker investigated this additional phenomenon and used an ear-vessel perfusion technique for bioassay of the 'platelet vasoconstrictor' factor. The concensus of a number of workers [367] on this problem is that disintegrating platelets liberate 5-hydroxytryptamine [399] which reacts with some plasma factor to become 'serum serotonin' (prob. 5-hydroxytryptamine creatinine sulfate) previously recognized by Rapport et al. [400]. We have some unpublished data showing low serum serotonin (platelet vasoconstrictor) values in cases with

hemophilia and other severe clotting disorders. The suggestion (p. 66) is that thrombin (or some factor in the thrombin-forming system) is needed for platelet 'release' of 5-hydroxytryptamine.

2. THROMBOCYTES.

The thrombocytes serve the hemostatic functions in lower animals, e.g. birds, fish, reptiles, amphibia, etc., much as the platelets do in mammals [185,189]. That they are not quite as efficient, in some ways, is suggested by the relative ease of preparing incoagulable, or very slowly clotting, plasmas from the thrombocyte-possessing bloods, by careful collection and centrifuging, perhaps even without use of anti-coagulants. This is very difficult in mammals, even with use of anti-coagulants, because of the ease of platelet breakdown. There are species differences, however, and equine blood has been used by a number of investigators [24].

3. PLATELETS AND CLOT RETRACTION.

a) Clot Retraction. In 1951, O. E. Budtz-Olsen [70], of Cape Town, published an outstanding monograph on this subject, with a very extensive bibliography. His theoretical approach may be quoted verbatim:

...."The blood clot consists of a three-dimensional network of fibrin threads"....."The serum is held in this network by capillary forces. When the serum artifically is pressed out of the meshes the network collapses and does not again expand when immerged in serum, due to adhesion between the individual fibers. This can be seen by manipulation of the network under the microscope. The capillary force holding the serum in the clot might conveniently be expressed by this formula: -

$$\text{Force:} \quad \frac{2\,y\,\cos\theta}{d\,g\,r}$$

where y: the surface tension of the serum

θ: the angle of contact of the serum with the fibrin which is an expression of the wettability of fibrin.

d: the density of the serum.

g: the force of gravity.

r: the width of the mesh.

which is simply the formula for any fluid in any capillary tube". Consideration of the force of gravity by this author is a new contribution....."When the clot is placed in rigid structures like glass tubes gravity tends to draw the serum out of the fibrin network against the capillary forces holding it back; an artificial "clot retraction" may thus be produced". To avoid this, Budtz-Olsen designed an ingenious and simple method ("the suspended clot method"). Under constant (37°C) temperature conditions, 5 ml of blood (or test mixture) are floated, free from the influence of gravity, in an inert oil mixture. The serum expressed during retraction of the clot floats to the surface of the denser oil mixture and can be measured. "It is shown that the size of the meshes in the fibrin network, and therefore the capillary forces holding the serum in the clot, depends mainly on the concentration of fibrinogen in the plasma"...."It unavoidably follows that clot retraction is inversely proportional to the fibrinogen concentration".... ...."of major influence on clot retraction is the volume of red and white cells in the blood. These cells play a passive, obstructive role, and it is demonstrated experimentally that an almost linear, inverse relationship exists between the volume of packed cells and the clot retraction".... "correction can be achieved by simply adding the haematocrit to the observed clot retraction".

...."Rigid control of the temperature is .... essential in all work on clot retraction".

...."Character of the clot, its firmness, rigidity and elasticity .... depend nearly exclusively on the fibrin concentration and the occurrence or absence of fibrinolysis. They are completely independent of platelets. ....Fibrinolysis and clot retraction are independent processes."

....Clot retraction is due to a special function of the platelets.... "viscous metamorphosis leads to the formation of long strands or pseudopodia of cytoplasm connecting individual platelets or platelet-masses. These strands subsequently contract producing a patchy fusion of platelets throughout the clot. As the platelets are adherent to the fibrin network of the clot the contraction of the cytoplasmic strands and the merging of the platelets result in a contraction of the whole fibrin clot with a squeezing out of the serum and a few of the red and white cells enmeshed in the fibrin network".

...."From the delicate platelet-mechanism producing clot retraction it would be expected that the force exerted by the process is very small. By a simple experiment (p.79)[70] this assumption is shown to be correct; the force (about 19 mm H_2O) is so insignificant that it becomes extremely unlikely that clot retraction serves any physiological purpose in mammalian life. It can certainly not draw together the walls of even the smallest vessels".

...."In the crab, clot formation and retraction take place in the complete absence of fibrin and are entirely cellular functions"...."It is suggested that the process is a redundant phylogenetic relic of no importance in higher animal life".

A critical review of Budtz-Olsen's work will not be attempted here,

but the present writer would raise some questions, e.g.

(1) platelets don't retract clots formed from fibrinogen with thrombin, but only with 'thrombin-forming' coagulant mixtures. The present author's experience is otherwise.

(2) clot retraction cannot draw together small vessel walls. Tocantins [468], who is cited by Budtz-Olsen, collapsed cellophane tubing by clot retraction.

(3) fibrinogen concentration is the chief determinant of size of fibrin mesh and hence of capillary forces. There are many discrepancies between the ineffectiveness of certain agents in modifying Budtz-Olsen's clot retraction measurements and data of physico-chemical and electromicroscopy studies which indicate they can greatly alter the fibrillar composition of the fibrin clot.

(4) Can fibrinolysis be critically ruled out in connection with clot retraction? (see below).

(5) accepting Budtz-Olsen's worthy critical review of Glanzmann's (1918) [188] case data on "hereditary hemorrhagic thrombasthenia", there are, contrary to the South African's opinion, undoubted cases of platelet anomalies (thrombocytopathias) in which these elements are deficient in some retractor factor (? Glanzmann's "retraktozyme"). Such platelets can be isolated and, compared with normal platelets, shown unable to induce clot retraction in normal platelet-free plasma [453]. Such a case will be described in the present thesis.

b) Role of Platelets. Budtz-Olsen [70] refers to Thackrah (1819) and others whose work clearly indicates that the fact of deficient clot retraction in association with certain purpuras was known at the beginning

of the nineteenth century. Hayem [215], in 1896, was the first to correlate poor clot retraction, in purpura, with definite reduction in the number of blood platelets. Among workers who have experimentally reduced platelet counts by centrifuging plasma and carefully correlated with clot retraction data, we may mention Tocantins [467].

LeSourd and Pagniez [289], Bordet and Delange [57], Opitz and Matzdorff [356], and others [70] have recorded inability to obtain clot retraction with platelet 'extractives', but only with 'intact' platelets. In recent electron microscopy studies, Bessis and Burstein [44,43] observed adherence of fibrin filaments only to spiculated 'altering' platelets and not to 'disintegrated' forms. Glanzmann's [188] suggestion of a 'retractozyme' (enzyme-like factor in platelets) has, therefore, not gained acceptance, until recently, when Fonio [170] claimed separation of the outer 'hyalomere' from the central 'granulomere' of platelets by differential centrifugation and the identification of 'retractozyme' with the hyalomere fraction. Very recently, 1956, Magalini and Stefanini [316] claim isolation of a 'retractin' both from platelets and from blood-free tissues. Their best method is to disintegrate and extract with ether-water (65:35) and then deposit in the cold at -20°C. Thromboplastic factor is eliminated by heating for 2 hrs. at 56°C and subsequent storage at -20°C. Tests for vasoconstrictor factor (5-hyroxytryptamine) are also negative. They conclude: "it could represent a lipoid substance".

How do platelets bring about clot-retraction? The weakness of the force involved in clot-retraction is stressed by Budtz-Olsen, as by previous workers. While it is usually sufficient to detach the fibrin strands from adherence to glass, this may not be the case in some

instances. Fibrin is more adherent to collodion [220] and Tocantins [467] observed failure of retraction in such vessels. However, clot retraction is not significantly better in non-wettable siliconed tubes. Budtz-Olsen reviews much of the older literature (e.g. Fuchs [179]) on the influence of the containing vessel's surface on clot retraction. Although he cites their paper in another connection (temperature effect), he doesn't include a very interesting experiment of Lampert and Ott [279], which the present writer has frequently reproduced for the benefit of his students. Thus, if blood is allowed to clot in a wide test tube paraffined down half of its side, the retraction of the clot will subsequently be away from the glass and to the paraffin. That fibrin is less adherent to paraffin than glass was shown by Fuchs (1931), and many workers have used paraffined vessels in clotting experiments because of its 'non-wettability'. It is difficult to explain the Lampert and Ott experiment except on the basis that the glass in some way 'activates' the retraction-producing factor.

Observing certain similarities between retraction, e.g. of plasma clots containing platelets and thrombin-fibrinogen clots, which do not normally retract but can be made to withdraw from the glass tube and 'clump' when known to contain very small amounts of trypsin or fibrinolysin, Ferguson and Erickson [149] revived the idea [219] that clot retraction may be related to fibrinolysis. This suggestion is not easily dismissed by the arguments of Budtz-Olsen, whose experiments give no heed to the quantity of fibrinolytic enzyme. It may also be necessary in the most recent work [316] to present conclusive evidence to rule out all possibility of traces of active fibrinolysin being present or formed in the test systems. The present author does not insist on 'the fibrinolytic

theory', but merely believes that it should be convincingly ruled out.

This being so, it is appropriate to consider some peculiar 'retractility' of platelet 'processes'. This will be done in the present thesis, especially in terms of lipoid-water phase relations. It can be stated, with fair certainty, however, that platelets do not extend and withdraw true 'pseudopods', comparable to those in leukocytes, etc. Budtz-Olsen's data are very weak on this point. This author, however, may be referred to for a good comparison between physical 'syneresis' of certain gels and the 'clot retraction' phenomenon. The differences are numerous and support the conclusion: "...the fundamental mechanism of the two processes is probably entirely different".

4. CLOTTING FACTORS IN BLOOD PLATELETS.

a) Need of platelets for blood clotting. There was much doubt about the blood platelets being essential for normal blood clotting until Brinkhous [64], using the newly introduced siliconed surface glassware of Jaques et al. [251,288], showed that plasma recalcification clotting-times and prothrombin consumption (utilization during clotting) were delayed, the more the platelet count was reduced by centrifugation. Successful experiments in obtaining a non-clotting platelet-free plasma will be cited (pp. 120-121) in the body of this thesis. The thromboplastin generation test is discussed on p. 80.

b) Erroneous ideas. In the earlier literature, about what the platelets can contribute to the blood clotting mechanism, appear the following:

1) platelets act as a complete 'coagulant' for fibrinogen, (a) in the presence of calcium, by allegedly providing a 'tissue fibrinogen' (Wooldridge [508] 1886; Mills [338] 1930); (b) in still earlier ideas of a similar kind, platelets were not separated from the 'white corpuscles', but only from red cells (Mandl, 1842; Buchanan,

1843 [68] -- cited by Anderson [16] 1844).

2) platelets contain prothrombin (Morawitz [346] 1905; Bayne-Jones [32] 1912; Christie, Davies and Stewart [85] 1927; Fuchs [177] 1930; Howell [238] 1935 -- cited by Tocantins [469] 1938). Disproved by Bordet [55], Mills [338] and Eagle [110].

3) platelets directly activate prothrombin through their zymoplastic substance (A. Schmidt [420,421]).

4) abnormal 'stability' of platelets is the cause of hemophilia (Sahli [418] 1905; Minot and Lee [341] 1916; Morawitz [347] 1925; Howell and Cekada [240] 1926; Christie, Davies and Stewart [85] 1927; Fonio [169] 1932): See present thesis. Hayem [216] 1923, suggested that whatever is responsible for the stability of hemophilic plasma also accounts for the stability of platelets in that plasma.

c) Platelet 'thromboplastic' factor (prothrombin 'activator'). Bordet and Delange [57] 1912, implicated platelets as one source of their tissue clot-accelerating principle or 'cytozyme' and extracted a thermostable lipid material, soluble in alcohol, ether, and chloroform, but little soluble in acetone, which agreed with Howell's [231], 1912, identification of the thromboplastic factor as cephalin. Because cephalin was less potent than crude platelet (or tissue) extracts Howell [231] and Rumpf [416] believed the true thromboplastin to be a cephalin-protein (i.e. lipoprotein) 'complex'. Eagle [110] also noted that the lipid extract from platelets had only about 1/10 of the original activator potency. Chargaff, Bancroft, and Stanley-Brown [78] prepared platelets from horse blood and isolated their various lipids. A phosphatide fraction was found to contain a potent activator. They concluded: ...."present methods for the separation of phosphatides are still much

too crude to permit the isolation of compounds in a state even approaching purity. It may very well be that a cephalin containing highly unsaturated fatty acids, and, therefore, more soluble in ethyl alcohol, is the real activator of blood clotting". As in their later work on lung thromboplastin, Chargaff et al. [78] also noted that the 'defatted' material after platelet extraction was clot-inhibitory. In 1945, Chargaff [77] subjected normal human oxalated plasma to high-speed (150 min. at 20,000 r.p.m.) centrifugation and lengthened the recalcification clotting-times 3x or more. In the following year, further studies [81] added information that the 'pellet' sediment from such centrifugation had thromboplastic properties similar to those of the 'macromolecular lipoprotein' similarly obtained from lung extracts. Flynn and Standley [167] reported to the Macy Conferences (1949) that their confirmatory observations also included microscopic examination of stained films, which clearly showed "...that the pellet obtained by high speed centrifugation of human plasma is composed of platelets and, occasionally erythrocytes". Chargaff was mistaken, then, in identifying a 'plasma thromboplastin', and was really just confirming Brinkhous' (1939) observations of the effects of platelet removal on plasma clotting.

In 1935, the present author, knowing only of a short note of Haurowitz and Sladék [211], 1928, containing lipid analyses of platelets, visited the Research Laboratory of the Children's Fund, Detroit, Michigan, where a group of workers, under Dr. I. G. Macy, were performing some excellent lipid (and other) fractional analyses of erythrocytes. After preliminary work on some horse platelets, prepared for the author by the Mulford laboratories, the Michigan group completed a study on "the lipid distribution of human platelets in health and disease" [117], shortly

after the appearance of Chargaff's work. 68% of the total platelet phospholipid analyzed as cephalin and it was computed that, if this were all liberated, it could furnish 100 ml of blood with some 5-10 mg of cephalin. This could be very significant in the light of the quantitative clotting data of Spagnol [447] and of Ferguson [129].

d) Platelets and the activation of prothrombin. Eagle's [110] data were as follows:

(1) platelets (like cephalin) can participate in the activation of prothrombin (cf. [328]) to thrombin. Since Eagle's prothrombin activated slowly (about 1 hr.) with $CaCl_2$ alone, he concluded that "platelets and cephalin accelerate coagulation by increasing the rate of thrombin formation, without affecting the quantity produced". However, Eagle may not have tested sufficiently small amounts. His weakest cephalin (0.0001 cc. of 0.1% suspension) does not quite reach '100%' activation in his Fig. 3 [110].

(2) Prothrombin prepared from hemophilic plasma and compared with a similar preparation from normal plasma showed, in the presence of a concentrated platelet suspension, considerably slower thrombin formation, but a normal (100%) eventual yield. With calcium alone, Eagle claimed ultimately 'normal' thrombin yields (actually his curves, Fig. 1, ref. [110] show 80-90%), but very slow rates, particularly in the hemophilic. Simple plasma recalcification clotting-times showed that hemophilic platelets were as good as normal platelets in accelerating clotting, in a series of strengths of the platelet suspensions, in both (a) normal plasma and (b) hemophilic plasma. However, the stronger platelet suspension (both types) worked less well on hemophilic plasma and the weakest platelet strength did not work at all. In comparable experiments,

cephalin speeded up the clotting of normal recalcified plasmas, but had so little effect on the hemophilic (Table III, of [110]), as had previously been shown by Mills [333], that Eagle dismissed it as "unaccountably ineffective".

Addis [3] had demonstrated, in 1911, that hemophilic plasma was not deficient in prothrombin, but that its conversion to thrombin was delayed, which led him to the hypothetical suggestion of some "qualitative defect" in the hemophilic prothrombin. Addis prepared 'prothrombin' by a method similar to that later used by Eagle, and Howell [238] pointed out that both studies could be subjected to the same criticism, namely, that their prothrombin contained thromboplastin and that this might be what is lacking in hemophilia. Using his acetone method, Howell, with Cekada [240] could not find any difference in quantity or reactivity between the prothrombins of normal and hemophilic blood. Howell prepared a 'plasma thromboplastin' and found less of it in hemophilic plasma. Purified preparations (after glycerol extraction) were said to be protein-free (ninhydrin and biuret reaction) but Howell could not obtain enough material for conclusive analyses and turned to the study of lung thromboplastin, also known to be able, experimentally, to restore normal clotting-times in hemophilic plasma, independent of platelets. Howell did think that his 'plasma thromboplastin' might come from platelets and he was able to prepare active thromboplastic material from platelets, both normal and hemophilic (p. 19 of ref. [240]).

Fonio [169], by cold centrifugation in paraffined tubes was able to obtain hemophilic plasma which clotted very slowly (4-8 hrs. at room temp.), but rapidly on adding washed platelet suspension from normal blood, or less rapidly with washed hemophilic platelets.

Other early workers, including those we have dismissed under 'erroneous ideas', argued for 'more stable' or 'less active' platelets in hemophilia, but it is not profitable to go into this any further. All recent work supports the conclusion that it is not the platelet which is at fault in hemophilia, but some plasma factor (AHF) which works with platelets to 'generate thromboplastin' for participation in the activation of prothrombin to thrombin (see p. 80).

e) Platelets and prothrombin consumption. In 1939, Brinkhous [63] used the two-stage prothrombin method to assay residual prothrombin in serum, after various intervals, and thus determine 'prothrombin conversion rate'. This was much reduced in hemophilic plasma, compared with normal. It could be brought to normal, however, by adding lung thromboplastin. In the careful preparation of the plasmas, immediately centrifuging the citrated blood at low temperature, the prothrombin conversion was found to be slower than in an earlier experiment. Conversely, when hemophilic blood was kept in citrate for 24 hrs. before removing the plasma from the cells, the subsequent test showed great improvement in the prothrombin conversion, as well as in recalcification clotting-times.

Eight years later, with the advent of the silicone technique [251], Brinkhous [64] repeated efforts to obtain a platelet-poor plasma and clearly showed that, in normal plasma, recalcification clotting-times and 'prothrombin consumption' (or -'utilization') depended on the numbers of residual platelets. A platelet poor plasma became 'quasi-hemophilic', in these respects, but normal platelet-poor plasma corrected the defect when added to the hemophilic. This was not so, however, when the hemophilic plasma was also 'platelet-free'. He concluded: "in hemophilia there is a plasma factor required for platelet utilization".

In further pursuit of the prothrombin utilization problem, Brinkhous and colleagues [281] compared data obtained by the 2-stage test with the Quick 1-stage prothrombin method, in both dogs and human, each being (1) normal, (2) hemophilic, (3) platelet-poor (by centrifugation), (4) blood-clotting in silicone. While both methods revealed the defective prothrombin consumption in (2), (3) and (4), the 1-stage values were not only higher, but ran much above the original (0 time) value, even in the normal. This would "appear to be due to the evolution and persistence of the recently recognized serum factor (convertin-see p. 44) which accelerates thrombin formation". Evidently, the 2-stage test is the more reliable measure of the prothrombin.

Quick [385], in 1947, also approached the prothrombin consumption problem via hemophilia, and modified his 1-stage 'prothrombin' test, (see p. 98), by addition of fibrinogen, later substituting $Ca_3P_2O_8$ adsorbed plasma [386,387], in order to apply it to (1 hr.) serum (cf. Soulier [444]). Like Brinkhous, Dr. Quick and his colleagues showed deficient prothrombin utilisation in hemophilia and in platelet-poor (centrifuged) plasma [391]. They also studied the prothrombin consumption defect in (a) cases of thrombocytopenia [391,392], (b) a case of labile factor deficiency [390], and (c) after dicumarol [388]. In one thrombocytopenic, splenectomy raised the platelet count and restored the prothrombin consumption [392,388]. Stefanini and Crosby [452] have extended these observations.

f) Platelets and thromboplastin generation: Intermediates. A new avenue of exploration of the blood clotting field, particularly in relation to clinical bleeding disorders, was opened up recently by the work of the Oxford University pathologists in the laboratory of

Prof. R. G. Macfarlane. In 1952, Dr. Rosemary Biggs [47] observed the development of a plasma thromboplastin in the platelet-rich recalcified plasma from a rare case of congenital prothrombin deficiency. Working with Dr. Douglas and Prof. Macfarlane, she showed that three components, namely, (1) platelets, (2) $Al(OH)_3$-treated plasma (AHF; factor V), and (3) aged serum (PTC; factor VII), in the presence of calcium interacted to generate this thromboplastin. Samples of the above mixture, removed from the pre-incubate at minute intervals, are added to platelet-poor plasma (prothrombin; fibrinogen, etc.) and observed to give progressively shorter clotting-times, through a period, which is noted, before they get longer again. This is the thromboplastin generation test of Biggs and Douglas [48]. The final clot-timing resembles, in principle, the Quick prothrombin time test (p. 98), but the preincubated mixture described above is substituted for the usual tissue (brain) thromboplastin.

The Oxford group and investigators from other laboratories have applied the thromboplastin generation test system to the study of very many problems, among which we may list the following:

1) testing of platelet functions in blood clotting. The platelet suspension may be tested (a) before and (b) after activation by plasma factors [225] (see pp. 46-49), in normal subjects, in thrombocytopenias [49] and in cases with functional platelet disorders (thrombasthenias or thrombocytopathias) [1,445].
2) substances able to substitute for platelets in the thromboplastin generation test include (a) a weak tissue thromboplastin [38], (b) cephalin [354], (c) chylomicra suspensions [406].
3) diagnosis of hemophilia and related disorders [313,378]

(a) when the patient's $Al(OH)_3$ plasma gives a poor test, the other reagents all being normal, the result is presumptive evidence for hemophilia. Ordinarily, AHF persists in fresh $Al(OH)_3$ plasma, along with AcG (factor V) whereas PTC (Christmas factor), prothrombin, proconvertin (factor VII), and probably factor X, are removed by the adsorption.

(b) when the patient's aged (overnight) serum gives a poor test, the other reagents being normal, the result suggests PTC-deficiency (Christmas disease). PTC ordinarily survives in the serum along with factor VII, whereas AHF and factor V are gone, as is prothrombin (consumed) and thrombin (removed by the natural antithrombin).

4) certain circulating anticoagulants interfere with the test (Hougie and Fearnley [227], Hougie [226]). The present author and colleagues [152] have recently proved that these inhibitors are specifically anti-AHF and anti-PTC, respectively. We prefer our specific tests, based upon the prothrombin consumption technique (p. 79), because they make the distinction between the two inhibitors. The thromboplastin generation test is unable to do this. In other respects, however, it may be more sensitive than prothrombin consumption test methods. A careful comparative study of the two methods has not been made and there is, as yet, no true yardstick for evaluating the validity of <u>quantitative</u> data of these and also other (e.g. [282]) methods.

5) the question of intermediates:

(a) Biggs, Douglas, and Macfarlane [50] found evidence for participation of factor V (AcG) and factor VII (proconvertin) in thromboplastin generation from platelets. Macfarlane and Biggs [314] also showed

an increasing potency when an already active brain extract was preincubated with factors V and VII (cf. [320,319,318]). This raises considerable question as to terminology and Macfarlane et al. suggest that the term 'thromboplastin' should really apply to the product of the preliminary reactions which, with calcium, participates directly in the conversion of prothrombin to thrombin. Since Howell (p. 12), although not strictly in that author's sense, the term thromboplastin has been widely used to designate any tissue extract used in activating prothrombin. Brain thromboplastin, for instance, is the key reagent in the Quick prothrombin time test (p. 98). Brinkhous and colleagues (p. 80) distinguish between tissue thromboplastin as a 'complete' thromboplastin and cephalin or platelets as 'incomplete' or 'partial' thromboplastins. They apply these ideas in their two-stage prothrombin consumption test (p. 79) and partial thromboplastin AHF assay [65,66].

Actually, the historical term (p. 10) for the postulated activator, with calcium, of the prothrombin conversion was 'thrombokinase'. Hence, there is much merit in Milstone's [339] suggestion to re-define this term for the present purposes. Milstone's work, while confined to experimental test systems, essentially anticipated much of the basic concepts advanced by the more recent English investigators. Other American researchers, notably F. D. Mann and associates [320,318], Flynn and Coon [165,166], have pursued the idea of intermediate complexes in the thrombin-forming reactions, although not emphasizing quite the same viewpoint as the above. The distinction between thrombin generation [312,377] and thromboplastin generation is by no means clearly established

at this time, however, (see p. 86).

(b) Hougie [225] has studied the shortening of the recalcification time of platelet-rich plasmas from cases with (1) circulating anti-coagulant, (2) hemophilia, (3) Christmas disease, (4) 'dindevan' (phenylindanedione; related in action to dicumarol and tromexan), and (5) normal bloods. He concludes that there is another important intermediate at a later stage in these complex reactions. The following summary of these ideas was kindly supplied by Dr. Hougie, in a personal communication: According to Bergsagel and Hougie [40], AHF and PTC undergo a reaction with calcium to form an intermediate product (Product I). Product I reacts with platelets causing them first to agglutinate and then to lyse (Bergsagel) resulting in "a sedimentable thromboplastin complex" believed to be a complete thromboplastin. This (a) differs from tissue (e.g. brain) thromboplastin (b) in that the one-stage prothrombin time of stored and dindevan plasma, considered to be deficient in both factor V (proaccelerin) and factor VII (proconvertin), respectively, is normal with (a) but delayed with (b). This is true even when (a) is washed and re-sedimented and used in high dilution. The complete thromboplastin loses its activity on heating for 10 min. at 56°C. However, after heating for 5 min. to 100°C and re-treating with Product I, the activity is restored and may again be recovered in the (ultra)centrifugal sediment. The heat-stable component is thought to be cephalin, and the heat labile component to be Product I. Product I loses its activity after decalcification by Amberlite (ion-exchange resin) and, therefore, calcium is believed to be an essential component

of Product I and the complete thromboplastin. The last named is moderately stable in simple preparations but loses activity in serum or when citrated or oxalated. The participation of factor V and factor VII is presumed in the formation of Product I and hence of complete thromboplastin, but details of these relationships need further investigation.

(c) Hörder has studied the defect in thromboplastin generation in a case with a specific inhibitor of factor V [223], and has data on the influence of factor V upon plasma thromboplastin and hence thrombin formation [224].

Discussion on intermediates. It appears to the present author that present methods are yielding conclusions which are suggestive rather than definitive. The reagents used in the cited experiments are crude materials for the most part and this raises some questions. In Macfarlane and Biggs' [314] experiments, for instance, it was concluded that factors V and VII increased the potency of tissue thromboplastin. What, if any, is the evidence that the potencies of factor VII and factor V are not being increased? Serum contains much proconvertin as well as convertin, and pre-incubation with calcium and thromboplastin can activate the former to the latter and abolish the lag phase in the conversion of prothrombin (factor VII-free) to thrombin [295]. Similarly, a very minute trace of thrombin can activate proaccelerin to accelerin, with similar abolition of the lag phase in purified thrombin-forming systems [290]. Can the English workers state the complete absence of traces of thrombin in their test system? These are debatable matters, and the important point is that the experimental test systems leave the results open to divergent interpretations. The final answers must await more definitive

experiments. It would seem that the distinction between the thromboplastin-forming and the thrombin-forming reactions is not yet wholly established.

Conclusion. Despite a diversity of approaches and viewpoints, modern investigators in this field are providing evidence of similar basic concepts and of a common goal of enquiry. It would now seem to be established that the many factors which participate in the thrombin-forming reaction do so by a series of complex and inter-related reactions, in which the formation of certain intermediates would seem to be a logical necessity. Many, but not all, of the data can be interpreted in terms of thromboplastin generation.

g) Platelet factors related to clotting, etc. At an April 1955 Conference of the National Research Council's Coagulation Committee, meeting in Washington, D. C., W. H. Seegers [426,104] listed 11 experimentally demonstrable actions of platelets or platelet fractions in the blood clotting system. We quote his list verbatim:

"1. Platelet factor 1 (Ac-globulin like property)
2. Platelet factor 2 (Thrombin-fibrinogen interaction)
3. Platelet factor 3 (Prothrombin activation with plasma cofactor(s))
4. Platelet factor 4 (Antiheparin)
5. Clottable factor
6. Antifibrinolysin activity
7. Antithromboplastin activity
8. Clot retraction
9. Vasoconstriction (serotonin)
10. Snake venom factor
11. Ac-globulin stabilization."

Stefanini [449] includes some detail as to platelet factors in his 1953 review. Ackroyd's recent review [1] cites "platelet factors 1-4" and these are so titled and discussed in much of the current work in this field. We shall not attempt any comprehensive review of all the literature on this topic, but will present our own attempt to order the experimental facts in some logical order, with a few selected references to each category.

1) PLATELET "THROMBOPLASTIN" (PREFORMED). The best evidence for this is Chargaff's [78] preparation of 'platelet thromboplastin' as a lipoprotein centrifugate. From this he recovered 'platelet thromboplastic phospholipid' (? cephalin) in lipoidal extracts. That this is a weak and 'incomplete' thromboplastin is evident from the data of Quick [387], Ware, Fahey, and Seegers [490], Travis and Ferguson [476], and others.

2) PLATELET COMPONENT (platelet Factor 3) in generation of plasma thromboplastin. Lenggenhager [287] used the term 'thrombokinin' and Quick [387] the term 'thromboplastinogenase' in looking at this from one point of view, namely, as if the platelet component were the activator of the plasma components (see AHF = "cofactor I" and PTC = "cofactor II, etc. - p. 46). It is really semantic (or tautological), but most other workers prefer to present the problem from the opposite point of view, namely, as if the plasma co-factors activate the platelet precursor of a thromboplastin. Bergsagel and Hougie's views as to probable 'intermediates' in these reactions have been stated (p. 84). Alkjaersig, Abe, and Seegers [12] have attempted to purify "platelet factor 3" and guardedly state that it is unlikely to be a protein. In their

best preparations it has a N:P ratio of 8.4:1. No discussion of the reactions which these authors claim for "platelet factor 3" can be adequate without going into the many details of Seegers' current theories, which we do not propose to review in the present dissertation.

3) SNAKE VENOM FACTOR, according to Lee, Johnson, and Seegers [285] refers to some platelet factor, which, acting in conjunction with Russell's viper venom (itself a 'thromboplastin', with certain restrictions of potency [398,436,500]) is able to activate purified prothrombin rapidly in experimental systems. Interrelations between venoms and phospholipids is a topic with a considerable literature and we shall not explore this any further.

4) ANTIHEPARIN ACTION (platelet Factor 4); claimed by van Creveld and Paulssen [481] and recovered in a separate fraction by Deutsch et al. [104]. Other recent references [261,490].

5) ANTITHROMBOPLASTIC ACTION: ref. Jürgen's [261], thermolabile factor sedimentable in the ultracentrifuge.

6) PLATELET "ACCELERATOR" (Platelet Factor 1): was discovered by Ware, Fahey, and Seegers [490] and probably accounted for some of the data of Quick [385] and of Mann, Hurn, and Magath [320]. Travis and Ferguson [476] found only traces in well-washed platelets. Allowing for the possibility of loss due to lability of the AcG-like factor, we inclined to the view that it might merely represent some of the plasma AcG adsorbed on to the platelets. This view is strongly supported in a recent study by Hjort et al. [222].

7) A FACTOR CONTRIBUTING TO "AcG INSTABILITY": was incidentally noted by Ware, Fahey, and Seegers [490].

8) A FACTOR CONTRIBUTING TO "THROMBIN STABILITY": was incidentally noted by Travis and Ferguson [476].

9) A "FIBRINOPLASTIC FACTOR" (the present author's term, see p. 27): aiding the thrombin-fibrinogen reaction, was claimed by Ware, Fahey, and Seegers [490], and also reported by van Creveld and Paulssen [481] and by Jürgens [261]. Travis and Ferguson [476] could not confirm this, however. "Platelet Factor 2" (Seegers) denotes this alleged activity.

10) A "CLOTTABLE FACTOR" [490] refers to the 'clot-like' reaction of platelets to the addition of thrombin. The author's (unpublished) dark-field observations indicate this to be a variant of the 'viscous metamorphosis' (p. 61) of platelets and not any true clotting phenomenon.

11) PLATELET VASOCONSTRICTOR or "SEROTONIN" has also been considered separately (p. 67). Also ref. Correll et al. [94] and Humphrey and R. Jacques [243]. Magalini and Stefanini [315] could not confirm Fenichel and Seegers' claim [121] for a clot retraction effect of serotonin.

12) AN ANTIFIBRINOLYTIC FACTOR was noted by Seegers' colleagues, Johnson and Schneider [257], and confirmed by Stefanini [450]. Also see p. 212.

Conclusion. This is obviously a rich field for further investigation to determine the exact identity of the factors involved and the way in which platelets can participate in the phenomena mentioned.

h) Erkelens' thesis. A recent comprehensive thesis [118] of "the role of platelets in the coagulation process", by A. D. Erkelens (Rotterdam) has appeared in Holland. Its chief conclusion is that plasma changes

precede and are probably responsible for platelet breakdown. In platelet-free plasma, coagulation can be induced by kaolin and other particulate matter. "As a result of the secondary structural changes of the thrombocytes, after the formation of the first fibrin, a thermostable thrombocyte factor is liberated, stimulating the formation of thromboplastin. Therefore an accelerating and finishing function in the process of blood coagulation is assigned to the thrombocytes but no initiating function".

PART II. EXPERIMENTAL

A. GENERAL PURPOSE.

The following experiments seek further knowledge concerning the role of lipoids and platelets in the blood-clotting mechanisms. Blood, its platelets, plasma and plasma fractions, and a number of tissue components are analyzed with regard to the various factors which they contain and which can be demonstrated to play a part in the interactions involved in these mechanisms. The significance of the platelets and some of the other factors is considered in relation to hemorrhagic disease. In vitro test systems are devised to demonstrate the modes of action of the various factors and agents studied, many experiments being performed by this means.

B. GENERAL METHODS.

The materials used in these investigations are for the most part derived from mammalian blood, namely, dogs, humans, and beef cattle.

1. BLOOD COLLECTION.

1a) Dog. Blood samples of some 10-50 (or more) ml may often be obtained from the jugular vein of the unanesthetized dog, with minimal discomfort, using a syringe and needle technique, as in the case of humans (see 1b). For large amounts of blood it is desirable to sacrifice the animal, under intraperitoneal sodium pentobarbital (nembutal) or similar anesthesia. The carotid artery is cleanly exposed and cannulated with a siliconed glass cannula or polyethylene tubing. Controlling the vessel with a soft rubber-tipped

bulldog clamp, and avoiding tissue contact, e.g. by discarding the first few ml of blood, collection is made into anticoagulant, or otherwise, as needed, using glass (pyrex) Erlenmeyer flasks or siliconed bottles. Oxalation or citration requires 9 volumes of blood to 1 of 0.1 molar sodium oxalate or trisodium citrate. Sequestrene-Na_2 (EDTA or 'Versene') is reserved for special collections, e.g. for platelet studies, the routine being to receive 5 ml of blood into a siliconed 1 oz. bottle containing 50 mg of the powdered chelating agent; cf. p. 276 of ref.[453].

1b) Human. An extensive testing routine[172] may be performed on 20 - 40 ml of blood obtained aseptically from the antecubital vein, using a siliconed syringe with a sharp new short-bevel No. 20 needle. Arquad 2-C (2 percent solution) coating of needles (p. 283 of ref.[453]) has little additional advantage. The blood is immediately transferred into anticoagulants, as in 1a, and 4.5 ml of blood is also clotted in a small (Wassermann - type) tube held in the water-bath at 37°C. This is later centrifuged in order to recover the serum. As a routine, for prothrombin consumption determination, 0.5 ml of 0.1M sodium oxalate is added after exactly 1 hr., immediately before centrifuging.

$1b_1$) Special blood donations, e.g. normal, hemophilic, PTC-deficient, etc. are secured through cooperation of the Blood Bank and the citrated or oxalated plasmas are reserved or fractionated for particular test purposes. Frozen storage at -20°C ('Deep-Freeze') is a routine.

1c) Beef. Ox blood is obtainable at a cooperative slaughterhouse, where it is possible to arrange for suitable large scale collections

With suspension of the cleansed animal and skill on the part of the butcher, there is such a rapid flow of blood that very little objection can be raised to its slight contact with the wounded tissues. Several gallons of blood may be secured in thoroughly clean galvanized pails containing the appropriate amount of anti-coagulant. The mixture is quickly transferred to large pyrex bottles, kept chilled in buckets of ice, for transport to the processing laboratory.

2. PREPARATION OF SERUM, PLASMAS, PLATELETS.

2a) Natural serum. Whole blood is clotted in tubes (or bottles) held in the water-bath at 37°C for 1 hr. The serum is then separated from the retracting clot by suitable centrifugation.
Oxalated serum. This is described in 1b., p. 92 .
2b) Plasmas, e.g. oxalated or citrated, are secured by centrifuging the blood containing the anticoagulant in glass, lusteroid, or polyethylene tubes. The refrigerated (4°C) 'International' centrifuge is recommended and it is best to perform the operation in two stages, namely, 15 min. at 1500 r.p.m. and recentrifugation of the supernatant cell-contaminated plasma at 3000 r.p.m. for ½ - 1 hr.

2c) Platelet-rich plasma. Versene-treated blood, p. 92 , is transferred to siliconed tubes and centrifuged at 800 r.p.m. for 20 min. The supernant plasma is rich in platelets, and if only the upper portion is removed there will be a reduced contamination with leucocytes and erythrocytes.

2d) Platelet-poor plasma. The top 2/3, only, of the above (2c) platelet-rich plasma is carefully removed and measured with a siliconed pipette. The measured volume is transferred directly

into the 6 ml capacity siliconed tubes of the 'multispeed' attachment of the refrigerated 'International' centrifuge and spun for at least 45 minutes at 20,000 r.p.m. The clear supernatant, avoiding the lowermost portion, is all but free from platelets.

2e) Platelets. The sedimented platelets from 2d are washed 6X with 0.85 percent NaCl (saline) containing 0.2 percent Triton (WR 1339, Rohm and Haas, ref.[453]) and finally resuspended in 1/10 the volume of the original platelet-rich plasma.

3. $BaSO_4$ adsorption, etc.[98,463,410,914]. 10 min. adsorption of plasma with pure $BaSO_4$, e.g. Merck's 'reagent', at 100 mg per ml, removes prothrombin, proconvertin, PTC, etc., leaving most of the fibrinogen, AcG, AHG, etc.

Other adsorbents, used in some of our earlier work, include: $Mg(OH)_2$[176,442] which is too alkaline in our opinion; type C[∝] $Al(OH)_3$ gel[381,52]; $Ca_3(PO_4)_2$[56,387].

4. Fibrinogen. For purposes of the experiments described in this thesis, the most satisfactory fibrinogen is prepared from dog oxalated plasma which has been adsorbed with $BaSO_4$, as in 3. The Fibrinogen is salted-out at 1/4 sat. $(NH_4)_2SO_4$, the centrifuged precipitate dissolved in saline and dialysed against saline containing 0.005 M trisodium citrate. Some purer fibrinogens will be described in certain experiments.

5. Prothrombin preparations.

5a) Howell-type[71]. This was much used in our earlier studies, before the advent of the modern types of preparation. It was made from Berkefeld-filtered citrated dog plasma (see Expt. 22, p. 162) by means of acetone precipitation (after heat - defibrination),

collecting the precipitate on filter papers, washing with ether, and drying. For details of preparation of solutions see p. 162 .

5b) Seegers-type. These are the purest prothrombin preparations to date. They are secured by adsorption of citrated plasma (bovine) with $Mg(OH)_2$ and subsequent elution with CO_2 under pressure, see p. 22[435,428]. We have had some personal experience with this type of preparation, but have used many that were kindly prepared for us by Dr. Seegers, ref.[156].

5c) Eluate-type. In recent work, including many of the experiments in this thesis, we have obtained a prothrombin-rich solution from dog, human, or bovine oxalated plasma by adsorbing with $BaSO_4$ (see 3 above) and subsequently eluting the twice-washed (with distilled water) sediment with 1/3 volume 0.2 M trisodium citrate, see p. 104. Analyses on these eluates will be described in several of the experiments.

C. REAGENTS.

1. Commercially available products.

Chemicals, such as various salts, solvents, etc. usually require choice of the best 'analytical' grade and careful and accurate preparation of solutions.

Special materials, which are currently available, include:

1) Armour & Co.'s bovine fibrinogen, plasma 'Fraction I', lyophile dried, the preparative methods being based on the Harvard Plasma Fractionation Commission's cold ethanol technique[114]. We routinely remove traces of prothrombin, etc. by $BaSO_4$ adsorption. 1 g /100 ml of 1% solution of B.F. is sufficient.

2) Human fibrinogen, prepared by this method, and available for restricted distribution by the American National Red Cross.

3) 'Incubation mixture', for performance of the two stage prothrombin assay (according to Ware and Seegers)[495], obtainable from the Difco Corp. (Courtesy of Dr. C. W. Christensen).

4) 'Simplastin' (Ca-thromboplastin) is available from the Warner-Chilcott Co., and is reliable for prothrombin time tests.

5) 'Soluplastin', Schieffelin and Co. (Courtesy of Dr. E. W. Blanchard) is the commercial thromboplastin used for most of the tests in the present study.

6) Bovine thrombin, Upjohn & Co. (Courtesy of Dr. J. T. Correll) is preferred.

7) 'Thrombin topical' (bovine), Parke, Davis & Co. (Courtesy of Dr. E. A. Sharp), also is good, but is often unduly contaminated with fibrinolysin.

Many individual investigators have provided us with samples of their materials, which will be acknowledged in appropriate sections. Our own procedures and preparations will also be described and critically discussed in connection with their use in various experiments.

2. TESTING OF REAGENTS.

While the special qualitative and quantitative characterization of reagents will entail testings which are part of the individual experiments, there are some general test procedures which are appropriately reviewed at this time. The following selected techniques represent the fruits of many years of experience. They embody experimental criteria based upon many lines of

of progress by a host of modern workers in this field. However, many of them were not available in the earlier periods of our study, when some factors, much less techniques, were still unknown. Hence these test methods are presented as recommendations in the light of which old experiments may be reviewed and re-evaluated and sometimes profitably repeated, perhaps with modifications suggested by the newer ideas.

a) Fibrinogen assay. (I) Gravimetric. The common clinical routine[453,473] is quite useful for approximate determinations on isolated fibrinogen solutions. Add 0.5 ml bovine thrombin (500 units/ml) to 0.5 ml solution (e.g. plasma) diluted with 5 ml imidazole-buffered saline. Imidazole buffer[331] of pH: 7.3 is made by dissolving 1.72 g imidazole (Edcan Labs.) in 90 ml 0.1 N HCl and diluting with dist. water to 100 ml. 1 vol. imid. buff. is added to 9 vols. saline (0.85%NaCl). The fibrin clot is carefully and completely wound off on a tared glass rod, washed with saline followed by distilled water, and dried to constant weight, i.e. overnight at 85°C, and weighed.

(II) Colorimetric[348]. Our[60] preferred method for highly accurate assays of purified fibrinogen is to recover the thrombin-fibrin, obtained as in (I), on a mounted dissecting needle, washing, digesting with a modified Kehl's biuret reagent, then diluting and reading the color intensity with the Klett-Summerson photoelectric colorimeter, using No. 540 filter. The instrument is previously graduated against the above gravimetric (dry weight) and macro-Kjeldahl (fibrin N) determinations.

(III) Turbidimetric. In 1942, the author[135] used the Evelyn photoelectric colorimeter, with No. 540 filter, as a turbidimeter. Supplementary to other studies, it was noted that the turbidity of clots formed by adding thrombin to a 1:10 dil. plasma could accurately measure the addition of fibrinogen in amounts as small as 7.5 mg/100 ml. A recent micro-method[512] is based on these principles.

b) The prothrombin time determination, patterned after the original one-stage test of Quick et al.[393] is performed as follows: To 0.1 ml plasma is added 0.2 ml of Ca-tpln. (equal volumes of 0.02 M $CaCl_2$ and tissue thromboplastin, e.g. 'Soluplastin, p. 96) and the appearance of fibrin timed, with a stopwatch, at 37°C.

c) Thrombin-forming factors, assayed by specific ONE-STAGE methods. By modifying the above technique we (and others) have overcome the non-specificity of the original test and introduced specific bioassays for (1) prothrombin, (2) (pro)accelerin, and (3) (pro)convertin.

The three methods differ in the choice of substrates (see below). They all follow the common procedure of adding 0.1 ml of 1:10 (or suitable) dil. plasma (or serum, or other agent) to 0.1 ml of the appropriate substrate, in a 13 mm. diam. serological tube in a 37°C water-bath, then adding 0.2 ml Ca-tpln. and timing the clot (film at edge) with a stop-watch.

Ca-tpln: equal vols. 0.02 M $CaCl_2$ and 'Soluplastin' (p. 96) or other suitable tissue thromboplastin. A normal, designated 100 'per cent', is the mean of a statistically significant number of assays on plasmas from healthy normal adult human subjects. From (a) such a normal or, better, (b) a hyperreactive pool from

a number of cases at or near the upper limit of normal variations, a series of saline dilutions (preferred to other diluents) is made and the data are plotted as a standard reference curve. Values up to about 120-130 per cent of the statistical norm are included in the curve obtained by method (b), and most plasma unknowns will fall on this reference curve. In the case of reagent testing, variable dilutions are necessary.

To overcome day-to-day variations of reagents and test conditions, it is recommended that a 'control of the day' be selected from available personnel whose assay point on the standard curve has been repeatedly established. This test will then orient the standard curve for the particular set of tests as shown in the follow example:

Patient 'X' gives a 1-stage clotting-time (say, for prothrombin) corresponding to 63 per cent on the standard reference curve. The day's control, known to be 90 per cent on the standard curve, today gives a clotting-time which reads at 81 per cent. Hence the corrected UNITAGE of the unknown 'X' is $63 \times \frac{90}{81} = 70$, expressed as 'percentage' of the standard normal. In the present study, reciprocal prothrombin times are also expressed, percentagewise, in terms of a standard normal 'activity'. Figures 9, 10, 11, 12 show correlation graphs of four methods, namely, 'prothrombin' time, Method I (specific 1-stage prothrombin assay), Method II (orthodox 2-stage prothrombin assay), Method III (eluate assay method for prothrombin). They represent over 300 tests on plasmas from human cases, both normal and with clinical variations to the limits of the test methods.

The excellent correlation (= 0.95) of prothrombin data obtained by the specific 1-stage (Method I) and the orthodox 2-stage (Method II, see below) is graphically illustrated in Figure 10. Incidentally, as shown in Figure 9, there is a very much poorer correlation (= 5) of Method II and the unmodified prothrombin time test, due to the now recognized fact that the Quick test is influenced by variables other than prothrombin. All three specific methods show excellent correlations.

d) Method I: Specific 1-stage prothrombin assay. Our modification of the method of Owren and Aas[365], uses as substrate a $BaSO_4$-adsorbed beef plasma, fortified with 1/10 vol. of a bovine serum proconvertin preparation. This proconvertin is prepared from ox serum by the same method, essentially, as we[295] used in the case of human plasma. The serum was obtained by recalcifying oxalated beef plasma and centrifuging after standing for 18 hrs. at 4°C. Interestingly, this reagent, although prepared from serum, is mainly unchanged proconvertin. We deduce this by observing that it does not abolish the 'lag phase' in prothrombin conversion as compared with convertin (proconvertin pre-incubated with $CaCl_2$ and thromboplastin), which does.

The prothrombin substrate is designed to contain the normal plasma amounts of fibrinogen, proaccelerin, and other possible factors not adsorbed on the $BaSO_4$. The $BaSO_4$ has removed the prothrombin (hence its suitability as a test substrate), the proconvertin (hence the readdition of this factor), PTC, and possibly other materials which are not believed to play any role in the conversion of prothrombin to thrombin in the presence of Ca-tpln. Frozen

stored, in 5 ml lots, at -20°C, this prothrombin substrate remains serviceable for many months. Occasionally it loses significant amounts of AcG, although this is far more stable in bovine than in human plasma. In such case, the substrate should be assayed for prothrombin by the two-stage method and, if this is satisfactory, the substrate can be restored to usefulness merely by using a suitable dilution of AcG, instead of saline, in the (1:10) dilution of the test 'unknown'.

Serum AcG (accelerin), as used for this and other (see 2-stage, etc.) purposes, is prepared according to the directions of Mare and Seegers[495], i.e. ox serum, aged several hours, is adsorbed with 4g/100 ml $BaCO_3$. $BaSO_4$ can be substituted for the carbonate.

e) Specific 1-Stage proaccelerin assay: uses as substrate 'aged' human oxalate plasma, as originally recommended by Quick and Stefanini[395]. For some time (e.g.[297]) we recommended $BaSO_4$ adsorption (the usual 100 mg/ml) of the unknown plasma before the routine 1:10 dilution prior to adding to substrate, etc. However, any additional prothrombin which would be carried over in the diluted unknown to the normally prothrombin-rich substrate can be shown to have a negligible effect, particularly when the reference standards are handled in the same way. The easiest method, therefore, is to employ untreated 1:10 'unknown' plasma, etc., as in the other specific 1-stage methods. In reagent (e.g. AcG) assays, there is again no need for pre-treatment, unless the material contains a large amount of prothrombin.

'Aging' of the substrate plasma is best accomplished by freezing and thawing, (which always reduces the labile proaccelerin) and

storage in the ice-box at 4°C for 1 or 2 wks. (or longer), until the regular prothrombin time test (0.1 ml plasma + 0.2 ml. Ca-tpln.), at 37°C, gives clotting-times in excess of 60 seconds.

Aged plasma should contain normal amounts of fibrinogen, prothrombin, proconvertin, etc. Frozen stored, it is serviceable for many months and if a small amount of sediment appears on thawing this can be removed by centrifugation and the residual supernatant found to be little affected in most instances. A very fresh normal plasma must be used for the standard of reference and for the daily control. We have found it best to use a single well-known 'normal' for both.

f) Specific 1-stage proconvertin assay: uses as substrate bovine plasma twice filtered through special '20%' asbestos (Seitz-type) pads, obtained from the St. Gallen Co., Switzerland, according to the original recommendations of Owren and Aas[365]. This filtration is designed to remove nearly all the proconvertin, but to leave a sufficient amount of prothrombin. Our tests usually reveal about 60% of the original prothrombin and a control prothrombin time test (with 0.1 ml saline, instead of test plasma) of 120 sec. or longer. A new recommendation[2] to employ a wood charcoal 'L-50' (Springfield Facing Co., Williamsnsett, Mass.) may be noted. We have, at times, supplemented our substrate, with proconvertin-free bovine prothrombin, prepared by the method described for human plasma fractionation[295]. Fibrinogen and other factors are satisfactory in this substrate but, like the prothrombin substrate (p.101), it may lose AcG and require supplementation of this in the test plasma dilution.

g) Method II: The orthodox TWO-STAGE prothrombin assay. The original two-stage assay[496] has been modified[495]. Ferguson et al.[153] have recently made a critical restudy of the two-stage prothrombin assay method, both as to technical considerations and in application to the investigation of bleeding disorders. Our 'improved' test adds 1% bovine proconvertin (see above) as well as AcG (the 'modified' method). This is actually essential only in very rare instances, when testing plasmas, etc. which are extremely low in proconvertin, e.g. some congenital hypoproconvertinemics. Briefly detailed, the method requires four steps, namely,

Step 1. Defibrination: 0.5 ml plasma + 0.4 ml saline + 0.1 ml thrombin (bovine, Upjohn's, 20u/ml): remove clot with glass rod over 15 minutes.

Step 2. Dilution: Appropriate (e.g. 1:30) dilution of 0.1 ml(1) with 1:100 (saline dil.) AcG + proconvertin.

Step 3. Activation: 0.6 ml (2) + 1.8 ml 'incubation mix', consisting of 11.5 saline, 1 imidazole buffer, 4 purified (Ca-free) acacia 15% (Wills Corp.), 0.6 'Soluplastin' (Schieffelin & Co.), 0.9 $CaCl_2$ (0.1 M).

Step 4. Clot-timing: 0.4 ml samples of (3), removed at 1 min. intervals, are tested with 0.1 ml fibrinogen at 28°C (25°-30°), noting the minimum reached. This should be in the 13-17 sec. range.

Our best standard fibrinogen is simply prepared from oxalated dog plasma, $BaSO_4$-adsorbed as usual (see p. 94), precipitated at 1/4 saturation $(NH_4)_2SO_4$, redissolved in 1/3 orig. vol. saline and dialyzed against citrated saline (0.005 M sodium citrate).

Frozen stored (-20°C) in 10 ml lots it keeps well for many months with a remarkably constant reactivity (see p.109). Occasionally a small amount of sediment appears on first thawing, but this can be centrifuged off and the bulk of solution remains clear thereafter, even through repeated freezings and thawings.

Computation of unitage: E.g. If a 30x dil. is used in stage (2) and the minimal clotting-time (reached, say, in 3 or 4 min. of step (3) incubation) is 14 sec. and hence the 'correction factor' from Hare and Seegers' Tables[495,256] is 1.1:

1.1 x 5/4 x 4/1 x 30/1 x 2/1 (i.e. respective dilutions at steps 4, 3, 2, 1) = 330 units/ml.

h) Method III: $BaSO_4$-citrate ELUATE techniques. a new two-stage method, developed in the author's laboratory.

METHOD: 3 ml oxalated (not citrated) plasma is adsorbed 10 min. with 300 mg $BaSO_4$ (Merck's) and the sediment, recovered by centrifugation, is washed with distilled water and then eluted for 10 min. with 1 ml of 0.2 molar sod. citrate. The eluate, recovered by centrifugation, contains essentially all the prothrombin, proconvertin, and PTC of the original plasma. It lacks fibrinogen, proaccelerin (except for traces), AHF, and most of the plasma inhibitors (e.g. antithrombin), usually.

i) Eluate assay (Method III) for prothrombin, by new two-stage: 0.1 ml eluate is incubated at 28°C (or approximate room temperature) with the following activator system: 3.6 ml saline (0.85% NaCl), 0.5 ml (i.e. 1/10 vol.) imidazole buffer (pH: 7.3) 0:1 ml bovine proconvertin (see p.100), 0.1 ml AcG ($BaCO_3$ bovine serum, see p.101) diluted 1:8, 0.1 ml 'Seluplastin' (Schieffelin & Co.'s thromboplastin), and finally, 0.5 ml 0.15 M $CaCl_2$.

At 1 min. intervals, 0.2 ml samples are tested with 0.2 ml fibrinogen (Armour's bovine 'Fraction I', 1%, treated with 1g $BaSO_4$/100 ml). Clotting-times recorded with a stop watch, usually reach a minimum within 5 minutes, and because of lack of inhibitors are typically stable or nearly so, for many minutes thereafter.

Minimum clotting-times are converted into prothrombin 'percentage' of normal by reference to a standard curve of data obtained from dilutions of a normal pool of plasmas, as described under the 1-stage assays. A 'standard of the day' is also routinely used, in the manner previously outlined (p. 99). Illustration of the technique used to assay standard eluates, is seen in Expt. 10 (p. 134).

Correlation between Method III and the orthodox 2-stage (Method II) prothrombin assays for the same large group of (300) human cases as studied in the other comparisons of prothrombin methods, is shown to be very satisfactory in Figure 12.

j) A 'Proconvertin Index', by modification of the eluate method:

By omitting the 0.1 ml of proconvertin from the activator system in the previous test, and securing the 1st minute test without it, a clotting-time value is obtained which, using the reference curve, indicates the initial velocity of the prothrombin activation. This is due chiefly to the proconvertin present in the plasma eluate. By then adding the 0.1 ml proconvertin and continuing the usual incubation and testing, the prothrombin activation is carried to completion. The ratio of percentage units in the 1 min. test divided by the final (e.g. 5 min.) percentage is a

"proconvertin index", which we have found to give a fair degree of correlation with the routine 1-stage specific proconvertin assay (p.102).

k,l) Eluate Method of assaying (1) accelerator or (2) thromboplastin. A STANDARD **eluate** is made, essentially by the method described for human plasma, but using bulk volumes of either (a) dog or (b) bovine oxalated plasma. Using this as a substrate, the prothrombin conversion may be studied at 1 min. or other suitable intervals, with the regular activator system modified by substitution, for the cited (1) accelerator globulin or (2) 'Soluplastin', of any other accelerator or thromboplastic material it is desired to study.

k_1, k_2) The new two-stage AcG assay: can be used, for example on (k_1) **fresh** plasma ($BaSO_4$-adsorption is not necessary), or (k_2) **platelet** suspensions (fresh), to assay their 'accelerator' property. See p. 94 for method of preparing washed platelets. Standards for the accelerator factor assays are a series of activation curves obtained with several dilutions of a comparable normal, whether (k_1) plasma or (k_2) platelet suspensions, after the manner of the Ware and Seegers two-stage AcG assay[493].

l_1) Thromboplastic factor assay by the eluate method: Using essentially the same procedure, but substituting for the regular thromboplastin ('Soluplastin'), any material containing a thromboplastic factor can be assayed, e.g. tissue thromboplastins (l_1).

l_2) Platelet thromboplastic factor: is routinely assayed in our laboratories by this technique. It involves platelet preparation

(as above) and normal platelet standards, with the reference series of dilution curves (activation curves).

Cephalin and a variety of lipoids and tissue thromboplastic extracts will be studied, by this method, in experiments to follow.

D. INHIBITOR MECHANISMS.

These will barely be mentioned in the present thesis, but it must be noted that certain inhibitors of the thromboplastic mechanisms, or perhaps of other components in the prothrombin conversion reaction, can be demonstrated by delay in thrombin formation (compared with inhibitor-free controls) in such test systems as these of the eluate method. Other prothrombin activation tests (showing inhibitory phenomena) will also be used in the present investigation.

1. Heparin (with its cofactor) is an important experimental addition in some of the present test systems.

Testing for heparin in blood or plasma: is usually done by (a) whole blood clotting-time tests or (b) protamine titration (with which we prefer to use a plasma recalcification system), but details of these techniques will be omitted.

2. Antithrombin tests.

a. Plasma thrombin clotting-time test. The clotting-time (at 37°C) of a test plasma mixed with a standard solution of thrombin, and compared with a similar experiment using a normal plasma, is often suggestive evidence of some 'immediate' (or rapidly acting) antithrombic factor. An example is seen in the new born infant[172].

b. Serum antithrombin: refers to the 'progressive' loss of thrombic activity when a standard thrombin is incubated with serum (or other fibrinogen-free material) and retested with fibrinogen at successive time intervals. Saline may be substituted in the control.

This test is performed with many of our test materials, when we wish to demonstrate the presence, or absence, of antithrombin. An example is seen in Table VII.

Often, however, it is merely enough to retest a thrombin-forming mixture at intervals to observe definite antithrombic action. This is always seen, for instance, in the orthodox 2-stage prothrombin test and raises some theoretical questions as to the true end-point. In practice, at the high dilutions tested, this is usually ignored, however.

E. STUDIES ON ARTIFICIAL CLOTTING SYSTEMS.

1. TEST METHODS. In general, test systems are quantitative, employing accuate measurement of amounts of reagents, including use of volumetric (e.g. serological) pipettes and other glassware, always kept scrupulously clean with a cleansing routine involving detergent (Dural H), sulfuric acid-chromate solution, distilled water, and drying oven. Timing of many procedures is facilitated by electric chronometers and mechanical stop-watches. Clotting-times are used as 'end points' in the great majority of experiments.

2. CLOTTING-TIME, as discussed on p. 28, is an empirical observational datum which becomes significant only under strict conditions, which will be defined for the individual experiments.

3. THE THROMBIN-FIBRINOGEN REACTION. The key reaction in the physiological mechanism of blood clotting is the conversion of fibrinogen into fibrin through the agency of thrombin. The first step in our investigation, therefore, must be an evaluation of the time relationships of the thrombin-fibrinogen reaction.

a) Fibrinogen and clotting-time. Fibrinogen concentration, over a considerable range[60] has remarkably little effect on clotting-time (c.t.). Of much more significance is the reactivity of the particular fibrinogen solution, meaning the particular clotting-time it will show with a given thrombin preparation under the defined test conditions. There is no real information, to date, upon which we can predict the reactivity of a fibrinogen. It is also not exactly known what determines the potency of a particular thrombin preparation. However, any of the modern highly purified and stable thrombin preparations may be relied upon to give a definite clotting-time, reproducible, within a small limit of experimental error, in many repetitive tests conducted with a given good fibrinogen solution. Under carefully controlled and standardized experimental conditions, repeatedly using the same substrate fibrinogen, comparative clotting-times afford a valid, though relative, measure of thrombin potencies. Actually, two variants of method have both been widely used. In the first, the thrombin concentration is varied and the respective clotting-times used as a measure of relative thrombin potency. An example is given in Figure 5, in the introductory section. In the 'method II', (p. 103) the thrombic material is diluted to give a standard clotting-time, and relative dilutions are used as the measure of thrombic activity.

b) Standardization of thrombin. The National Institutes of Health (Bethesda, Maryland) provides a thrombin standardized by this second method, the original (revised) procedure being that of Smith, Warner, and Brinkhous[443], as modified by Ware and Seegers[495]. The method was originally (1934) developed to assay prothrombin, in terms of the thrombin unitage developed after maximal activation to thrombin. The unit, whether for thrombin or activated prothrombin, is defined as that potency of the product which gives a 15 second clotting-time of a particular standard fibrinogen, under the stipulated experimental conditions. Difficulties in securing a standard fibrinogen are insuperable, as previously mentioned, but a practical solution has been to have many testings of a particular lot of thrombin and then to retest many times when the original fibrinogen needs replacement with a new batch. It is in this way that the N.I.H. standard is maintained.

c) Standardization of fibrinogen. It is reasonable to standardize every batch of fibrinogen by repeated clotting-tests with a standard thrombin, as performed at N.I.H. In our laboratories this is not attempted. Either, the particular batch of fibrinogen, unstandardized, is regarded as the 'standard' for a particular set of experiments, relative c.t. values being acceptable in the absence of any evidence of fluctuations. Or, when a particular fibrinogen is used for routine two-stage prothrombin assays of plasma from normal subjects and human clinical cases, sufficient experience is secured with the fibrinogen to characterize its reactivity, using a statistical mean of cases with normal plasma

prothrombin unitage[153]. We have prepared many dog fibrinogen preparations (p. 94) with which the two-stage prothrombin assay gives close to 300 units/ml human plasma, which is a very acceptable value.

d) Clotting-times and thrombin concentration. Returning to the first method, while this has no value in terms of any absolute unitage, it, nevertheless, gives perfectly valid relative values, if some reference series of thrombin dilutions is used, under the same experimental conditions. The fundamental principle is that the shorter the clotting-time under standard conditions, the more the thrombin activity or relative concentration. This is here put into practical use, being validated, as mentioned, only for a given set of comparative experiments. By experimentally obtaining the data and plotting graphically as a 'thrombin dilution curve', e.g. Fig. 5, the observed clotting-times, in any variation of the experiment, may be read off as a 'percentage' of the original (strongest) thrombin. Much valuable information does not even require exact quantitation in this way. For instance, a very long clotting-time is clearly a matter of a very low thrombin concentration and two such tests may be far below the quantitative limits of the method and yet be significantly different by simple inspection of the data.

4. THE INVERSE LAW. In the example given in Figure 5 (p. 28), quite by chance, a very fair approximation to 'the inverse law'[141,162,20] was obtained. In our experience, the true variables responsible for the usual divergence of data from this 'law' have not been

defined, and we do not usually attempt to secure this exact type of linear reference curve. A recent (unpublished) study of one possible variable, however, is illustrated in the following experiment.

F. EXPERIMENTS.

1. EXPERIMENTAL CONDITIONS MODIFYING THE THROMBIN-FIBRINOGEN REACTION.

The thrombin-fibrinogen reaction has been discussed on pp. 25 et seq. in the introductory part of this thesis. Among the data there reviewed are those of Boyles, Ferguson and Muehlke[60] dealing with a number of experimental variables in the thrombin-fibrinogen reaction. The first two experiments of the present thesis add new data, obtained by the author recently, which deal with certain aspects of the serum (fibrin) factor (cf. p. 33) problem.

EXPERIMENT 1 (1956). TABLE II. Figure 13.

PURPOSE. To investigate possible relationships of thrombin-fibrinogen clotting-times to the 'serum (fibrin) factor' of Laki and Lorand.

REAGENTS AND METHODS.

Preparation of 'serum factor'-free fibrinogen. (ref. Laki and Lorand[277]; Lorand[304,305,307], Shulman[439]. Stock fibrinogen: 1% Armour's bovine (plasma) Fraction I, adsorbed with $BaSO_4$ to remove traces of prothrombin, etc. (p. 95)

Fibrinogen I(Control): Stock fibrinogen dialysed 2 hrs. against 2M sodium chloride, followed by overnight dialysis against 0.85% NaCl (room temperature). Thrombin clots of this solution remained insoluble in equal volume of 3 M urea, whether NaCl or $CaCl_2$ was added.

Fibrinogen II. Stock fibrinogen dialysed 2 hrs. against 2 M sodium bromide, followed by overnight dialysis against 0.85% NaCl. The bromide treatment removed much of the serum factor but the test clots were still only partly soluble in urea in the presence of

NaCl, and negligibly soluble in the presence of $CaCl_2$;

Fibrinogen III. Fibrinogen II, a week later, was diluted 20x with distilled water and iscelectrically precipitated with 0.1 M acetic acid, with maximal turbidity at pH: 5.5. The precipitate was recovered by 30 min. centrifugation at 350 r.p.m. and redissolved in 0.85% NaCl to original volume. Thrombin clots were now nearly all soluble in 1.5 M urea, even with added calcium, only a trace remaining after 24 hrs.

Testing fibrin clots for solubility in urea. 0.2 ml Fibr. 0.05 ml. 0.1 M $CaCl_2$ (or 0.85% NaCl) + 0.05 ml thrombin (bovine, Upjohn's: 10 units/ml); treated 10 min. later with 0.3 ml 3 M urea.

Clotting-time tests, with the above three fibrinogens. Clotting-times are measured with a stop-watch, at constant temperature (25°C), on adding, to 0.2 ml of the respective fibrinogens, 0.2 ml of bovine (Upjohn's, p. 96) thrombin, in siliconed tubes, which are used in order to avoid any loss of thrombin by adsorption on to glass. The thrombin was actually added at twice the strength of the final concentration stated in Table II.

RESULTS: are shown in Table II and in Figure 13. In the graphic presentation in the cited figure, relative thrombin concentrations are plotted against the reciprocal of the clotting times (1/c.t.).

CONCLUSION. There appears to be a much better linearity in III, and some improvement in II, as compared with I (Fig. 13), suggesting that the 'serum factor' of Lorand may indeed by a variable in causing deviations from 'the inverse law'. It is not known how valid the criterion of urea solubility of recalcified clots is in determining

freedom from the Lorand factor. It is possible, therefore, that further progress may be made along the present lines. There is an unexplored possibility that disturbing factors, other than the Lorand factor, may also have been removed in the isoelectric method of purification. Arguing for only a trace impurity is the observation that the $(NH_4)_2SO_4$ purification of Armour's fibrinogen gave a 97% clottable preparation which failed to obey the inverse law in the experiments of Boyles and the author[60]. Ammonium sulfate fractionation does not eliminate the Lorand factor[439].

EXPERIMENT 2. (1956). TABLE III.

PURPOSE. In the light of the cited recent work[307,439] clearly relating the Lorand 'serum factor' to calcium and the opacity, rigidity, and urea insolubility of fibrin clots, is it possible to explain the anomalous action of calcium salts, in a limited concentration range, namely, shortening the clotting-times of thrombin-fibrinogen mixtures, on the basis of the reactions involving the serum factor?

TEST. Under constant volume, and fixed thrombin and fibrinogen (I-III) concentrations, $CaCl_2$ was added to the final molarity noted and clotting-times determined, as in the preceding experiment.

RESULTS: are given in Table III. They show the usual (p. 30) accelerating effect of certain small concentrations of calcium ions.

CONCLUSION. The usual acceleration of clotting in a certain range of calcium salt concentrations, here 0.001 to 0.01 molar, is evident. There is a definite optimum at .005 M, with all three fibrinogens. It must be concluded, therefore, that the Lorand 'serum factor' is not significantly concerned with these effects of calcium ions on thrombin-fibrinogen clotting-times.

2. EXPERIMENTAL EVIDENCE THAT CALCIUM IS NOT AN ESSENTIAL COMPONENT OF THE THROMBIN-FIBRINOGEN REACTION.

Since the careful work of Hammarsten[206], see p. 10, it has been reasonably established that calcium salts, i.e. Ca^{++} ions, are not essential to the thrombin clotting of fibrinogen. Howell's comment[238], that there might still be room for doubt, however, indicated the need for final proof that the trace of (protein-bound) calcium in the clotting reagents (fibrinogen as well as thrombin) is without specific influence on the clotting reaction.

EXPERIMENT(S) 3. (1937-56). (cf. TABLE XX).

PURPOSE. To study the clotting of a completely decalcified thrombin-fibrinogen system.

METHODS. (A). In a 1937 experiment[128], the author prepared thrombin from Howell-type prothrombin (p. 161) by maximal activation ('ripe', after several hours) with calcium and cephalin. In three experiments, this thrombin was electrodialyzed for 2 hrs., 8 hrs., 13 hrs., respectively, in a Bradfield 3-compartment electrodialyzer with collodion membranes. The 110-volt direct current reached a maximum amperage of 0.3 to 0.4 amps. in 1/4 hr. and fell to 1 or 2 milli-amps. in about 2 hrs. Distilled water was run continuously through both electrode (end-) chambers. The thrombin began to flocculate out at the anode membrane of the middle chamber in about 15 min. and to sediment. Later it underwent partial migration to the cathode membrane. The diphasic behavior was explained by a change in pH of the unbuffered material over to the acid side of the isoelectric point of the globulins present. The final precipitate was recovered by centrifugation and redissolved in dilute

Ca-free KOH to pH 7.5 (phenol red indicator). The water-clear supernatant always showed some, relatively weak, thrombic activity. Neither the redissolved precipitate nor the supernatant showed the least trace of turbidity on adding test reagent pot. oxalate, and spectroscopic tests for traces of Ca were negative. The test fibrinogen was obtained from Berkefeld-filtered (see p. 122) citrated dog plasma, 'deprothrombinized' by $Mg(OH)_2$ adsorption[176], and precipitated (x3) at 1/4 sat. $(NH_4)_2SO_4$, reprecipitating at 1/2 sat. NaCl and redissolving in dist. water. We were unable to electrodialyse the fibrinogen without denaturation, but relied upon excess of oxalate or citrate to decalcify it, as indicated by a negative spectroscopic test for calcium. The most crucial test was performed after 18 hrs. incubation of both (a) the 13 hr. electrodialyzed thrombin, and (b) the purified fibrinogen, with equal volumes of (1) N/1 pot. oxalate, (2) N/1 sod. citrate. On mixture of 1 ml amounts of thrombin and fibrinogen, thus treated, and still in the presence of the strong inhibitory and decalcifying salt(s), firm clots were obtained in about 1/2 hr.

An interesting incidental observation was clot lysis, after several hours, indicating activation of the fibrinolytic enzyme system contaminating the thrombin preparation.

(B). Decalcification of proteins has subsequently become very simple with the advent of the ion exchange resins (p. 275 of Stefanini and Dameshek[453]). We have repeatedly subjected both thrombin and fibrinogen to the cationic exchange resins, Amberlite and Dowex "50" (sodium treated), and observed perfectly normal clotting of the subsequent mixtures (see Table XX).

CONCLUSION. It is, therefore, concluded that protein-bound calcium, while contributing (like sodium, potassium, etc.) to the charge patterns on the protein molecules, is not essential for the thrombin fibrinogen reaction and that thrombin in no true sense is a calcium compound.

3. EXPERIMENTS ON THE CLOTTING OF PLATELET-FREE PLASMA.

EXPERIMENT 4 (1949).[147] TABLES IV,V.

PURPOSE. To study the clotting properties of platelet-free plasma.

METHOD. Citrated dog plasma was obtained with the siliconed technique, after 16 hr. centrifugation at 20,000 r.p.m. in the refrigerated 'Multispeed'. Equal vols. of each plasma sample were tested, at 38°C, for clotting-times with 0.025 M $CaCl_2$, (G) in glass and (S) in silicone. Series I plasmas were kept in silicone, but tested on both types of surface. Series II were (G+) treated with powdered glass for 1 hr. at 34°C, in order to obtain a large measure of action of wettable surface. The control series II (S) were held in silicone for the same period, at the same (34°C) temperature. Besides the Ca, the following additives were tested: (1) saline, (2) intact platelets (carefully preserved in silicone), (3) platelet extract (frozen, thawed, and triturated with saline), (4) brain cephalin. In another series of experiments, simply conducted in glass (pyrex tubes), (1) was repeated, and tests were also made with (5) tissue thromboplastin('Soluplastin'), (6) crystalline trypsin (100γ), (7) Loomis'[302] bovine fibrinolysin (4mg). The data are shown in Tables IV and V.

RESULTS. Table IV: In (1), with Ca and saline only, no clots were obtained in 24-48 hrs., whether in glass or in silicone. This

unequivocal result is difficult to achieve and was repeated on only two or three occasions. In many more similar experiments clotting did occur in glass in from 1 to many hrs.; and longer in silicone.

In (2) and (3), clotting was restored, with insignificant differences due to the manner of treating the platelets. There was, however, a consistent and significant difference between the tests on plasma only in contact with silicone and those exposed, either (G) immediately or (G+) over a period, to 'wettable' glass surfaces. The glass clotting-times (170"-210") may be compared with the silicone clotting-times (435"-660"). The somewhat longer c.t.'s in the 1 hr. expts. probably mean some loss of labile factor (AcG). That the plasma is actually altered by the surface contact is shown by the finding of the shorter clotting-times also in the tests in silicone (II,S) after transfer to such tubes of the (G+) glass-incubated samples.

In (4), cephalin is shown to act in much the same way as platelets. Actually, it is even more effective in the concentration studied. The accelerator effect of glass on some component of the platelet-free plasma is also evident in the cephalin experiments.

In the Table V data: (1) the non-clotting control is repeated: (5) tissue thromboplastin is observed to give the very rapid clotting-time of 7 sec., exactly corresponding, in fact, to the typical 'prothrombin time' (Quick test) on ordinary dog plasma; (6) trypsin and (7) fibrinolysin are also effective. That the two proteolytic enzymes, of these last tests, show similar 'thromboplastic' properties is an interesting observation, which will require further investigation (see later).

DISCUSSION. None of the earlier efforts to establish the essential role of platelets in normal blood clotting were sufficiently convincing. Fuchs[178], in 1930, claimed the obtaining of a spontaneously incoagulable human plasma by high speed centrifugation in paraffined glassware, but Feissly (1934) and Smith, Warner, Brinkhous (cited[64]) were unable to confirm this work. Introducing the silicone technique, in 1946, Jaques, Fidlar, Feldsted and MacDonald[251] noted the prolongation of natural and recalcification clotting-times as platelet counts were more and more reduced by increasing centrifugation in siliconized tubes. However, they were unable to get rid of all platelets and did not obtain an incoagulable plasma. In the following year, Brinkhous[64] did succeed in obtaining a platelet-free human citrated plasma, with the silicone technic, refrigeration, and repeated centrifugations over a total period of 1350 min., using speeds up to 14,000 r.p.m. The reported final preparation, recalcified in glass at 28°C, did not clot in 30 hrs. Prothrombin conversion was delayed in these 'quasi-hemophilic' plasmas, but prothrombin assays were normal. 'Thrombin topical' and 'thromboplastic extract from beef lung' clotted them as rapidly as normal whole blood. Addition of platelet suspensions reduced the clotting time to 6-9 min. They destroyed added thrombin normally. The platelet-free normal plasma was able to correct the clotting defect of hemophilic whole blood, but not of recalcified platelet-free hemophilic plasma. Correction did occur in the plasma mixtures, however, if some platelets, whether normal or hemophilic, were provided. Hartmann and Conley[209], after many failures, finally succeeded in obtaining a platelet-free incoagulable canine plasma.

Patton, Ware and Seegers[369] used a special technique in collecting blood, without anticoagulant, from a dog for siliconed-tube centrifugation in the refrigerated 'Multispeed' at 22,000 - 23,000 r.p.m. for 5 min. - 5 hrs. Some of the 'native' plasmas thus obtained "remained fluid" for longer than 72 hrs. at room temperature. "There would usually, however, be some fibrin formed", whether in glass or in silicone "with about equal frequency". Prothrombin analyses, immediately after the centrifugations, showed normal values, compared with an oxalated plasma sample obtained at the same time as the special blood collection. The more highly centrifuged samples retained the same assay levels of prothrombin for several hours. Special (indirect) tests, however, showed the formation of traces of thrombin. The fibrin noted represented only "a small portion of the fibrinogen".

CONCLUSIONS. Our experiments[147], therefore, confirm the few previous workers' evidence pointing to the fact that platelets are normally essential for blood clotting. The data on 'surface' effects causing some alteration of the platelet-free plasma are original, as is our use of cephalin and the enzymes, in this type of experiment. The fact that a lipoid (cephalin) and the other cited reagents can replace platelets in what we believe to be a 'thromboplastic' type of action (see later), opens up some very intriguing questions.

ADDENDUM. Owing to the technical difficulty in obtaining large quantities of completely platelet-free plasma, the author (and others) have not progressed to the logical procedure, namely, to use such material as the starting point for the attempted preparation of plasma clotting factors free from all traces of natural 'thromboplastin' (at least of platelet origin). Here 'platelet-poor'

plasma is insufficient for satisfying this rigid criterion, although the greater the success in this direction, the better would seem to be claims for purity in preparing prothrombin, proconvertin, proaccelerin, antihemophilic globulin, PTC, and other factors. As a member of the Coagulation Committee of the Harvard Plasma Fractionation Commission, the author has followed the recent use of the ADL Cohn Fractionator in the preparation of deplateletized human plasma[460] and has tested some of the fractions (e.g. prothrombin, accelerator globulin, etc.) which Dr. Surgenor is preparing from it. It must be reported that the advances, to date, while encouraging, still leave much to be desired. Nevertheless, this should turn out to be a promising line of investigation leading toward the goal of purified individual blood clotting factors for specific therapeutic use in human deficiency cases.

4. EXPERIMENTS WITH THE BERKEFELD (BACTERIOLOGICAL) FILTER TECHNIQUE. (cf. Expt. 22).

In 1913, Cramer and Pringle[95] claimed that the passage of citrated plasma through a Berkefeld filter rendered it incoagulable on subsequent recalcification, but restored to clotting again on the addition of platelets. They concluded that they had (a) discovered a simple technique for 'deplateletizing' plasma and (b) proved that platelets were essential for normal clotting.

W. H. Howell at once set one of his pupils, C. H. Goddard (1914)[192] to repeating this experiment. On carefully perusing the English data, Goddard noted that some of their Berkefeld-filtered plasmas did clot on simple recalcification, although delayed, perhaps for several hours. One dog that had been fed three hours prior to

bleeding gave a filtrate which clotted readily in 5 min., i.e. practically a normal recalcification clotting-time. Goddard performed many Berkefeld filtrations and divided the filtrates into (1) 1st portion, (2) intermediate, and (3) last portion. It was found that only the first portion, which was slow in appearing through the fine pores of the diatomaceous filter, showed marked clotting defects. In fact, it was often incoagulable even with thrombin, proving that most of the clotting factors, even the fibrinogen, had been removed. The last portions always clotted readily on simple recalcification. The intermediate portions varied, and not uncommonly would clot poorly, if at all, on adding calcium, but quite well if platelets, brain extract, or cephalin were also added. Prothrombin could be recovered by Howell's acetone method, even from some of the 'intermediate' filtrates which did not clot satisfactorily with Ca alone.

For a number of years the present author[126,130] routinely employed the Berkefeld filtration of plasma prior to preparing Howell-type prothrombin. By the simple bacteriological laboratory trick of inverting a large test tube over the candle, the filtration was invariably rapid and satisfactory. Test results confirmed Goddard, in the finding of good clotting on simple recalcification. Clotting-times might be as long as 10-12 min., instead of the usual (unfiltered) plasma's 2-3 min., but could easily be restored to normal, or better, by addition of a small amount of cephalin[86].

CONCLUSIONS. The previously cited more recent work with the silicone technique and high speed centrifugation undoubtedly proves that the present author's views of an earlier period were erroneous in believing that all platelet material could be removed by Berkefeld filtration.

We were looking for some plasma thromboplastin of other than platelet origin, and well remember Howell's caution (p. 480 of ref. 233) that platelets may very well disintegrate when roughly handled in ordinary centrifugation and in Berkefeld filtration. There were important errors in Howell's other views, however.

5. EXPERIMENTS WITH THE BENZENE-EXTRACTION TECHNIQUE AND ITS RELATION TO 'AVAILABLE' CEPHALIN.

Apart from the errors in his work on platelets and lung extracts, C. A. Mills[335,336] correctly controverted Howell's view that calcium alone could activate prothrombin. Mills insisted on an essential role for cephalin, acting along with calcium, as a 'thrombokinase' in the sense of the Morawitz[345,346] theory, see p. 10. His evidence[336] included the following experiment:

Dog plasma was precipitated at 1/4 sat. $(NH_4)_2SO_4$, for fibrinogen, followed by 1/2 sat. with the ammon. sulphate, for a globulin fraction which served as a 'prothrombin', after both fractions were dialysed free from excess salt. On extracting his prothrombin by shaking with benzene (C_6H_6), Mills obtained a preparation which, when mixed with fibrinogen and calcium salt, no longer gave a clot. Coagulation was restored, however, on further addition of cephalin.

EXPERIMENT 5 (1934)[123].

The present author's first modest essay into the blood coagulation field, in 1934, was an attempt to confirm this experiment. We were able to do so, however, only after benzene-extracting (b.e.) the fibrinogen also. With considerable difficulty, the recalcified mixture of b.e. fibrinogen and b.e. prothrombin could also be induced to clot by (a) restoring the benzene extractives (recovered

by evaporation) in a watery suspension of the mere trace of material recovered or (b) layering the benzene extract on the top of the test mixture.

CONCLUSION. Of course, an elementary knowledge of the lipid chemistry of the blood precludes any notion that this simple use of benzene can possibly extract more than a very small fraction of the fatty materials in the plasma fractions. Indeed, the paucity of material in our evaporated extracts bears witness to this. Nevertheless, the experiment does show the removal of some plasma property which can be restored by added lipoid (cephalin) and which would appear to fit the definition of a 'thromboplastin' (see later). Re-evaluation, from the modern viewpoint, raises at least one important possibility, namely that the test materials are perhaps deprived of some labile clotting factor (? proaccelerin) not necessarily related to the partial lipoidal extraction. This possibility will be explored in the present thesis.

EXPERIMENT 6. (1956). TABLE VIII, with assays on ELUATES (TABLES VI,VII).

PURPOSE. To observe the clotting properties of an artificially isolated system of identifiable coagulation components, after subjection to benzene-extraction.

REAGENTS. Fibrinogen (Fibr.): Precipitated at 1/4 sat. $(NH_4)_2SO_4$, from $BaSO_4$-adsorbed oxalated dog plasma, redissolved in 1/3 orig. vol. of saline (0.85% NaCl) and dialysed against saline containing 0.005 M sod. citrate. Fibr. (b.e.): Fibrinogen shaken for 15 min. at room temp. with an equal vol. of benzene (C_6H_6), in a separatory funnel, recovering the clear bottom layer. No turbidity noted.

Eluate: Sediment from the $BaSO_4$ adsorption (100 mg/ml) of the above plasma, No. 126, washed 2x with dist. water and then eluted for 10 min. with 1/3 orig. vol. of 0.2 M sod. citrate. Aliquots of this eluate were (a) preserved, frozen, untreated, (b) dialysed 3 hrs. against saline, and (c) benzene-extracted (b.e.), in a similar manner to the b.e. fibrinogen (see above). Another portion (d) was benzene-extracted without dialysis and then (e) a fraction of this was dialysed. Specific assays of components of eluates (a) - (d) were obtained by the routine methods previously described. The data are given in Table VI and are expressed as a unitage/ml, compared 'percentagewise' with the statistical normal human plasma, by which each test is standardized, cf. Fig. 16. These eluates (1) lack fibrinogen and thrombin (no clot in 3 days in control tests), (2) contain very small amounts of AcG (proaccelerin), but are rich in (3) prothrombin and (4) proconvertin. The dialysis and benzene-extraction did not alter the prothrombin and proconvertin values significantly. The high ionic strength of the 0.2 M citrate is inhibitory in clotting tests, unless the eluate is highly diluted. It may be recalled that it is routinely diluted 50x in our eluate method III assays (p. 104). Some incubations with 40 u/ml thrombin were performed with the undiluted eluates and failed to show any significant inactivation of the added thrombin. There was, however, a 2-3x lengthening of clotting-times, from the start, as compared with a 0.85% NaCl control. With 0.2 M sod. citrate as the control, however, the clotting-times (of the order of 25"-30") were comparable. A more significant antithrombin assay was, therefore, conducted on the b. e. dialyzed eluate (e). The data are given in Table VII,

with details of method appended. There is no more than the merest trace, if anything, of progressive increase in clotting-times in the test (2) with the eluate, which is nothing like the rapid activation in defibrinated plasma or serum, or in (3) the untreated AcG preparation I, used as a positive control (in Table VII) and compared (4) with a partly purified AcG^{Φ}, described in Expt. 10, on p. 134.

AcG: Our regular $BaCO_3$-treated bovine serum (p.101) assays about 1200 percentage units of (pro)accelerin/ml. When assayed at full strength by the 'improved' 2-stage method, p.103, it showed a barely assayable 1 unit/ml of thrombin yield. It is probable that traces of both thrombin and prothrombin persist in barium carbonate-treated bovine serum, but they are quite negligible in the dilutions usually employed. However, we did attempt to 'purify' this AcG preparation by an additional $BaSO_4$ adsorption. This reduced its AcG unitage to 900 percentage units/ml, but the traces of thrombin and prothrombin persisted, as shown by the control tests (1-4) in the experiments of Tables X and XI.

Ceph.: 'Cephalin' was prepared from acetone-dried dog brain (cf. Eagle[110]) by several days extraction with ether, removing the acetone-soluble fraction and resuspending the dried acetone-insoluble lipoids in saline as a stock 3% suspension, uniformly dispersed with aid of a mechanical homogenizer. Dilutions (e.g. 0.5%) from this stock are stable and uniform. The stock 3% solution, like other reagents, is stored frozen at -20°C in the 'Deep Freeze'. This cephalin served as the 'standard' in many experiments (e.g. p. 175).

Ca.: The optimal $CaCl_2$ concentration was carefully determined for the eluate clotting tests by a preliminary series of mixtures,

corresponding to (8) in Table XI, but with varied calcium concentration. The cited amount of $CaCl_2$ was found to be optimal.

EXPERIMENT. Clotting test mixtures of fibrinogen (b.e.) and dialysed eluates (as described above) constituted, in essence, a synthetic plasma system of known composition and free from overt inhibitors. Upon such, the effects of benzene extraction could be studied in the clotting tests summarized in Table VIII.

RESULTS. The most significant finding was the much poorer clotting-time (10054") in (5), with the b.e. eluate activated with (added) Ca alone, than in the same eluate (9) before benzene extraction (354"). Moreover, the "+" sign indicates the timing of the first wisp of fibrin and many more minutes were required for a solid clot in (5), whereas (9) progressed to a solid 'gel' within a minute or two. Recalcification in the presence of b.e. AcG ($BaSO_4$-treated) (7) considerably reduced the c.t. (210"), showing that AcG lack was significant in the eluates, as was known from the analyses in Table VI. Test (8) showed cephalin to be a good (? partial) thromboplastin in the presence of Ca and AcG. In comparison with (5), test (6) was equally significant in showing that cephalin could accelerate clotting even in a system very poor in AcG. In fact, the 221" c.t. in (6) was better than the 354" c.t. in the simply recalcified untreated eluate (9). All four tests with the untreated eluate (9-12) were definitely better than the corresponding tests (5-8) with the b.e. eluate. The differences between 11 (107.5") and 7 (210") are especially significant, because both are performed in the presence of adequate accelerator factor. It should be noted that the 90 percentage units of AcG supplied in the 0.1 ml introduced

into to the total 1 ml mixture, corresponded very well to the level normally encountered in plasma. Actually, our synthetic system was hypoprothrombinemic (about 17'per cent') and hypoproconvertinemic (about 11 'per cent'), compared with a normal plasma. The 16.7" c.t. in (13) and 15.7" in (14), where tissue thromboplastin ('Soluplastin') was added, with AcG and Ca, and the temperature raised to 37°C, are of the order of magnitude that would be expected, if we were testing a plasma of similar composition by the regular 1-stage 'prothrombin time' test. Tests 1-4 are, essentially, controls for thrombin and prothrombin in the AcG preparation. Thrombin is really absent from the eluate (see Table VI).

CONCLUSION. These new experiments afford significant evidence of a diminished coagulability after benzene-extraction. It is essentially independent of the presence or absence of abundant accelerator factor (AcG), although this has its own effect on clotting-time. It is not an inhibitory phenomenon. It can be corrected, in fact over-corrected, by the addition of cephalin or tissue thromboplastin. May it not, therefore, be concluded that: (a) benzene extraction removes some 'thromboplastic' factor associated with the complex proteins of the clotting system, and (b) benzene being a lipoid-solvent, may not this factor be a thromboplastic lipid related to cephalin?

EXPERIMENT 7 (1956). TABLE IX.

PURPOSE. To study further the clotting phenomena in 1-stage systems, such as those of the preceding experiment, and to test for clot-promoting activity in the benzene extractives.

PREPARATION OF EXTRACT (Extr.). Preliminary observations gained from evaporation of the benzene layer from the preceding extractions indicated the extremely minute yields of extractives. It was, therefore, attempted to obtain a more significant amount of material from whole plasma. About 33 ml of a citrated dog plasma which had kept well for many months in the 'Deep Freeze', was thawed, filtered (from trace of sediment), and extracted as usual with an equal volume of benzene. Centrifugation of the top layer separated 30 ml of clear benzene from a small foamy deposit. The clear extract was evaporated to dryness, in vacuo, and the residue extracted with 3 ml imidazole-buffered saline. Although this was definitely turbid, it did not contain a weighable amount of extracted material.

METHOD. In a 1-stage test series, similar to those of Experiment 6, the extract (extr.) was compared with simple recalcification of eluate + fibrinogen mixtures. An optimal amount of cephalin was substituted in another parallel test.

As an independent observation, the effects of b.e. AcG (a second extraction of the same reagent as used in Expt. 6 five days earlier) were studied, both with and without cephalin.

RESULTS: These are given in Table IX. In (A) an eluate which had been benzene-extracted and then dialysed 5 days previously was used. In (B) this same reagent was submitted to a further benzene extraction and tested twice, namely (I) within 15 min. and (II) after about 3 hrs. Both series of tests employed the same freshly benzene-extracted fibrinogen. Note the following:

(a) simple recalcification times show a significant prolongation after the fresh benzene extraction B, 2 vs. A, 2. However, there

is some reversion to a shorter clotting time in the 3 hr. test of B, 2 after standing several hours at room temperature.

(b) the extr. reduces the clotting-times, although not as well as cephalin does in the AcG-poor systems.

(c) AcG gives considerable aid to the clotting system, but only moderately so with Ca only and much more so when a relatively adequate thromboplastic action is restored by cephalin.

All tests are consistently shorter in the 'A' series. The precious extract was not tested with AcG, in order to conserve it for the more significant 2-stage experiments to follow.

CONCLUSIONS. Any conclusion from a 1-stage type of experiment must be tentative, but it does seem reasonable to conclude that at least four factors are contributing toward the observed clotting-times. These factors are (1) eluate (i.e. prothrombin + proconvertin), (2) Ca, (3) AcG, (4) a thromboplastic type of factor, which can be supplied rather well by cephalin and weakly by the benzene extract. The fact that benzene extraction does not result in a system completely unable to clot with calcium alone suggests that a small amount of some thromboplastic agent normally accompanies the reagents studied. That non-clotting, with Ca alone, may be recorded in other systems of benzene-extracted reagents (Expt. 5) may possibly be explained on two grounds, viz. (1) too great an ionic strength, i.e. inhibitory to weak thrombin formation; (2) antithrombic inhibitors in the system, able to remove any small traces of thrombin formation before they can induce invisible fibrin formation. The present synthetic system permits the detection of very weak thrombin formation by eliminating these two unfavorable circumstances.

In so doing, however, it still permits observation of a quantitative difference, which can be interpreted as an ability of benzene extraction to remove a small portion of the potential 'thromboplastic' material in the clotting materials. From the nature of the extraction and the similarity to a weak cephalin in restoration of the clotting deficit, may it not be surmised that we are dealing with a thromboplastic lipoid? It is also suggested, somewhat theoretically, that the reappearance of greater clotting activity on simple recalcification, at times, after this has been lessened by benzene extraction, may possibly indicate some 'free' form of lipid becoming liberated from the natural lipo-protein complexes, and being 'available' in this form for (a) the clotting reactions, on the one hand, and for (b) easy removal (in trace amounts) by the lipoid solvent on the other.

EXPERIMENTS 8 and 9 (1956). TABLES X, XI.

PURPOSE. To study the conversion of prothrombin to thrombin in variously activated eluates, by a two-stage test system with special reference to thromboplastic additives.

REAGENTS. The same as those used in the preceding 1-stage experiments.

METHOD. The 2-stage procedure is essentially that of our standard 'Method III' (see p.104). Note that the salt concentration (0.85% NaCl) and pH (7.3) are physiologically equivalent to plasma. Moreover, the 50-fold dilution (0.1 eluate in 5 ml thrombic mixture) reduces the citrate content to a point of non-significance. However, the chosen amounts of $CaCl_2$ are strictly optimal, according to careful preliminary tests on rapidly activating test systems.

EXPERIMENT 8 is performed on dialysed, benzene-extracted eluate: (1) with Ca alone; (2) with Ca + extr. (p. 130); (3) with Ca + extr. + AcG (b.e.).

The composition of the thrombic mixtures and the clotting-test data, after varying periods of incubation, up to 3-48 hrs. are given in Table X.

CONCLUSIONS. (1) There is extremely little thrombin formation in these systems, particularly with Ca alone. (2) The 'extract' adds a little, which is significant, and also indicates the extreme sensitivity of the test system. (3) Although tests (1) and (2) are intentionally conducted with only the trace of contaminant AcG (see analyses in Table VI) in the eluate, the provision of adequate accelerator in the form of 1:8 dil. b.e. AcG, still adds only a nominal increase in the trace of thrombin formation. The important conclusion is that thrombin formation is extremely poor in a system adequate with regard to prothrombin, proconvertin, calcium, and AcG, but deficient in 'thromboplastin'.

An incidental finding in these tests confirms the antithrombin tests of Table VII. Note the excellent stability over 3 hrs. of the very weak thrombins in tests (1) and (2), with eluate mixtures only, whereas the 2 and 3 hr. tests in the presence of the serum AcG are definitely beginning to lose potency, despite the 1:50 dilution of its serum antithrombin. There is some loss in all, in 48 hrs., especially in (3).

EXPERIMENT 9 is similar to the foregoing, but relies upon the dilution and a little extra calcium to overcome the citrate.

The test data given in Table XI show:

1) The AcG preparation has a minute amount of thrombin-yielding potency, even without any eluate (4).

2) Eluate, with Ca, yields very little thrombin, even with the adequate provision of AcG (5) (7).

3) When the system is completed by addition of a 'thromboplastin', whether (a) cephalin or (b) crude tissue tpln., thrombin-formation is rapid and optimal.

4) Cephalin is remarkably effective and compares well with tissue thromboplastin as to activity (shortest clotting-time) attained, but is definitely slower (7 min., instead of 2-3 min.) in reaching this optimum. In fact, the end-point comparison in (10) and (8) indicates practically complete thrombin formation in the cephalin experiment.

5) Extra proconvertin, in (9) and (11), affords no benefits, which agrees with the analytical findings (Table VI), showing that the eluate already contains it in adequate amounts.

CONCLUSION. The major conclusion from the foregoing experiments is that an extremely important clotting function must be assigned to factors playing a 'thromboplastic' role. Significant among such is the phospholipid cephalin.

6. EXPERIMENTS ON THROMBIN-FORMATION.

(6,A) REFERENCE STANDARDS.

EXPERIMENT 10 (1956) TABLE XII. Figure 14.

PURPOSE. To obtain reference assays, for thrombin yields, on a series of dilutions of 'standard' (canine) eluate, containing prothrombin and proconvertin.

REAGENTS. Eluate (No. 127): was prepared by 0.2 M citrate elution of the usual $BaSO_4$ adsorbate from oxalated dog plasma, as previously described (pp. 94,126).

Specific assays, on Eluate No. 127: (1) thrombin: 0; (2) antithrombin: 0; (3) prothrombin: 573 standard 2-stage (p.103) units, per ml., or 191 on basis of 'percentage' (of normal standard human plasma); (4) proconvertin: 36 (percentage' units, by 1-stage method (p.102); (5) AcG: 1-stage (p.101): trace only.

Fibrinogen (dog: standard): was prepared from the $BaSO_4$-adsorbed plasma of the same dog, by the usual technique (pp.94,125).

AcG$^{\Phi}$: The routine AcG preparation (p.101), namely, $BaCO_3$-adsorbed bovine serum, was partially purified by precipitation at 1/3 sat. $(NH_4)_2SO_4$, redissolving in original vol. of saline and dialyzing 2 hrs. against saline in cold room (4°C). A 1:5 dil. of AcG$^{\Phi}$, by preliminary test, proved optimal for tests of Expt. 10.

Assays of AcG$^{\Phi}$: (1) The data included in Table VII (p.127) show the very considerable removal of antithrombin by this procedure; (2) specific AcG assay (1-stage, p.101) shows considerable loss, viz. 555 'percentage' units, per ml., compared with an untreated sample of the original AcG preparation (1240 'percentage' units), but, nevertheless, a very serviceable preparation; (3) no proconvertin; (4) merest traces of (pro)thrombin.

Thromboplastin: Schieffin's 'Soluplastin' (p. 96).

Ca: 0.15 M $CaCl_2$, optimal, in amount used.

METHOD. Standard 'Method III', except for use of 1:5 AcG$^{\Phi}$ and varying amounts of eluate. Hence: Saline & eluate (3.8 ml), imid. buff. (0.5 ml), 1:5 AcG$^{\Phi}$ (0.1 ml), Soluplastin (0.1 ml), 0.15 M $CaCl_2$ (0.5 ml).

RESULTS. Are given in Table XII, showing the actual test data, and graphically, in Figure 14 , plotting 'end-point' (i.e. minimal) clotting-times against relative strength of eluate. 100 'per cent', in our standard technic, refers to 0.1 ml eluate per 5 ml (total) thrombic mixture (T.M.). Since this represents a 50-fold dilution of the eluate, it will correspond to 573 ÷ 50 = 11 5 standard 2-stage prothrombin units per ml of thrombin-forming mixture.

CONCLUSIONS. This assay series is very satisfactory for measuring differences in the thrombin yields between 150% and 1% ('percentage' units; relative) of eluate. As the amount of prothrombin is reduced, the thrombin yield, as measured by the minimal clotting-time endpoint, is reduced (longer c.t.'s). The required incubation period (3 ± 1 min.) is not significantly different, however, until the prothrombin is below 20 per cent (of standard). With very weak, e.g. 1 per cent, eluate, the lengthening of the incubation period is significant. This might be due to the usual salt concentrations becoming unfavorable at very low protein (specifically, prothrombin and proconvertin) concentrations. That relative excess of salts delays thrombin formation and lengthens clotting-times is illustrated, incidentally, in Expt. 12 (p. 140).

(6,B) ANTIHEMOPHILIC GLOBULIN IN RELATION TO THROMBIN FORMATION BY CEPHALIN AND BY TISSUE THROMBOPLASTIN.

EXPERIMENT 11 (1956). TABLE XIII.

PURPOSE. To study the effects of purified antihemophilic globulin (AHG) on thrombin formation, from eluate prothrombin, in relation to cephalin and to tissue thromboplastin.

METHOD. Method III two-stage procedure, as in the foregoing experiments. Standard volumes of reagents are given on p. 104.

REAGENTS. Eluate and standard fibrinogen, as in Expt. 10. The eluate provides prothrombin, proconvertin, and PTC, (? etc.). AcG: same purified preparation as described on p. 135, used in 1:5 dilution. AHG: A purified preparation of antihemophilic globulin from bovine plasma, for which we are indebted to our colleagues in the Pathology Department, Dr. R. H. Wagner et al.[448]. Our assays for its very potent antihemophilic activity and for a trace of PTC are described in Expt. 34 on p. 191 (Table XXXVII). The other specific assays showed no thrombin, prothrombin, or proconvertin, and test (1) of Table XIII proves that it lacks thromboplastic activity. It does contain citrate and a trace of fibrinogen. The last causes a very small amount of clot when used in the 1:100 dilution, of a 1% stock solution, during the incubation of the thrombic mixtures. It also interferes with tests for antithrombin. Ceph.: 0.1%, diluted from the same 3% stock cephalin solution (p.127) as in the preceding experiments. Tpln.: 'Soluplastin', Schieffelin's (p. 96), used (a) undiluted (1:1) or (b) at 1:5 dilution. Ca: 0.15% $CaCl_2$.

RESULTS. See data of Table XIII. The control (1) without added thromboplastin, but in the presence of AcG and AHG, shows very little thrombin formation. The trace activation is still continuing at 1 hr. but is not significantly increased at 18 hrs. (2) AHG, with thromboplastin (1/1), shows little, if any, difference from the 'AHG-free' control (3), i.e. the same maximal activation (15.9"c.t.) takes the same 3' time. (4) AHG,

with thromboplastin (1/5), again shows no significant differences from the control (5). Both require 10 min. for maximal activation and the slight difference in end-point clotting-times is experimental and actually not in favor of the AHG-containing mixture. The trace of fibrin in the T.M. could be removing a little thrombin. A very different result is seen in the cephalin experiments, (6) with AHG, and (7) without it. There is a definite improvement in activation rate (7 min. vs. 15 min.) and also a decidedly better (15.7" vs. 25.5") thrombin yield, when AHG is present.

DISCUSSION. The circumstances in test (6) permit the interpretation that we are, in basic principle, performing, by a new method, the essential reactions involved in the "thromboplastin generation test" (see p. 81). Cephalin is an 'incomplete' thromboplastin and does not yield thrombin formation either as fast as, or, in the present concentration (10γ/ml mixture), as completely as, tissue thromboplastin. With the AHG (etc.), however, it does complete the thrombin formation to the same (15.7 sec. c.t.) end-point, and in a time (5-7 min.) between that of the 1:1 tpln. (3 min.) and that of the 1:5 tpln. (10 min.). It is presumed that PTC (and possibly other plasma thromboplastic factors, see p. 46) are available from the eluate. See Expt. 34 (Table XXXVII).

CONCLUSION. This experiment gives some very important information about the thromboplastic effect of the phospholipid cephalin, in that it indicates how a weak partial thromboplastin may be converted into a more powerful complete thromboplastin, when AHG (antihemophilic globulin) participates in the reaction(s). There is evidence of

some antihemophilic factor contaminant of our dialysed eluate preparations (Table XXXVII, Expt. 34). This could explain why cephalin acts as well as it does, in test (7) for instance. It may very well be that this is the significant difference between our eluates and Seegers' more purified prothrombin (p. 259), with respect to activation by Ca + cephalin.

ADDENDUM. ROLE OF PTC? We do not at present have a suitable PTC preparation, to investigate its probable participation (according to other current knowledge) in the generation of 'complete' thromboplastin. We may suggest, however, that it should be possible to study this in much the same way, with the basic test eluate prepared from a very PTC deficient plasma. Some of our[298] human cases would be the most suitable source of such plasmas. It is just possible also that such might be prepared from a dog after administration of dicumarol, tromexan, or similar drugs. We have some unpublished data on PTC-lowering (cf.[350]) in several human cases undergoing such therapy. Low levels of PTC were found in all such cases tested, but it is questionable whether low enough levels could be thus obtained for the suggested experiment without too much loss of prothrombin and proconvertin.

($6B_1$) THE THROMBOPLASTIN GENERATION TEST.

We shall not include the experience we have ourselves had with the "thromboplastin generation test" (p. 80). We do not use it as a clinical routine because we believe our more specific AHG and PTC (and anti-AHG and anti-PTC) assays give more significant quantitative information. The thromboplastin generation test may be considered to have considerable practical value in differential

'spot' diagnosis of the hemophiliac diseases. The test has a value, in these disorders, comparable, perhaps, to what the Quick (prothrombin time) test has in the different group of prothrombin and related deficiencies. There are a number of current workers, both in England and in America, who are experimentally studying variants of the thromboplastin generation test in order to learn more about the basic mechanisms and possible 'intermediates' in these reactions (ref. Bergsagel and Hougie[40], cited p. 84). We are content, therefore, to leave to others this particular line of inquiry and, merely, to add our own confirmatory data, to what is now widely known[354] and used, namely, that cephalin can substitute excellently for platelets (see p. 81) in the performance of many variants of the thromboplastin generation test. We shall, however, review some of our own experiments of many years ago and some more recent confirmatory data on certain aspects of the problem of 'intermediates' in thrombin formation, with particular reference to the phosphatide cephalin and the role of calcium.

(6,C) A CALCIUM-CONTAINING 'INTERMEDIATE' IN THROMBIN FORMATION ?

EXPERIMENT 12 (1956). Table XIV.

PURPOSE. To decalcify a thrombin-forming mixture at various times from start of prothrombin activation and to observe the effects on thrombic activity. Sequestrene-Na_2 chosen as the decalcifying agent.

REAGENTS. Sequestrene-Na_2 ('Versene' or EDTA), Alrose Chemical Co., Providence, R. I., has a molecular weight of 372 and solubility, in water at 26°C, of 11.1 g/100 ml. 100 g EDTA 'sequesters' 10.5g of Ca^{++}. 3.72g dissolved in dist. water to 50 ml volume = 0.2 M

(stock solution). EDTA: 0.1 M was used in Expt. 12.

Eluate (No. 127) see p. 135.

AcG$^{\Phi}$, partially purified, see p.135 .

Ceph.: 0.1% cephalin, see p. 127.

Ca: 0.1 M $CaCl_2$.

Fibr.: std. (dog) fibrinogen, see p. 125.

METHOD. A special problem was presented by the high ionic strengths of the total salts in the mixtures used in Expt. 12. A compromise was made which permitted the obtaining of clotting-times, which were reliably readable. Although prolonged by the high salt content, they yet allowed the necessary control of the calcium.

Thrombic Mixture: 1.6 ml saline, 1.0 ml imidazole buffer, 0.2 ml AcG$^{\Phi}$ (1:5), 0.2 ml ceph. (0.1%), 1.0 ml eluate, 1.0 ml $CaCl_2$ (0.1 M). 28°C.

Series (A): 0.15 ml T.M. + 0.45 ml fibrinogen-sequestrene (i.e. 8 vols. fibr. 1 vol. 0.1 M EDTA). This is properly controlled to be equivalent in all salts to the final test mixtures in series (B).

Series (B): After 5 min. of incubation of T.M. (above), 1.2 ml was added to 0.6 ml sequestrene (0.1 M) and 0.2 ml samples tested on 0.4 ml (untreated) fibrinogen, 'immediately' (15 sec.) and at stated times thereafter.

Series (C): After 68 min. of incubation of T.M. (above), another 1.2 ml was treated with sequestrene (0.6 ml) and 0.2 ml samples repeatedly tested, as in (B).

RESULTS. Under these particular experimental conditions, maximal thrombic activity was attained in the T.M. after 15 min. incubation, according to test series (A), in which the T.M. was incubated alone

and the EDTA added (to control the salt mixture) only with the fibrinogen. In series (B), the initial 5 min. test, immediately after adding EDTA to the T.M., was only very slightly longer than the corresponding test of the (A) series. Thereafter, however, as incubation of the T.M. with the sequestrene continued, thrombic activity was not merely arrested, but actually underwent a very significant reversal to progressively longer and longer clotting-times. In series (B), the initial test was, if anything, slightly better than the minimal clotting-time of series (A), but the small difference may have been due to use of a fresh tube of fibrinogen. On incubation with the sequestrene, followed for an hour, there was no trace of the 'reversal' phenomenon seen in series (B), but the T.M. retained its activity with excellent stability.

DISCUSSION: This experiment is a successful confirmation of an observation which the author first made in 1937[128], using Howell-type prothrombin + Ca + cephalin as the T.M., and repeated in 1947[156] with a T.M. composed of Seegers' purified prothrombin Ca + 0.25% tissue thromboplastin (from brain, Squibb's). These data are reproduced on pp. 146-149. 1 M oxalate or 1 M citrate were used as 'decalcifying' agents. The former produced a heavy precipitate of CaC_2O_4 and the latter a translucency of clots, which gave difficulties in the reading of end-points. The clot-timing was carefully performed, however. The sequestrene experiments have good end-points. An extremely important conclusion was drawn from the first presentation (1937) of this experiment, namely, that the results can only be explained by postulating an intermediate Ca-containing 'complex' (of prothrombin + thromboplastic factor, and

we should now, doubtless, include the probability of the accelerator and convertor 'co'-factors also). This 'intermediate' is demonstrable only during the earlier phases of prothrombin conversion. After a time, thrombin formation is complete and the "ripe" thrombin can be decalcified without altering its clotting potency. Some evidence of a true reversal, back to inactive prothrombin in the presence of excess of the decalcifying agent, was allegedly adduced in the 1937 experiments (see pp. 147-148, but cf. p. 153).

Ignoring the citrate in the eluate (which isn't quite fair, since it amounts to 0.04 molar in the 5 ml T.M., containing 1 ml of 0.2 M sod. citrate used in the eluate preparation), the following computation indicates the excess (equivalents) of EDTA over the added (1 ml of 0.1 M) calcium: In 3 vols. of EDTA-treated T.M., the 2 vols. of T.M. supply 2/3 x 0.02 M (1/5 of 0.1 M) Ca, while the 1 vol. EDTA supplies 1/3 x 0.1 M sequestrene-Na_2. The relative molarities do not tell the full story, however. Because of the ability of Ca to displace some of the Na in the chelating agent, 100 g EDTA actually 'sequesters' 10.5 g Ca^{++}. Therefore, the above amount of EDTA, namely 24.8 milligrams per ml, is actually enough to sequester 2.5 mgm Ca. The added Ca per ml of mixture, however, is only 0.53 mgm. Hence there is nearly 5 times as much sequestrene-Na_2 added as would be required to remove all the added calcium, even if the 0.04 M citrate played no part in the removal of Ca-ions. Even a slight insufficiency of EDTA, leaving a small part of the calcium still ionized, results in failure to show the 'reversal' effect. Instead, slow continued activation occurs in Series (B), under these conditions.

CONCLUSION. These experiments support the hypothesis that an 'intermediate' containing loosely-bound calcium is temporarily present during the activation of prothrombin to thrombin. The final ("ripe") thrombin can be decalcified without loss of potency.

EXPERIMENT 13 (1956). Table XV.

PURPOSE. To repeat Expt. 12, but with the following modifications: (a) use **dialysed** eluate, in order to provide more favorable ionic strength conditions, and (b) substitute tissue thromboplastin for cephalin. Further, to demonstrate whether the sequestrene-'reversed' mixture can be re-activated by subsequent restoral of calcium, etc.

METHOD: follows the same general principles as in the preceding expt. Temp.: 28°C.

Thrombic Mixture (T.M.): 7.2 ml saline + 1.0 ml imid. buff. + 0.4 ml AcC$^{\Phi}$ (1:5 dil.) + 0.4 ml 'Scluplastin' (1:10dil.) + 0.8 ml. dialysed eluate (No. 127) + 0.2 ml $CaCl_2$ (0.1 M).

Dial. EL.: The dialysed eluate assayed 90 percentage prothrombin units/ml, and proconvertin 216 percentage units.

SERIES (A): 0.15 ml samples of T.M. tested on 0.20 ml fibrinogen +0.05 ml 0.2 M sequestrene-Na_2, at stated incubation intervals.

SERIES (B): After 1½ min. incubation of T.M., an 8 ml aliquot was added to a waiting 4 ml of 0.2 M EDTA, and a second test series started at the 2nd minute, now using 0.2 ml samples of mixture (B) to 0.2 ml untreated fibrinogen.

SERIES (C): After 1½ hr., the remainder of mixture B was transferred to a Visking casing (cellophane) bag and dialysed 1 hr. against three changes of 0.85% NaCl. The casing membrane was tied so as

just to enclose the material to be dialysed, with the result that there was no volume change during the dialysis. Subsequently, a 3 ml aliquot was mixed with 1 ml of 'Calcium activator mix' (Ca.-mix: 3.0 ml imid. buff. sal. + 0.8 ml AcG$^{\Phi}$(1:5) + 0.8 ml 'Soluplastin' (1:10) + 0.4 ml 0.1 M $CaCl_2$) and 0.25 ml samples tested against 0.2 ml of untreated fibrinogen, after successive incubation intervals.

SERIES (D): As a control for test series (C), 0.08 ml dialysed eluate was substituted for saline in a 1 ml mixture similar to the 'Calcium activator mix' in proportions of other ingredients, and diluted with 3 ml of buff. saline just before adding the eluate and beginning the test series, viz. 0.25 ml samplings tested on 0.2 ml fibrinogen, as in (C).

RESULTS: are given in Table XV. Notwithstanding the more rapid and complete activation (in about 5 min. in (A) and in (D) series) by use of tissue thromboplastin, the thrombin formation could be interrupted after 1½ min. by adding 0.2 M sequestrene (1/3 total vol.) and 'reversed' in a manner qualitatively indistinguishable from that observed in Expt. 12. The initial clotting-time in the (B) series was 108.4". In an 8½ hr. test (not included in Table XV), the c.t. was 323". The attempt (series (C)) to reactivate incubate (B), by (a) Ca-addition (not illustrated) or (b) activator mix (Ca-mix), was unsuccessful. Within the few seconds of experimental error, all tests of the (C) series reproduce the (B) c.t. value (at 90 min.), with which the dialysis started. Only further c.t. lengthening (cf. (B) at 185 min.) was arrested. Nothing like the control series (D) was shown in (C).

DISCUSSION. Success in this rather tricky experiment depends upon careful consideration of (1) ionic strengths (total salt content), (2) amount of sequestrene, (3) rapidity of activation (depending upon amounts of AcG, thromboplastic agent, calcium, and eluate in the T.M.), and (4) choice of a period during activation at which the EDTA can start its action before completion of thrombin formation, since "ripe" thrombin (Expt. 12) is insensitive to sequestrene inactivation.

EARLIER DATA. The following data are selected from some 1937 experiments, published by the author[128].

EXPERIMENT 14 (1937). TABLE XVI.

PURPOSE. To study the effects of excess oxalate at various stages of thrombin formation.

METHOD. A two-stage technique, essentially similar to the foregoing.

REAGENTS. Pro.: Howell-type prothrombin, from Berkefeld-filtered citrated dog plasma (see pp.161-164). No attempt was made at the time to assay its prothrombin content, and nothing was then known about proaccelerin and proconvertin.

Ceph: 0.1% brain cephalin[126] prepared according to Howell[235].

Ca.: 0.1 N (i.e. 0.05 M) $CaCl_2$.

Oxal.: N/1 $K_2C_2O_4$.

Fibr.: Precipitated from $Mg(OH)_2$-adsorbed citrated dog plasma with 1/4 sat. $(NH_4)_2SO_4$, reprecipitated, and again a third time now with 1/2 sat. NaCl. This fibrinogen was prothrombin-free according to test with Ca. + Ceph., which gave no trace of clot, at 38°C, in 24 hrs[126,128].

T.M.: 40 ml Pro. + 4 ml Ceph. + 4 ml Ca. Test 0.5 ml samples with 1.0 ml Fibr., after various incubation periods. 38°C.

SERIES (I): After about 11 min. activation, 10 ml T.M. was diluted with an equal vol. distilled water (control test) and incubated and tested parallel with (II).

SERIES (II): After 12 min. activation, another 10 ml aliquot of T.M. was mixed with an equal vol. of N/1 $K_2C_2O_4$ and 1 ml samples tested at intervals thereafter with 1 ml fibrinogen.

SERIES (III): After 4 hrs. activation of T.M., a 3rd 10 ml sample was treated with oxalate and tested in a similar manner.

RESULTS: These are given in Table XVI.

EXPERIMENT 15 (1937). TABLE XVII.

PURPOSE. To study the effects of excess citrate at various stages of thrombin formation.

METHOD AND REAGENTS: As in Expt. 14, except for substitution of N/1 sod. citrate (for the oxalate).

RESULTS: are given in Table XVII.

DISCUSSION. (Expts. 15 and 16). The obvious differences between (1) 'fresh' thrombin (10-15 min. incubated T.M.) and (2) 'ripe' (several hours old T.M.) in the progressive inactivation of (1), but not of (2), with a 50-100 fold excess of oxalate or citrate were noted[128]. The only effect on 'ripe' thrombin, as in the initial (1/4 min) test on 'fresh' thrombin, is a prolongation of clotting-time (compared with the control) due to the immediate effect of the high salt content.

The cited publication (p. 758 of Ferguson[126]) includes mention of the following: "By dialyzing 'fresh' thrombin which had been progressively inactivated by citrate to a clotting time of about 1 hour, we have been able to check the inactivation, and by recalcifying to restore

the clotting-time to a value almost identical with a control consisting of untreated prothrombin with a like amount of calcium and cephalin." (cf. p. 153)

Electrodialysed 'ripe' thrombin (see p. 116) was also unaffected by oxalate or citrate except for the 'immediate' effect of the high salt concentration, which was exaggerated by the low potency of these particular thrombin preparations.

The thrombic mixtures (T.M.), untreated, were remarkably stable in these early experiments (p. 760 of Ferguson[126]).

CONCLUSION (cited from [126]) . . . "It is clearly indicated that calcium forms an intermediary complex (prothrombin-cephalin-calcium "compound"). This complex soon passes over into a stable thrombin. The intermediary can readily be deprived of its calcium with resulting inactivation, whereas the final coagulant can be prepared calcium-free without significant loss in potency" . . . . "The mode of action of the so-called "decalcifying" anticoagulants is now elucidated. In addition to their classical effect of preventing clotting by depression of the ionization of the calcium salts necessary for thrombin formation, they can progressively remove calcium from the "intermediary complex" . . . . "Once the thrombin is "ripe" or fully elaborated, however, calcium is now no longer an essential component and oxalation or citration ceases to affect its potency, except for the usual non-specific "immediate effect" . . ."

EXPERIMENT 16 (1947). Figure 15.

PURPOSE. To confirm the 1937 expts. (14;15) cited[126], but using one of Dr. W. H. Seeger's 'purified' prothrombin preparations and substituting a weak tissue thromboplastin for the phospholipid

(cephalin). This 1947 Expt. was presented graphically in three publications[156,141,147] and is reproduced in Figure 15.

METHOD. Essentially similar to the foregoing.

Series I: (T_1) 13.5 ml Seegers' bovine prothrombin (0.5%) + 0.75 ml tissue thromboplastin (0.25% Squibb's rabbit brain (commercial) preparation) + 0.75 ml 0.1 M $CaCl_2$. Clotting-tests: 1 ml 1% bovine fibrinogen (Armour's) +0.25 ml borate buffer, pH: 7.7 (45 vol. 2.5% H_3BO_3, 45 vol. 0.5% NaCl, 10 vol. $Na_2B_4O_7$, 10 H_2O) + 0.25 ml T_1.

Series II: Same T.M. but tested (second phase oxalate control) by substituting 0.25 ml M/1 $K_2C_2O_4$ for the buffer.

Series III: (T_2) 3 ml T_1 5 min. old + 3 ml M/1 sod. oxal. Clotting tests: 1 ml B.F. + 0.5 ml T_2.

Series IV: (T_3) 3 ml T_1 30 min. old + 3 ml M/1 sod. oxal. Clotting tests: 1 ml B.F. + 0.5 ml T_3.

EXPERIMENT 17 (1956). TABLE XVIII.

PURPOSE. Further study of the progressive inactivation of thrombin-forming mixtures, using cationic exchange resin as the decalcifying agent. Attempted reactivation.

METHOD. Essentially similar to the foregoing (see Expt. 13, p. 144)

REAGENTS: Same dialysed eluate (No. 127), 'purified' AcG$^{\Phi}$, etc. as in Expt. 13. 0.1% Ceph. (p. 127). B.F.: 1% Armour's bovine fibrinogen, adsorbed with $BaSO_4$ (p.95). Dowex '50': sulfonic acid resin (Dow Chemical Co., Midland, Mich.), charged in sodium cycle (p. 275 of Stefanini and Dameshek[453]). I.M.: 13 ml saline + 2 ml imid. buff. + 1 ml AcG$^{\Phi}$ (1:5) + 1 ml 0.1% ceph. + 1 ml dial. eluate + 2 ml 0.02 M $CaCl_2$. All clotting tests use 0.2 ml incubate + 0.2 ml B.F., at 28°C, or (D) equivalent specified.

SERIES (A): Control.

SERIES (B): After 10 min. incubation, 10 ml T.M. is added to 1g Dowex '50' and shaken vigorously at intervals thereafter, with testing of clotting potency of the clear supernatant.

SERIES (C): After 1 hr. incubation, another 5 ml of T.M. incubate is similarly treated with 0.5 g Dowex '50' and retested.

SERIES (D): After 20 min. shaking with Dowex '50', 2 ml of (B) is treated with 0.25 ml AcG$^{\Phi}$, 0.25 Soluplastin (p. 96), and 0.5 ml 0.02M $CaCl_2$. Thereafter, tests are made with 0.3 ml of (D)+ 0.2 ml B.F.

SERIES (E): Original eluate pre-treated with Dowex '50' (100 mg/ml) for 1 hr. (next day) and used in T.M. of same composition as (A).

RESULTS: Given in Table XVIII, show that the decalcifying resin gives an excellent 'progressive' inactivation, in (B) when the thrombin-formation is not quite complete, as seen from the control series (A). The similar tests after 1 hr. (series C) show much less effect of the decalcification. That a little progressive inactivation does occur in this series may be regarded as evidence that the thrombin formation is not yet quite complete. This is probably due to the use of the relatively slow-acting and 'incomplete' thromboplastic factor provided by the 0.1% cephalin.

It was not possible to demonstrate any re-activation by restoral of Ca and other 'activators' in series (D) although these clotting-times were somewhat shorter than in the (B) series, at the start of the (D) experiment. There is possibly a point of some minor significance in the data (e.g. 1st test in (B) and (C)) suggesting a slight 'immediate' inhibitory action of the Dowex '50', which might be due to some alteration of the ionic strength pattern in these mixtures.

Series (E), performed next day, clearly shows that Dowex '50' has no effect on the original eluate. After 1 hour's treatment with the resin, the eluate activates just as well (if not better-? experimental) than in the control series (A), particularly reaching the end-point in an identical (15 min.) incubation period. In other experiments (see Table XX), bovine thrombin and dog fibrinogen were subjected to prolonged treatment with Dowex '50', without significant changes in their reactivity.

(6,D) A LIPID-CONTAINING 'INTERMEDIATE' IN THROMBIN FORMATION ?

EXPERIMENT 18 (1956). TABLES XIX,XX.

PURPOSE. To study the effect of benzene-extraction during the conversion of prothrombin to thrombin in eluates (prothrombin proconvertin) activated by AcG, cephalin, and Ca-salt.

METHOD. Essentially similar to the preceding experiment, and using the same preparations of dialysed eluate, 'purified' AcG$^{\Phi}$ (1:5), cephalin (0.1%), and buffered-saline-Ca (0.02 M). Armour's bovine fibrinogen (B.F.), 1%, $BaSO_4$-adsorbed, was used as substrate: 0.2 ml fibr. + 0.2 ml (or equivalent) of test mixture, after successive incubation periods.

SERIES (1): Control, activation of the usual T.M., viz. 6.5 ml sal. + 1 ml imid. buff. + 0.5 AcG$^{\Phi}$ + 0.5 ceph. + 0.5 eluate + 1.0 $CaCl_2$.

SERIES (2): After 5 minutes activation of (1), an aliquot was treated with an equal volume of benzene in a separatory funnel, with vigorous shaking at intervals, and 0.2 ml samples retested after further incubation.

SERIES (3): After 70 min. inactivation of (2), a 1 ml sample was re-treated with an activator mix of 0.2 AcG$^{\Phi}$, 0.2 Ceph., 0.1 Ca and 0.3 ml samples tested thereafter.

RESULTS: are shown in Table XIX. The data clearly show a progressive inactivation in the b.e. mixture of incompletely-formed thrombin (B). It was not possible to reactivate the 70 min. sample of (2), either by the complete activator mix (C) or in other attempts (not illustrated) using (a) ceph., alone or (b) Ceph. + AcG$^{\Phi}$.

DISCUSSION. This new experiment is a most interesting piece of evidence to support the 1936-1938 suggestion[126,129] that 'free' or 'available' cephalin also participates in the postulated 'intermediary' during thrombin formation. The earlier benzene extraction experiments (pp. 125 et seq.) showed that fibrinogen, eluate (prothrombin and proconvertin) and the AcG preparation could be shaken with benzene without more than minor changes in potency. Control tests on a bovine thrombin (i.e. fully formed thrombin), shown in Table XX, likewise demonstrate the essential insensitivity of thrombin to the benzene-extraction technique. With these controls, therefore, the progressive inactivation of partially-formed thrombin (series (2)) is particularly significant.

CONCLUSION. The foregoing evidence, then, definitely points to a loose binding of 'free' phospholipid (cephalin) into some 'intermediary complex' during the conversion of prothrombin to thrombin. Removal of such lipid by benzene-extraction does not seem to restore the original prothrombin (activatable by Ca., Ceph., AcG), but to result in some inactive by-product (? 'autoprothrombin', in Seegers' sense).

SUMMARY OF DATA ON THE POSTULATED 'INTERMEDIARY' COMPLEX (Expt. 12-18).

That there is a progressive inactivation, not merely an arrest of further thrombin formation, when a partially activated thrombin forming mixture is treated with a decalcifying agent, has been repeatedly confirmed by experiments which we have made in 1937, 1947, and again in 1956. In all, we have used (a) three different types of prothrombin preparation (Howell-type, Seegers-type, and our own 'eluate', (b) four types of decalcifying agent (oxalate, citrate, sequestrene, and ion-exchange resin), and (c) both partial (cephalin) and complete (tissue extract) types of thromboplastin. On the other hand, the completely-formed or 'ripe' thrombin is essentially devoid of calcium and is not specifically affected by the decalcifying anticoagulants. The progressively inactivated mixture cannot be restored to prothrombin (activatable by the usual means, i.e. Ca, thromboplastin, proconvertin, AcG). Earlier (pp. 147-148) suggestion[128] that such reactivation might be possible must be reinterpreted as probably due (as was conceded at that time) to a considerable amount of the original prothrombin persisting unchanged through the test. We were always able to increase the potency of our electrodialysed thrombin by further recalcification, for instance. The tentative suggestion is that the decalcified intermediary changes to an inert by-product, perhaps similar to Seegers' 'autoprothrombin(s)'.

The new (1956) experiments demonstrate an apparently similar progressive inactivation when a partially activated mixture (prothrombin + proconvertin + AcG + free cephalin + Ca) is treated with benzene, whereas the final ('ripe') thrombin is unaffected by this lipoid solvent.

These data support the hypothesis, advanced by the author in 1937, namely, that the conversion of prothrombin to thrombin involves an intermediary step, during which the calcium and mobile phospholipid can be removed, resulting in a progressive inactivation phenomenon. The new addition to this theory is the suggestion that the removal of either Ca or cephalin causes the intermediary to alter its normal reaction pattern (toward thrombin formation) and be diverted into some side-reaction yielding an inactive by-product (? 'autoprothrombin(s)').

(6,E) FATE OF PROCONVERTIN DURING THROMBIN FORMATION.

Preliminary experiment. In a repetition of Expt. 17, 100 mg/ml Dowex '50' was added 5 min. after the start of activation of the usual (A) T.M. (same mixture as on p.149). Just after the addition of the resin (B) the test clotting-time was 20.9". After 1 hr. with the Dowex '50' it has lengthened to 106.4". Standard one-stage proconvertin assays (p.102) gave the following 'percentage' values: (A) i.e. untreated T.M. at start, 110%; (B), i.e. 1 hr. after adding Dowex '50', 54%. This could mean merely that about half the original proconvertin was consumed (utilized) in forming the 5 min. (? intermediate) thrombin and was surviving in the subsequent resin incubate. The answer is certainly negative to the purpose of the enquiry, namely, to see if there was any increase in proconvertin activity, as with some of Seegers' group's[13] experiments with 'autoprothrombin'.

It remains for future investigation to explore further the possible connections between our line of approach and the quite different methods used in Dr. W. H. Seegers' laboratories.

(6,F). QUANTITATIVE RELATIONSHIPS OF CEPHALIN (VARYING CONCENTRATIONS) TO RATE AND YIELD OF THROMBIN FORMATION.

When the prothrombin and proconvertin (in the eluate), the AcG, calcium, and saline-buffer are kept constant, our two-stage Method 'III', becomes a valuable quantitative technique for assay of thromboplastins, according to the effects which their additions have upon the rate and yield of thrombin, as determined by the clotting-times (for a standard fibrinogen) in the second stage of the test. Since these assays are relative, it is necessary, on each occasion, to prepare a reference series of dilutions of some 'standard' at the same time and with all the same reagents as in the tests with the unknowns. We choose as standards: (1) dog brain cephalin suspension (p. 127), when testing various lipid preparations, (2) 'Soluplastin' (p. 96), when comparing various tissue thromboplastin preparations, and (3) freshly obtained platelet suspension from a normal human donor (p. 94), when assaying a patient's platelets for 'preformed' thromboplastic component (factor '1', in our listing, p. 87).

EXPERIMENT 19 (1955). TABLE XXI.

PURPOSE. To prepare a standard 'reference series' of cephalin dilutions and to study the effects on rate and yield of thrombin formation by 'Method III', as previously described.

REAGENTS. The prothrombin and proconvertin eluate is described on p.126 , the 'untreated' preparation being used in routine assays. The AcG is described on p.101 and is further 'purified' (p.135) only for specially sensitive assays where it is desirable to minimize

any instability of the end-point). The usual saline, imidazole buffer (pH: 7.3), and $CaCl_2$ are used.

The standard T.M. (thrombic mixture): 3.7 ml saline + 0.5 ml imidazole buffer + 0.1 ml AcG (1:8) + 0.1 ml thromboplastic preparation (to be tested) + 0.1 ml eluate + 0.5 ml 0.15M $CaCl_2$. At successive incubation periods, 0.2 ml T.M. is tested for the clotting-times (at 28 °C.) with 0.2 ml fibrinogen (dog, p. 94 ; or bovine, p. 95).

RESULTS: are shown, for an illustrative experiment, in Table XXI.

DISCUSSION. Some of the test series were not carried to completion. They are, in fact, a typical reference series taken at random from one of our routine studies in connection with investigation of the thromboplastic potency of various lipoids (see p. 175). They include one or two small imperfections to illustrate the occasional experimental error, e.g. test series 4 is 'out of line'. By and large, however, they are reliable and serve to demonstrate the following:

(1) the smaller the concentration of cephalin, the slower the rate of thrombin formation. This is denoted by the sequence of clotting-times and, particularly, by the incubation period required to reach the minimal clotting-time end point.

(2) the less the cephalin, also, the less complete is the thrombin yield. This last is measured by the end-point (minimal) clotting-times, in a relative manner, and can be quantitated by use of reference standards of eluate dilutions, such as those given in Figure 11 and Table XII.

(3) while our cephalin is usually to be regarded as an incomplete thromboplastin, especially when comparing activation rates with

those in similar thrombic mixtures but substituting tissue thromboplastin (e.g. Expt. 9, p. 133), the final thrombin yield in our test systems is often surprizingly good, viz. 90-100 per cent in some cases and seldom under 50-60 per cent, if optimal strength cephalin is used. For suggested explanation see p. 139.

(4) that there is an apparent 'optimum' cephalin concentration is illustrated in Table XXI by the poorer yield (28.1" c.t.), despite the shorter (15 min.) incubation period, in the case of the 100 strength (test 1), as compared with the 50γ (test 2), viz. 22" minimal c.t. in 20 min. incubation.

That this is really due to the fact that cephalin is still a relatively crude preparation (see later) and contains some lipid inhibitor is now well recognised[73]. Similar inhibition has also been reported for excess or certain fractions of tissue thromboplastin[4,91,358,359,193] and even for platelet suspensions[261]. This immediately suggests, however, that our test can also be used to assay such antithromboplastic inhibitors. This we do simply by mixing the unknown material with an optimal (usually 50γ) cephalin and noting the reduced thromboplastic potency.

(5) The least cephalin concentration tested in this particular experiment was 0.78 or 1:1,280,000 (test 8). This is still very much superior to the control (0), with no added thromboplastin. It is obvious, therefore, that the method is extremely sensitive and is well able to detect thromboplastic activity in lipid suspensions (such as cephalin) diluted to something of the order of one in several millions. The self-imposed limits of the illustrated

test series easily suffice for screening tests on unknown lipids, such as those to be described in a subsequent section (p. 175)

CONCLUSION. These assays are an exceedingly sensitive measure of the relative thromboplastic potency of (cephalin-like) lipids. Alternatively, used with cephalin, they are equally sensitive indicators of antithromboplastic (anticephalin) inhibitors. The cephalin concentration determines both the rate of prothrombin activation and (with sub-optimal strengths of cephalin) the final thrombin yield.

EARLIER DATA. While the Howell-type prothrombin used in our earlier research was not as good and as well controlled as in the present recent studies, it did suffice to establish the same fundamental conclusions, as shown in the following data reproduced from a 1938 publication[129].

EXPERIMENT 20. (1938). TABLE XXII.

PURPOSE. To study the effects of varying cephalin concentrations on the rate and yield of thrombin formation.

REAGENTS. Fibrinogen, Howell-type prothrombin (Pro.), Ceph., and Ca(M/10), as described on p. 146.

METHOD. Essentially the same type of two-stage, i.e. (1) activation and (2) testing with fibrinogen, as in preceding experiments.

T.M.: 10 ml Pro. + 1 ml Ceph. (cited final dilutions) + 1 ml Ca.

Clotting Test: 1 ml fibr. + 0.5 ml T.M., at successive incubation periods.

RESULTS: are reproduced in Table XXII (Table 1 of cited ref.[129] in which the data are also presented graphically in its Fig. 2).

DISCUSSION. The results are essentially the same as those in Expt. 19. Instability of the end-point was often troublesome with Howell prothrombins (see later) and there was the slight possibility that the differences persisting after 3 hrs. incubation might be partly due to instability complications. However, this possibility could not explain the 4 hr. tests, where, after a 5 min. incubation with an additional 1/5 vol. of 1:1000 cephalin, all series attained a minimal c.t. of 12". The extra dilution of the T.M. in all probability, sufficiently accounts for the 12" value. It may be recalled that it was not until November 1939, that Mertz, Seegers, and H. P. Smith[332] published the results of their experiments, showing similar relationships of tissue thromboplastin. The two-stage assays of prothrombin and thrombin gave much weight to the findings of these reliable investigators. However, we continued to believe that relative clotting-times in carefully controlled comparative experiments, were equally valid evidence of the phenomena under consideration.

(6,G) EXPERIMENTS WITH TISSUE THROMBOPLASTIN.

In 1948, we again performed the foregoing type of experiment, but with highly purified prothrombin and using a tissue thromboplastin. The following data are reproduced from that work[156]:

EXPERIMENT 21 (1948). TABLE XXIII.

PURPOSE. To study the effects of varying concentrations of tissue thromboplastin upon the rate and yield of thrombin formation from highly purified prothrombin.

REAGENTS. Prothrombin(Pro. A) was prepared by ourselves from citrated dog plasma following closely the procedures (for bovine prothrombin) of Seegers, Loomis, and Vandenbelt[431]. It corresponded

to 'Product 4' (stage of purification) of the cited authorities and compared very favorably with a number of Seegers' own (bovine) preparations, with which we also experimented at that time[156].

Tpln. A: Squibb's (commercial) rabbit brain thromboplastin.

Fibr.: 1% B.F. (Armour's, bovine, - see p. 95).

METHOD. 5 ml vol. T.M., containing 2 ml Prc. A + 0.25 ml 0.1 M $CaCl_2$ + tpln. A (final concentrations stated in Table XXIII), in borate buffer, pH: 7.7 (see p. 149). Clotting tests: 0.5 ml B.F. + 0.25 ml T.M.: 25 ± 2°C.

RESULTS: are summarized in Table XXIII, stating (a) the final tpln. conc., (b) the end-point clotting-times (sec.), (c) the incubation period needed to reach the end-point.

DISCUSSION. Seegers-type prothrombins at that time undoubtedly contained proconvertin but probably only questionable traces of proaccelerin (AcG)[494]. However, these factors were not generally known at the time these experiments were performed. It was merely a demonstrable fact that in our experiments, Seegers-type prothrombins could be activated very slowly under unfavorable activation conditions, e.g. with Ca alone, or sometimes without any addition[146]. Cephalin was a poor activator of Seegers' prothrombin[146]. Nevertheless, the stability of our end-points often for 3-4 weeks at room temperature, in the bacteriostatic borate buffer solution, was indeed remarkable. The fact that small differences in the end-point clotting-times were apparent in the data (see Table XXIII), therefore, did seem to confirm the fact that " . . . below a certain "optimum", lessening the thromboplastin definitely reduces the final amount of thrombin formed, in addition to greatly slowing the rate of activation".

ADDITIONAL EXPERIMENTS, WITH THROMBOPLASTIN, tested on eluates (1956) qualitatively resemble the cephalin data (Expt. 19, Table XXI), and will not be cited in detail.

CONCLUSION (Expts. 19-21). The author's data (1938, 1948, 1956) repeatedly confirm the apparent fact that the amount of thromboplastic factor (whether cephalin or tissue thromboplastin), below a certain optimum (which may be a matter of contaminant inhibitor) determines both the rate of prothrombin conversion and the final thrombin yield. These ideas can be added to the 'intermediary' theory very nicely, but it does not seem appropriate to assume that the relationships are 'stoichiometric'. Even if this last could be demonstrated for an 'intermediate', it need not be true for the final thrombin.

(6,H) SOME MODERN ASSAYS AND TESTS ON HOWELL-TYPE 'PROTHROMBIN', WITH REFERENCE TO FACTORS WHICH MAY AFFECT THROMBIN FORMATION.

Because of some uncertainties, in retrospect, concerning clotting factors, other than prothrombin, which may have been present or deficient in our earlier preparations of Howell-type 'prothrombin' (with which many of the fundamental observations presented in this thesis were originally made), it seemed desirable to subject this reagent to the scrutiny of our modern assay methods.

PREPARATION. As in the past (1937, 1938), citrated dog plasma (p. 93) was subjected to Berkefeld-filtration (p. 122) and subsequently defibrinated by warming cautiously in test tubes held in a water-bath a 55°C for two minutes, then filtering through coarse filter paper. Prothrombin papers were prepared from 5 ml quantities of the filtrate by the acetone method of Howell[235] and his pupil Cekada[71]. This consisted simply of rapid precipitation with an equal volume

of acetone and immediate collection of the precipitate on a filter paper in a Büchner funnel, under suction-pump, and quickly washing with a test-tube-full (about 20 ml) of ether and finally drying in an air current vigorously applied with the aid of a jet or electric fan.

Prothrombin Solution (Pro.): is obtained by cutting up the dry papers and extracting them with a suitable volume of distilled water (usually 10 ml per paper) containing a drop or two of 0.5 per cent $NaHCO_3$, and subsequently filtering. The pH should be about 7.5 as tested with (a) phenol red indicator, (b) 'pHydrion' paper (Micro Essential Labs., Brooklyn, N. Y., or (c) the (Coleman) glass-electrode pH-meter. Borate buffer, pH: 7.7 (p. 149) or imidazole-buffered saline, pH: 7.3 (p. 97) are superior extracting agents for the prothrombin papers. The former was used in the following experiment.

EXPERIMENT 22 (1956). TABLES XXIV-A, XXIV-B.

PURPOSE. To assay the principal clotting factors in Howell-type prothrombin.

METHODS. (1) Fibrinogen test: 0.2 ml Pro. + 0.2 ml thrombin (bovine, Upjohn's, 20 units/ml, p. 96). (2) Thrombin test: 0.2 ml Pro. + 0.2 ml fibrinogen (p. 94). (3) Prothrombin assay: by 'improved' two-stage method (p. 103). (5) Proaccelerin assay: by specific one-stage method (p. 101). (6) Antithrombin test: incubate with thrombin as described on p. 126 (Table VII). (7), (8) PTC and AHF assays, p. 191, Expt. 34 (cf. Table XXXVII).

REFERENCE CURVES, for the 1-stage assays were obtained on normal dog oxalated plasma dilutions, and are shown in Figure 16.

RESULTS: are shown in Tables XXIV-A and XXIV-B, comparing the prothrombin (Pro.) solution with the precurser plasmas.

Fibrinogen: is absent from Pro. (4) and (almost) from the heat-defibrinated plasma (3), but, of course, is abundant in the original plasma (1), c.t. = 9.3 sec., and in the Berkefeld filtrate (2), c.t. = 10 sec.

Thrombin: traces are not infrequently (but not invariably) demonstrable in Pro. (4). In the cited test, a small trace of clot appeared in 3/4 hr. but was still very incomplete after many hours.

Prothrombin: The normal ('100%'; about 254 2-stage units/ml) was found in (1). Nearly half was lost in the Berkefeld filtration (2) and in the heat-defibrinated plasma (3). Only 10-15% levels were found in the final Pro. solution (4). Allowing for the double (cf. plasma) vol. of extract (10 ml per paper), the 'recovery' is about 20-29% of the original plasma prothrombin.

Proconvertin: The original plasma (1) assayed a normal 96%. About 1/3 was lost in the Berkefeld filtration (2) and about 1/2 after the heat-defibrination (3). A 20% level was found in the Pro. solution(4), which, correcting for the dilution, represents a 40% 'recovery' of the original plasma proconvertin.

Proaccelerin: The original plasma (1) assayed a normal 80%. This was reduced to 25% after the Berkefeld filtration (2) and negligibly more (22%) after the heat-defibrination (3). The amount (25%) in the Pro. solution (4), corrected for the dilution, represents a remarkably good 50% 'recovery' of the original plasma proaccelerin.

Antithrombin: The test in Table XXIV-B showed this to be essentially absent.

PTC: According to the assays reported in Expt. 34 (pp.191-193), (dog) Howell prothrombin (1:1) is better than a normal (human) plasma (1:5) in correcting a known PTC deficiency. These data suggest that the preparation contains most of the original plasma PTC.

AHF: was also assayed in Expt. 34 (pp. 191-192). It too is positive, but, in Pro. (1:1) was less active than in 1:10 normal human plasma. The data on these assays must be considered very significant.

CONCLUSION. Howell-type prothrombin preparations are not only rich in prothrombin, but also contain significant amounts of proconvertin, proaccelerin, PTC and AHF. They lack antithrombin and fibrinogen, but may contain traces of thrombin. These findings may very well explain why the old experiments with Howell-type prothrombin gave significantly correct answers to a number of basic questions concerning the conversion of prothrombin to thrombin.

(6,I) QUANTITATIVE RELATIONSHIPS OF CALCIUM IN THROMBIN FORMATION.

EXPERIMENT 23 (1956). TABLE XXV.

PURPOSE. To study the effects of decreasing calcium concentrations on the conversion of prothrombin to thrombin in dialysed eluate (prothrombin + proconvertin), in the presence of adequate AcG and tissue thromboplastin.

METHOD. Two-stage technique, similar to that used in previous experiments.

T.M.: 0.2 ml eluate (No. 127, dialysed) + 0.1 ml AcG** (p.135) + 0.5 ml 'Soluplastin' + $CaCl_2$ (volumes of 0.02 M solution cited) +

imid. buff. saline to total 10 ml vol. The stock (aqueous) $CaCl_2$ solution was 0.1 M and all dilutions were made with buff. saline.

Clotting-tests: 0.4 ml fibrinogen (dog) + 0.2 ml "adjusted" Ca (to give a final conc. of 5m M in the T.M. = F test mixture) + 0.2 ml T.M., tested after successive incubation periods. 26°C.

RESULTS: These are given in Table XXV.

DISCUSSION. The Ca conc. was varied between 8 and 0.5 milli-Molar in the T.M., keeping the other components constant. The clotting tests were all conducted at a fixed (final) Ca conc. of 5mM. The effects of reducing the Ca concentration during thrombin formation were (1) a definite slowing of the earlier (e.g. 1 min.) phases of activation, but (2) no significant effect upon the final thrombin yield — all c.t. end-points were within a few tenths of a second of each other (42.5 ± 0.3 sec.).

The negligible activation of the control (6) may just be the trace of (pro)thrombin in the AcG preparation, as noted on p.135 .

EXPERIMENT 24 (1956). TABLE XXVI.

PURPOSE. To study the effect of increasing calcium concentrations on thrombin formation and to investigate whether there is a Ca-optimum for this reaction.

FOREWORD. In the author's[129] 1938 experiments on this subject, using Howell-type prothrombin and cephalin as the thromboplastic agent, the effects of the variable amount of calcium carried over into the final thrombin-fibrinogen reaction (cf. Expt. 2, p.115) were not controlled. This led to some just criticism, especially by Wöhlisch[506], who cited Weitnauer's[499] controlled experiments. In Expts. 23, 24, 25, we have avoided this error by adjusting

the final Ca Conc. in the clotting test mixture to a fixed value. This is conveniently done by including a carefully computed additional buffered Ca-salt solution with the fibrinogen just before the final clotting test. With high concentrations of calcium in the thrombic mixture (T.M.), this results, unavoidably, in some excess of Ca in all the final tests. However, this is a relatively minor point, compared with the control achieved in validating the results of the main experiment.

METHOD. Essentially the same, and with the same reagents, as in Expt. 23, except for the increased amounts of $CaCl_2$. 1 Molar stock (14.7% $CaCl_2$, $2H_2O$) was dissolved in imidazole buffered saline.

RESULTS: These are given in Table XXVI.

DISCUSSION. These experiments were very carefully performed with excellent reagents and apparently homogeneous and only slightly opalescent mixtures. Clotting-times were accurately determined with a stop-watch and the end-points were sharp. The results, however, show some unexplained variability, which is considerably more than in experiments at lower (calcium) salt concentrations. We like to think our experimental error in clot-timings is well within 10 per cent, and indeed, it is usually within a few tenths of a second in literally thousands of tests, in a variety of two-stage systems. We are not quite sure, therefore, whether the variability of the end-points (minimal c.t.'s) in Expt. 24 has any real significance. Accepting a ±10% experimental error, the end-points of I-IV were all within 36 ± 4 sec., and of V (at 45.4") just a little longer than these limits. There was certainly no very striking and consistent reduction in the thrombin yield on increasing the Ca in the T.M.

from 8 to 25 m M. The 8 m M Ca level was best in this series (increasing Ca conc.), as in the decreasing Ca series of Table XXV. There is, therefore, some evidence of a calcium optimum at this 8 m M level. Above this level, the most consistent effects of increasing the Ca are the longer c.t.'s in the one minute test, just as is the case (Expt. 23) in reducing the Ca conc. below this optimum level. In the 5 min. test, however, there appears to be some slight improvement with increasing Ca.

CONCLUSION. It would seem that the main effect of varying the calcium level in our thrombin-forming mixtures is to modify the initial rate of thrombin formation. Effects on the final thrombin yield are very questionable at Ca levels above the optimum and negligible (if any) at sub-optimal Ca concentrations.

EXPERIMENT 25 (1948), TABLE XXVII.

PURPOSE. To study the effects of varying amounts of Ca^{++} during prothrombin activation, using Seegers'[431] 'purified' prothrombin, in presence of tissue thromboplastin.

FOREWORD. This experiment of the author's was published in 1948[156]. In retrospect, we were dealing with a very stable prothrombin, undoubtedly containing proconvertin, but very poor in AcG (hence the slow activation). The proper second phase controls were included.

METHOD AND RESULTS: are given in Table XXVII. They demonstrate:

1) No activation (in 2 hrs.) in the absence of added Ca;

2) slower rate of activation if the Ca conc. in the thrombic mixture is too low (2) or too high (4;5); but

3) no effect on the final (2 hr.) thrombin yield, i.e. identical (4 sec.) minimal c.t. end-points.

CONCLUSIONS (Expts. 23-25). The cited experimental data permit the important conclusions that: the rate of thrombin formation from prothrombin, in the presence of adequate thromboplastin and co-factors (proconvertin and AcG) is determined by the calcium concentration in the activating (T.M.) mixture. Too little or too much calcium retards the thrombin formation, and there is a definite Ca-optimum at about 0.008 M, in protein-poor mixtures at pH = 7.3 and 0.15 ionic strength. However, there is no evidence that the final thrombin yield is affected by varying the Ca, in the range studied. This could mean that any calcium, even a trace, could convert prothrombin to thrombin via the postulated 'intermediary' (p. 148) and then be released again to convert another portion of the prothrombin.

EFFECTS OF TRACES OF CALCIUM IN PROTHROMBIN PREPARATIONS.

Unless ion-exchange resins are used, it is extremely difficult to be sure that all traces of calcium are removed from any plasma fraction, including prothrombin. There is always the question of protein-bound Ca and the possibility of some free Ca^{++} released in attaining the equilibria studied by Nordbö[352]. We theorize, therefore, that the so-called 'spontaneous' activation of prothrombin, e.g. (1) in the experiments of Howell[233] and his pupil Cekada[71] and (2) in our own experience[156] with some of these and with Seegers' highly purified prothrombins are consistent with this ability of traces of Ca contaminant (and, likewise, traces of thromboplastin and co-factors), slowly but significantly to convert the prothrombin to thrombin by mechanisms which are identical in their essential nature with the processes going on in ordinary optimal activations.

(6,J) THE QUESTION OF 'SPONTANEOUS' ACTIVATION OF PROTHROMBIN TO THROMBIN. TRACE CONTAMINANTS ? STABILITY OF PROTHROMBIN AND THROMBIN.

EXPERIMENT 26 (1948). TABLE XXVIII.

PURPOSE. To investigate 'spontaneous' thrombin formation in highly purified (Seegers') prothrombin.

METHOD. This experiment was actually the chance outcome of keeping a solution of Seegers' purified prothrombin (Prep. H, cited[146]), for 9 months in the ordinary (4°C) refrigerator and re-testing out of curiosity, at the end of this period. It was found to be active thrombin. A dilution series (II) was prepared with the same amounts of calcium and tissue thromboplastin (not incubated) as had produced maximal activation of the original prothrombin solution 9 months earlier. At that time, such a dilution series had been tested and the data (I) were available for comparison.

RESULTS: These are given in Table XXVIII.

They clearly show identity of the thrombic potencies at successive dilutions, with minor divergence only at the highest dilutions, which are always subject to some experimental error. It is remarkable that as short a clotting-time as 4 sec., accurately measured with a stop-watch, has real meaning as a measure of thrombic potency. With such matching of serial dilutions, it is an inescapable conviction that the two thrombins are indeed identical.

CONCLUSION. It must be concluded, therefore, that any 'spontaneous' conversion of prothrombin to thrombin may very well be due to trace contaminants of calcium and other activators. These trace

amounts may be far too little to analyse. Nevertheless, given sufficient time, they may complete the conversion to a thrombin of potency identical with that ordinarily obtained after a short incubation period with optimal calcium and other activators. This obviously suggests that the calcium and other activators may be used over and over again in transforming the prothrombin bit-by-bit to the final end product. From one point of view this type of reaction may be regarded as 'catalytic'. This idea would seem difficult to apply to so simple an ion as Ca^{++}, were it not for an explanation on the basis of an 'intermediary' complex, such as we have argued for, on the basis of a variety of other experiments.

ADDITIONAL CONSIDERATIONS. It is valid to draw from the above experiment the following further conclusions:

1) prothrombin and thrombin are remarkably stable proteins in the absence of any factors which can alter them.

2) our purified system is particularly free from inhibitors. It is possible to visualize a less pure system, containing antithrombin, for instance, in which very slow conversion of prothrombin to thrombin may be proceeding but with the thrombin being removed, as fast as it is formed, by the action of antithrombin. Such a system would merely show a gradual loss of the ability of the remaining prothrombin to yield the original amount of thrombin, in successive samplings tested after the conventional activation.

3) At the dilutions studied, viz. about 1000-2000 two-stage prothrombin (Seegers) units/ml, the thrombin formed and in contact with the residual still-unaltered prothrombin for weeks or months showed no evidence whatsoever either of (a) 'autocatalytic'

acceleration of the prothrombin conversion or of (b) any destruction of the prothrombin by the thrombin (cf. [494]). We have repeatedly mixed fully formed thrombin with a prothrombin-activating system and failed to find any acceleration of the activation process, with only one exception, namely, in the presence of platelets. This exception will be studied further in connection with the platelet experiments (p. 196).

4) Either minimal traces or significant small amounts of thrombin could be detected in all 8 highly purified prothrombins, viz. 7 made by Dr. Seegers and 1 by ourselves, which were studied very exhaustively in 1948 investigations[156]. Some of these data, bearing on stability questions, will be reproduced, as follows:

EXPERIMENT 27 (1948). TABLE XXIX.

PURPOSE. To investigate the stability of prothrombin in solution.

REAGENTS. Pro B: One of Dr. W. H. Seegers' very highly purified bovine prothrombin preparations kindly provided for us, with the information that it contained 15,200 units/mg tyrosine N, or over 2000 units/ml of the 0.2% solution used in the present experiments. Tpln. A: Rabbit brain thromboplastin (commercial, Squibb's); Tpln. D: Extract, made with saline, from frozen dog brain.

T.M.: 5 ml., in borate buffer (p. 149), pH: 7.7, with additions, as stated, of 0.25 ml 0.1M $CaCl_2$, with or without 0.25 ml thromboplastin. Clotting-tests at 25±2°C, on 0.5 ml 0.5% B.F. (p.95)+ 0.25 ml T.M., after stated incubation periods, up to 7 days.

RESULTS: are given in Table XXIX.

DISCUSSION.

1) initial tests, about 2 hrs. after making up a 0.2% solution (in borate buffer) of the lyophilized prothrombin B, showed a trace of active thrombin estimated to be less than 0.5 per cent of the total potential thrombin yield (according to dilution tests given in the cited publication).

2) the relatively slow activations in these 1946 experiments, we believe, may very well be attributed to lack of sufficient pro-accelerin.

3) the 1 min. tests chiefly represent active thrombin in the solution, since very little thrombin will be formed in this very short period, under the slow activation conditions noted in (2). The first test in series (3), therefore, indicates a considerable increase in thrombin contaminant spontaneously appearing in the 2 months storage at 4°C, in the ice-box.

4) Although the activation is much slower in (1), with Ca alone, it finally (in 4 days) reaches a stable end-point, c.t. = 3 sec., identical with that reached in a couple of hours in the other two series.

5) While this prothrombin solution was unstable in the sense that it slowly changed to thrombin on simple keeping, yet it was amazingly stable in the sense of yielding the same potency thrombin and in the same incubation time when activated by Ca and tissue thrombo-plastin (2 different preparations) in tests two months apart.

6) Calcium alone gave an identical end-point and it must be conceded that this, although requiring a 4 day incubation, is just as complete a thrombin formation. Hence, it must be assumed that the 'purified'

prothrombin still contains significant traces of thromboplastic and other necessary activators.

EXPERIMENT 28 (1948). TABLE XXX.

PURPOSE. To study further the stability of prothrombin, in the presence of Ca^{++} and thrombin.

METHOD. Since previous experiments had shown the very slow formation of thrombin on addition, to highly purified (Seegers') prothrombin, of Ca salt alone, it was evident that such thrombin as was slowly formed would have every opportunity over several days to exert any possible effect on the still-unaltered prothrombin. Were there any such effect it would be expected to show up in the final (end-point) clotting-times of test samples removed at intervals and rapidly (within 1 hr.) activated by addition of tissue thromboplastin.

T.M.: 15 ml 0.35% Pro. F (Dr. Seegers' preparation, assaying 13,700 units per mg tyrosine N or 2450 units (2-stage) per ml of solution) + 5 ml borate buff. +1 ml 0.1M $CaCl_2$.

Test series (A): 0.5 ml 0.5% B.F. + 0.25 ml diluted T.M., i.e. 2 ml + 0.25 ml buffer, to serve as control for (B).

Test series (B): 2 ml T.M. removed after stated periods and treated with 0.2 ml thromboplastin (various tissue preparations), with successive testing of 0.25 ml samples on 0.5 ml B.F. (0.5%), until reaching the minimal clotting-time end-point noted.

RESULTS: These are given in Table XXX. Note that, in every case, no matter what the age of the recalcified T.M. or which type of tissue thromboplastin was used, the (B) series end-points were all exactly 4 sec. and identical with that reached sometime between the 3rd. and 8th. day in series (A).

CONCLUSION. This experiment completely fails to demonstrate any destruction or other effect of thrombin co-existing for days with its original (partly unchanged) prothrombin (cf.[494]). These observations are important in ruling out possibilities of interfering reactions in the type of experiment relied upon for most of the data in the present investigations.

(6,K) TESTING FOR (A) THROMBOPLASTIC AND (B) ANTI-THROMBOPLASTIC ACTIVITY IN VARIOUS LIPOIDAL MATERIALS, ESPECIALLY CEPHALIN AND SYNTHETIC PHOSPHATIDES, AND THEIR CONTAMINANTS.

EARLIER DATA, (TABLE XXXI.) Using T.M. containing Howell-type prothrombin (cf. Expt. 20, p.158), data were obtained by the author, in 1943, on four fractions obtained by Dr. J. Folch, of the Rockefeller Institute, N. Y., in the re-purification of brain cephalin[168]. Following brief preliminary mention[138], we included these data in the publication of the Transactions of the Second Conference on "Blood Clotting and Allied Disorders" of the Josiah Macy Jr. Foundation[144]. They are reproduced in Table XXXI.". . . Data concerning fractions, the percentage referring to yield in gm. per 100 gm. original "cephalin" mixture: V = phosphatidyl ethanolamine (15%); III = phosphatidyl serine (27%); I = inositol phosphatide (22%); IV = unidentified mixture (8%). In personal communications, Dr. Folch stated that III and I could contain about 5-10% of V (true cephalin) and IV "may very well turn out to be either identical with or closely related to phosphatidyl ethanolamine". Thus the biochemical criteria were not fully adequate for a definite correlation with the thromboplastic activity qualitatively demonstrated by our very sensitive test, which as shown in the table, detected

such activity in all four fractions, although none of them was as potent as a weak buffered suspension of cat brain thromboplastin. Moreover, all the preparations supplied were dried and brownish from months of exposure to the atmosphere and must have lost a considerable amount of their thromboplastic potency. Inconclusive as they are, these tests nevertheless add some evidence to indicate that true cephalin is a potent thromboplastic phospholipid. Any other conclusion will need further advance in the field of phospholipid chemistry.

CURRENT (1956) DATA. (TABLE XXXII). For the past few years the author has been using the preceding test system for the thromboplastic and antithromboplastic (anticephalin) assay of a number of synthetic phosphatides. These agents have been prepared and chemically studied by colleagues, Drs. C. E. Anderson and C. L. Yarbro of the Biochemistry Department at the University of North Carolina. A report to the 1953 annual meeting of the North Carolina Academy of Science recorded the finding of neither such activity in the testing of an acetal-phosphatide (test 5, Table XXXII). Tests on other current materials are listed, as to results, in Table XXXII.

METHOD: Our two-stage (Method III) technique, as previously described (p. 104). Several dog 'eluates' (p. 126) were prepared for use in these studies. The AcG (1:8), see p.101, was a single batch, as was the 'standard' (dog brain) cephalin (p. 127). The 'unknowns' were supplied to us identified only by a code number, and the descriptive data given to us by our biochemical colleagues only at the (temporary) conclusion of these investigations. The following criteria were used for degree (+ or -) of activity.

(a) ++++ : end-point clotting-times of < 20 - 30 sec. (at 25 ± 3°C), equivalent to >10 γ/ml obtained in incubation periods of <10 - 30 min.

(b) ± : slight enhancement of the std. ceph., but no significant thromboplastic activity alone.

(c) ——: end-point clotting-times, in 30 min., with std. ceph. (50 γ/ml) co-addition, of over 5 min., i.e. essentially complete inhibition.

(d) —: end-point (½ hr. incub.) c.t. of 3 - 5 min.

(e) –: end-point (½ hr. incub.) c.t. of 2 - 3 min.

(f) -: end-point (½ hr. incub.) c.t. of 1 - 2 min. (<0.1 γ)

DISCUSSION (ref. Table XXXII). The significant findings, to date, are:

1) synthetic cephalins, containing only saturated fatty acids, e.g. (tests 2, 3, 4) 1, 2-distearoyl; 1, 2-dipalmitoyl; or 1, 3 dimyristoyl, phosphatidyl ethanolamines ('α' in the first two, and 'β' type in the third), are devoid of thromboplastic activity. This confirms earlier data by Grün and Limpächer[202] and Kabashima and Suzuki[263].

2) efforts to synthesize cephalins containing unsaturated fatty acids are currently under way, but not yet available for presentation in this thesis.

3) a typical 'acetal' phosphatide, but again with only saturated fatty acids, viz. stearal-acetal-phosphatidyl-ethanolamine (test 5), (p. 175), is inert in our thrombin-forming test systems; so is the corresponding palmital-compound (test 8), except for an extremely doubtful ± .

4) a myristal-acetal-phosphatidyl-ethanolamine (Test 6), which was 85-90% pure, assayed quite inhibitory (anti-thromboplastic). This looked like a possible lead toward the identification of a lipoid antithromboplastin. However, when this synthetic compound was further purified, it (test 7) lost the inhibitory properties.

5) All efforts to run down the real inhibitor gave only the equivocal answer that it was residual in one or other trace fraction of the mother liquors (tests 9 - 12). None of the possible components which could actually be identified, turned out to have significant effects in our test system and we are unable to suggest its true nature. There was a similar trace of inhibitor in the supernatants (fraction I, test 17) in preparing the palmital acetal P-lipid. Another trace contaminant (II), test 18, however, gave only the negligible ± test. Some of the trace fractions (tests 13, 16) gave increased inhibition when added back to the purified phosphatide (No. 7).

6) One possible lead resulted from these efforts, namely, that the trace contaminants (9 - 11, not 12) showing the most significant effects were mixtures of the less active ones. Indeed, we were able to demonstrate this (15, 16) by actually mixing two of the less inhibitory fractions. This enhanced inhibition by the mixtures suggests a possible synergism.

CONCLUSIONS. These new efforts to identify (a) thromboplastic lipid(s) and (b) antithromboplastic lipid(s), via the approach of synthetic organic chemistry, are still very preliminary. They do lead to the conclusions that:

1) the ethanolamine base is not the primary determinant of thromboplastic activity;

2) known cephalins, containing saturated fatty acids, only, are inactive whether in the α or β form.

3) acetal phosphatides do not have either thromboplastic or anti-thromboplastic activity, associated with their acetal structure.

4) Anything more than this is speculative at the present time. Thus,

5) it is merely a tentative hypothesis, but not yet superceded, that the physiological thromboplastic lipid must be, either (a) a true cephalin of some specific type (? containing unsaturated fatty acids), or (b) a contaminant, or, possibly (c) some metabolic by-product, of the known cephalin group of phosphatides.

Hence this thesis will rest with the current status of this field of knowledge and continue to identify the thromboplastic (phospho) lipid(s) as cephalin(s), for want of a better working hypothesis.

ADDENDUM. Dr. K. M. Brinkhous kindly supplied us with a few milligrams of Dr. E. Baer's (Connaught Labs., Toronto) synthetic preparation[28,29] of 1,2-dimyristoyl-phosphatidyl-α-ethanolamine ('X'), with which our Pathology Department colleagues[282] had reported a feeble thromboplastic action. In our routine tests, a thrombic-mixture, which gave a minimal clotting-time of a negligible 22 minutes (over 1 hr. incubation), was able to give only a 6 min. c.t. with 2.0 ml of 0.1% suspension of 'X', and barely improved to about 4 min. c.t. with 2.0 ml of 'X' (per 5 ml T.M.). These are negligible results and point only to the most minute trace of something that can hardly be regarded as thromboplastic. We must conclude, therefore, that this compound, like our own and others[202,263], is essentially devoid of true thromboplastic properties.

(6,L) ACTIONS OF HEPARIN, WITH SPECIAL REFERENCE TO THE THROMBIN-FORMING REACTIONS.

EXPERIMENT 29 (1939). TABLE XXXIII.

PURPOSE. To study effects of heparin on the formation of thrombin from recalcified prothrombin, in the presence of various thromboplastic agents.[131]

METHOD. The usual two-stage technique, similar to previous experiments.

T. M.: 4 ml Pro. (Howell-type, p. 161) + 0.5 ml $CaCl_2$ (0.05 m.eq.) + 0.25 thromboplastic agent (varied; see below) + 0.25 dist. water or heparin (0.5 mg., equiv. to 55 Toronto units).

THROMBOPLASTIC AGENTS:

1) brain cephalin (p. 127), 100γ per 5 ml T.M.;

2) tpln.-Q., a rabbit brain thromboplastin (dried preparation) prepared and kindly supplied by Dr. A. J. Quick. The present solution analysed: Protein: 104 mg %; Total P-lipids: 11 mg %, incl. 4.6 mg cephalin.

3) brain P-lipid: Quick's thromboplastin was extracted with alcohol-ether (3:1), the acetone-insoluble P lipids being recovered in petroleum ether, dried, and prepared in a 1:10,000 aqueous solution. The amount added in tests 3, 4 represented the equivalent, in phospholipid, of the original tpln. Q. used in tests 5, 6.

4) crystalline trypsin, 125γ : a preparation kindly supplied by Dr. M. Kunitz of the Rockefeller Institute, Princeton, N. J.

The T. M. mixtures were incubated at a cool temperature, 15°C., in order to minimise any instability, but clotting tests, after the stated incubation periods, were made at 38°C, with 0.5 ml T.M. + 1 ml fibrinogen (p.146).

RESULTS: These are given in Table XXXIII.

DATA and DISCUSSION. Any cofactor (see later) which the heparin may need must have come from the Howell-type prothrombin preparation. With the amounts of agents tested in this particular experiment, the heparin proved completely inhibitory to the cephalin (test 2) and practically so to the isolated phospholipid fraction (test 4) from the thromboplastin. Distinctly different was the result of test 6 in which the heparin showed no effect upon the tissue thromboplastin, except in the 1 min. test. Trypsin (see p. 42) was also a good thromboplastic agent in these experiments (7) and resembled tissue thromboplastin in the very minor and early stage inhibition by heparin.

Even though the thromboplastin preparation (5) had been kept (at 4°C) for 3 days, pending analyses and preparation of (3), it was distinctly more potent than its equivalent of the extracted P-lipids.

EXPERIMENT 30 (1941). TABLE XXXIV.

PURPOSE. To demonstrate the significance of a heparin cofactor in its inhibitory effects during prothrombin conversion to thrombin.[151a]

METHOD. Similar technique to the foregoing.

REAGENTS. Pro: Howell-type prothrombin (p.161).

Tpln.: Glass-wool filtered saline suspension of frozen dog brain.

Ca: 0.1 N $CaCl_2$.

Hep.: 5 Toronto units/ml, diluted in saline, from a Connaught Lab. (Toronto) preparation, kindly supplied through the courtesy of Dr. C. H. Best. Stock solution, in saline at pH: 7.5, 0.1%.1 mg dry wt. represented 110 Toronto units.

Cofactor: This agent was a crude albumin obtained by precipitating citrated dog plasma between 50 and 100 per cent saturation with $(NH_4)_2SO_4$, dialysing away excess of salt, and obtaining a final solution in

0.9% NaCl at pH: 7.5.

T.M.: 4 ml Pro. + 5 ml cofactor (or saline, in (1)) + 0.5 ml hep. + 0.25 ml tpln. + 0.25 ml Ca. Incubated at 7.5°C, to avoid any deterioration.

Clotting-tests: Respective T. M.'s (0.5 ml) added to 1 ml fibrinogen (p. 146) + 0.5 ml of saline (tests 1-5) or saline mixture containing amounts of hep., cofactor, or both, required to make tests 6, 7, 8 equivalent, in the final clotting mixtures, to 2, 3, 4, respectively. Tested at 38°C. Tests 5, 6, 7, 8 were made with T. M. (1), at maximal (60 min.) activation.

RESULTS. See Table XXIV. Note the following:

DISCUSSION. (a) The chosen amount of heparin, alone, had a negligible effect on the fully formed thrombin (6), but did inhibit thrombin formation (2) in the early phases.

(b) This particular cofactor ('albumin') was very slightly inhibitory to the 1 hr. T. M. (1), in test 7, and to the later phases of T. M. (3). It was, however, slightly helpful to the earlier phases of activation in (3). In other experiments of a similar nature, we have found either a little inhibition or a little aid in this early phase, differing with various albumin preparations.

(c) The combination of Hep. + Cof. (4) was completely inhibitory to thrombin formation in tests extending over 2 hrs. This is the more significant because the test (8) on fully formed thrombin showed very little in the way of antithrombic action.

CONCLUSION. A cofactor, present in crude albumin fractions, is, therefore, able very greatly to increase the inhibitory effect of heparin during thrombin formation, even when the combined effects after full

thrombin formation are almost negligible. These data support the view that cofactor is needed for the action of heparin in preventing prothrombin activation, and this phenomenon is distinct from the established [242,382] antithrombic action of heparin + cofactor, which is exerted upon fully formed thrombin.

ADDENDA. (I). Brinkhous, Smith, Warner, and Seegers [67], in 1939, presented the first evidence that a serum cofactor is needed for heparin to prevent the conversion of prothrombin to thrombin. Their data may be briefly reviewed. One of Seegers' [435] early purified prothrombin preparations yielded 363 two-stage units after 2 hrs. activation by Ca and rabbit brain thromboplastin and practically as much (343 units) when heparin was also added. With heparin plus serum (cofactor), thrombin formation was blocked and almost all the prothrombin remained unchanged for two hours. With the serum alone, all but 52 units of prothrombin were converted into thrombin in the same time period. This experiment shows incidentally that serum antithrombin acts only on fully formed thrombin. By and large, this experiment proved the new observational fact. It is certain, however, that the two hour incubation period masks any effect which the heparin and serum, alone, might have on the rate of thrombin formation. Because of this doubt, we performed the 1941 experiment [151a] cited above which, we believe, established the validity of the Iowa workers' conclusion beyond any reasonable question, besides adding some new information as to what is going on during the activation period.

(II) The "antithrombic" action of heparin + cofactor: was first observed in the original experiments of Howell and Holt [242]. Quick [382] found the cofactor to be associated with the albumin, rather than the globulin,

in crude plasma fractions. Astrup [20] has reviewed the heparin studies made in the Carlsberg Foundation Laboratories (Copenhagen), including a 1939 confirmation of the Iowa discovery (above) and an important suggestion [21,22] that ...."the co-factor necessary for turning heparin into an antithrombin, which we have called 'thrombin coinhibitor', is a far more delicate substance than hitherto assumed. It disappears from plasma during clotting and is therefore not found in serum, whether prepared by addition of thrombin, by spontaneous clotting or by recalcification. It is further inactivated completely by heating to 56°C for 5 minutes".

(III). Since the topic of this thesis is primarily designed to explore the clotting functions of certain lipids (e.g. cephalin) and complex materials (thromboplastin) in which they occur, experiments with heparin are relevant when they deal with antithromboplastic inhibitions of the thrombin-forming reaction. To go into antithrombic actions, however, would unduly extend our enquiry. We shall, therefore, omit some otherwise valuable experiments, e.g. (1) on heparin + cofactor as antithrombin [131] and (2) some unpublished data, confirmatory of Astrup and Darling [22], which show, by a different method, that serum is devoid of heparin cofactor, but because of progressive removal of added thrombin by the classical serum antithrombin (Astrup [20]), this thrombin becomes so weak in the serum mixtures that is now very sensitive to the immediate inhibitory action of heparin in an effect which requires no cofactor.

EXPERIMENT 31 (1948). TABLE XXXV.

PURPOSE. To demonstrate that the mode of action of heparin in delaying the conversion of prothrombin to thrombin is 'anti-thromboplastic' in nature.

FOREWORD. In the previously cited 1941 publication [151a], the author (with Dr. A. J. Glazko) activated Howell prothrombin with Ca and tissue thromboplastin (in 1/1, 1/2...1/32 dilutions) with and without heparin. The significant observation in the heparin experiments was that the **rate** of thrombin formation was delayed by the heparin the more, the weaker the thromboplastin. The final thrombin yields (end-point c.t.'s) were essentially similar, however.

Another experiment [156] performed about 1948 with one of Seegers' purified fibrinogens, essentially confirmed the earlier finding and was especially convincing because of the adequate controls. This will be reproduced, as follows:

METHOD. Two-stage testing of prothrombin activation by method essentially similar to preceding experiments.

T. M.: 4 ml Pro. E. (ref. [156]) + 0.5 ml borate buffer (p. 149), in control (1), or 0.5 ml heparin (100 units) in series (2), + 0.25 ml tpln. A (rabbit brain, Squibb's, 0.25%) + 0.25 ml 0.1 M $CaCl_2$. 25 ± 2°C.

Clotting tests: (1a) 0.25 T. M. (1) + 0.25 buff. + 0.5 B. F., 1% (p. 95).

(1b) 0.25 T. M. (1) + 0.25 hep. (10 unit) + 0.5 B. F., 1%.

(2) 0.25 T. M. (2) + 0.25 buff. + 0.5 B. F., 1%.

RESULTS: These are shown in Table XXXV.

DISCUSSION. The data show:

1) complete activation to 4 sec. end-point in 8 hrs., in (1a), with complete stability of thrombin for a week thereafter.
2) some inhibition of the fully formed thrombin by the heparin, as shown in (1b). This is very slight, a mere 1/2 sec. (real) difference, after completion of the T. M. activation. The smaller amounts of thrombin in the earlier phases of activation are more significantly

inhibited (delayed) by the heparin, however.

3) inhibition of thrombin formation, in the presence of the heparin, is clearly shown by comparing (2) and its control (1b).

4) However, the final end-point, although it took a week to attain, is identical ($4\frac{1}{2}$") in the (2) and (1b) series.

CONCLUSION. Comparing this result with effects of a minor reduction in amount of thromboplastin (Table XXIII, also reproduced from the 1948 paper) [156], the heparin inhibition of thrombin formation may be regarded as due to an interference with the action of the thromboplastin. Using a potent tissue thromboplastin, the inhibition refers to a delay in rate of thrombin formation, with no effect on the final thrombin yield.

CONSIDERATIONS REGARDING MODES OF ACTION OF HEPARIN.

It should be emphasized that, in these experiments, no 'cofactor' was added and was unlikely to be present as a contaminant of the highly purified prothrombin. Further, the heparin was added in relatively high concentration. It may therefore, be reasonably concluded that these are some special experimental facts pertaining to high concentrations of heparin (itself highly purified). They may have no real connection with a more physiological system in which a much smaller amount of heparin inhibits thrombin formation by acting in conjunction with a plasma cofactor. However, similar results were obtained with physiological amounts of heparin, in Expt. 47, p. 213. The physiological significance of heparin has been reviewed on pp. 51, 52. Our researches have added a little to the understanding particularly of one of its possible modes of action, namely, to prevent prothrombin activation by functioning as an 'anti-thromboplastin'. Study of this phenomenon, in turn, adds to the general knowledge of the thromboplastic mechanism and its very

fundamental role in physiological thrombin formation.

We are endeavoring in this thesis to draw only those conclusions that are well-supported by experimental fact and wish to avoid theoretical speculation as much as possible. In the heparin problems, we are still on insecure ground and the following suggestions are very tentative, but may be worth presenting as a possible lead for future investigations. Heparin (with its sulfuric acid moities) and cephalin (with its phosphoric acid groups) are both acidic substances known capable of combining with proteins and basic protein split products such as protamine (Chargaff and Olson) [80]. May it not be possible, therefore, that the more acidic heparin can block or displace the essential thromboplastic phosphatide from certain protein combinations which are normally essential for the P-lipid to participate in the process of thrombin formation (prothrombin activation) ? May it not further be postulated that the cephalin is thereby deviated to some other protein, say, the cofactor altered (? made receptive) by the action of heparin? This could explain the 'antithromboplastic' action of heparin. The 'antithrombic' action, in somewhat similar manner, could be a deviation of thrombin to some cofactor + heparin complex (Astrup and Darling's [22] 'thrombin inhibitor') which, according to Fitzgerald and Waugh (cited p. 51), functions as a somewhat special type of antithrombin.

(6,M) THROMBOPLASTIC ENZYMES IN RELATION TO THROMBIN FORMATION BY THROMBOPLASTIC LIPOIDS AND (?) LIPOPROTEINS.

FOREWORD. 1939 DATA. The 'thromboplastic' action of crystalline pancreatic TRYPSIN was briefly reviewed on p. 42, and experimental evidence of this phenomenon was incidentally included in Expt. 4 (p. 118) and Table V (on completely platelet-free plasma) and in Expt. 29 (p. 179)

and Table XXXIII (on heparinized thrombin-forming systems, in which trypsin was substituted for tissue thromboplastin), much as Eagle (cited p. 42) had substituted it for Ca + platelets. We shall not cite the experimental details, but merely refer to the conclusions of our early work [150,149,136] viz.

1) trypsin can clot oxalated or citrated plasma and activate (Howell-type) prothrombin, even in the absence of added calcium and cephalin.
2) sufficient excess of the decalcifying anticoagulants can prevent these actions of trypsin.
3) added calcium, and, even better, Ca + cephalin greatly enhance the effects of trypsin. Hence,
4) the author's (with Mrs. Betty Nims Erickson's) 1939 conclusion [149] that trypsin is not a thromboplastic agent in its own right (as Eagle and Harris [112] erroneously concluded), but merely makes Ca^{++} and 'free' cephalin <u>available</u> for the prothrombin activation, presumably by releasing them from otherwise inert protein combinations. Mrs. Erickson and the author were never quite able to provide the convincing proof for this attractive theory and such is still unavailable. However, a 1948 experiment, with B. L. Travis and E. B. Gerheim (graduate students), ref. [156], did seem to add some new evidence on the need for both calcium and thromboplastin in order to obtain the actions of trypsin in thrombin formation. These experiments, therefore, will be recapitulated:

<u>EXPERIMENT 32</u> (1948). Figure 17.

PURPOSE. To demonstrate the need for Ca^{++} and thromboplastin in the trypsin-activation of purified prothrombin.

REAGENTS. <u>Pro. C.</u> (ref. [156]), purified by Dr. W. H. Seegers, 0.3% solution.

Tryp.: Crystalline trypsin, prepared by Dr. M. Kunitz [323], 40 units/ml.

Tpln. A.: Squibb's rabbit brain thromboplastin, 0.25% suspension.

Ca: 0.1 M $CaCl_2$.

T. M. (thrombic mixtures):

(I) 4 ml Pro. + 0.75 ml borate buff. (p. 149) + 0.25 ml Ca;

(II) 4 ml Pro. + 0.25 ml buff + 0.5 ml tryp. + 0.25 ml Ca;

(III) 4 ml Pro. + 0.5 ml buff. + 0.25 ml tpln. A. + 0.25 ml Ca;

(IV) 4 ml Pro. + 0.5 ml tryp. + 0.25 ml tpln. A. + 0.25 ml Ca.

Clotting tests: at 25°C, on 0.5 ml B. F. (p. 95) + 0.25 ml T. M., after successive incubation times (i.t.).

Thrombin 'percentages': were obtained by comparing the observed clotting-times with a dilution series of maximally activated (24 hr.) T. M. III. The dilution data are plotted as 1000 ÷ c.t. (sec.) in the dotted line of Figure 17.

RESULTS: are depicted graphically in Fig. 17 originally published [142] in the Ann. N. Y. Acad. Sci., 1948. Note the following:

1) Ca, alone, produces a very slow activation, complete in 50 hrs.

2) Trypsin, with Ca, is not significantly better.

3) Ca + tpln. gives adequate prothrombin conversion, but is rather slow under the particular experimental conditions (? lack of AcG) being about 50% complete in 4 hrs. and 100% in about 24 hrs.

4) Trypsin significantly improves the Ca + tpln., causing the reaction to be over 90% complete within 1 hr. and delayed (16-18 hrs.) only in the penultimate stages.

CONCLUSION. Trypsin does not act alone or with calcium only, but requires a source of thromboplastic agent. With this further addition, the enzyme has a significant effect in accelerating the thrombin-forming reactions.

EXPERIMENT 33 (1948). TABLE XXXVI.

PURPOSE. To study further the role of trypsin in systems of activating purified prothrombin.

METHOD. Essentially similar to Expt. 32, including computation of 'percentages' (of maximal activation).

REAGENTS. Pro. D. (ref. 156), another of Dr. Seegers' purified bovine prothrombins, 0.2% solution.

Tpln. D.: saline suspension (decanted) from frozen dog brain (p. 180), 0.1%. Two amounts were tested.

Tryp.: Same crystalline trypsin, 40 units/ml.

T. M.'s: all contain 4 ml Pro. D. + stated amounts of activators + borate buff. to total 5 ml vol.

Clotting tests: 0.5 ml B. F. (1%) + 0.25 ml T. M., incubated as stated. 25°C.

RESULTS: are given as 'percentage' activation in Table XXXVI.

DISCUSSION.

1) with Ca, only, activation is very slow and measurable only after 1-3 hr., but is complete (100%) in 48 hrs.
2) with trypsin, alone, activation is slow and poor, with best thrombin yield (20%) in 6 hrs. The decline, thereafter, points to a possible thrombinolytic action of trypsin.
3) with Ca + trypsin, a fairly good activation is noted with 100% completion in 3 hrs.
4) with Ca + tpln., at the higher concentration, gave good thrombin formation, complete in 30 min. This was used as the reference standard.
5) with Ca + tpln., at the weaker (1/3) concentration, was much poorer

and required as long as (I), viz. 48 hrs., to reach 100%.

6) with Ca + tpln. (1/3 str.) + trypsin, the activation was much improved, and complete in 3 hrs. However, this is only a little better, in the earlier stages, than in (III).

CONCLUSIONS (Expts. 32; 33). Both the cited experiments prove that the enhancing (accelerating) effect of trypsin upon the conversion of 'purified' prothrombin to thrombin depends upon the presence of other activators, namely calcium and thromboplastin. It is particularly when the calcium is adequate and the thromboplastin sub-optimal, that trypsin (experimentally) shows its most marked effects. It may be further concluded, with some slight reservations because of the lack (and insuperable difficulty in obtaining) exact evidence, that these highly purified prothrombins still contain traces of activator impurities. The differences in the results of the two experiments, while slight, may be significant suggestive evidence that (1) Pro. C (Expt. 32) contains insufficient trace of thromboplastin for the trypsin to add anything to the very minor effect (by and large) of added calcium, whereas (2) Pro. D. (Expt. 33) contains sufficient traces of some thromboplastin impurity for trypsin to exert a very definite effect with Ca. The further addition of the weak (1/3 str.) tpln. D. doesn't add much to the postulated thromboplastin contaminant, so that its inclusion (series VI) gives little better activation than in (III).

ADDENDUM. These, and other studies in the cited paper [156], give some new insight into the possible significance of 'trace contaminants' in even the best modern prothrombin preparations. It is perhaps, unfortunate, that Dr. Seegers has never seen fit to report on Micro-Calcium (spectroscopic) and P-lipid analyses of his prothrombin preparations, but merely

to assert (with reasonable pride) their 'purity' on the basis of potency, per weight of protein (or tyrosine N).

(6,N) PLASMATIC THROMBOPLASTIC COMPONENTS CONCERNED WITH THROMBIN FORMATION. PTC AND AHF.

Because thromboplastic function, particularly of platelets, is believed to be associated with a thromboplastin-generating reaction (pp. 80 et seq.) in which PTC, AHF, and probably other factors are involved, the following PTC and AHF assays were performed:

EXPERIMENT 34 (1956). TABLE XXXVII.

PURPOSE. To test for PTC and AHF in (I) Dialysed eluate (p. 126), (II) Howell-type Prothrombin (p. 161), and (III) Dr. R. H. Wagner's [488] 'purified' AHG (antihemophilic globulin, p. 137), using (IV) a normal human plasma (D.C.F.) as the factor control.

METHOD. Uses the specific one-stage prothrombin assay (p. 100) to test the improvement, over saline (substrate control: 0), of the prothrombin consumption of recalcified substrate plasmas (R. J.: PTC-deficient [294]; W.B.: AHF-deficient [152]; human clinical cases).

TEST: 0.2 ml substrate + 0.05 ml 'additive' are recalcified with 0.2 ml 0.025 M $CaCl_2$. After 1 hr. in the case of the AHF tests, and after ½ hr. in the case of the PTC tests (see Discussion), 0.05 ml of 0.1 M sod. oxalate is added to check further prothrombin utilization. 1-stage prothrombin assays are performed on (1) the original mixtures and (2) the 'serum' after oxalation and centrifugation (to remove clots).

Amounts of 'additives' are specified in Table XXXVII.

RESULTS. Table XXXVII gives the experimental details and test findings. Using reference curves, similar to those illustrated in Figure 16 (I), but obtained from human (instead of dog) standard normal plasma, the

1-stage clotting-times (c.t., at 37°C) are converted into the cited prothrombin (pro.) values, expressed as per cent of these normal standards.

Prothrombin consumption (%) = $\frac{\text{original pro (\%)} - \text{serum pro (\%)}}{\text{original pro (\%)}} \times 100.$

DISCUSSION. The PTC control (0) showed no clotting within 30 min. (37°), whereas all the additives caused clotting well within that period. At the time of these tests, our substrate subject (R.J.) did show some consumption of prothrombin, but gave significant assays when the incubations were restricted to $\frac{1}{2}$ hr. R. J. also presented another somewhat complicating problem in that he had a small amount of inhibitor (anti-PTC ^{294,152}). This made his plasma less sensitive in our assays, but still serviceable, as the results indicate.

The AHF control showed a trace of clot in 22 min., whereas the additives caused good clotting in less than 220 sec. The 10% (1 hr.) prothrombin consumption in the AHF control (0) is a severe deficiency. All the materials tested showed the presence of both PTC and AHF, in varying degree.

CONCLUSIONS. Our 'prothrombin' reagents, both (I) eluate and (II) Howell-type prothrombin are quite rich in PTC. They also contain significant amounts of AHF, about the same in the eluate and in the Howell prothrombin. In the case of the eluate, this must mean that some AHG is co-adsorbed on the $BaSO_4$ and resists the distilled water washings (p. 126). The traces of PTC in the 'purified' AHG may be due to the use of $Al(OH)_3$ in the preliminary plasma adsorption. This is not quite as effective as $BaSO_4$ in removing PTC. That the detected amounts of PTC were only traces is evident from consideration of the concentration of the AHG tested (1%). In the AHG assays a 0.001% AHG addition gave an almost

identical correction as 1:10 normal plasma, which indicates about a hundredfold concentration of AHF in the stock 1% AHG solution.

ADDENDUM. Later, another PTC-deficient case (W.S.) presented the opportunity for re-testing of the Howell prothrombin and the AHG preparation. This substrate, in the control (0), showed a 37% prothrombin consumption in ½ hr. at 37°C. A 1:10 normal plasma (D.C.F., p. 191) corrected this to 80%, whereas the AHG (1%) gave 96%, and the Howell prothrombin 100%. This is excellent confirmation of the Expt. 34 data, on an inhibitor-free substrate. It definitely confirms the trace of PTC contaminant in the AHF preparation.

7. TESTING OF CLOTTING FACTORS IN BLOOD PLATELETS.

The clotting factors in blood platelets are reviewed on pp. 74-89. The following experiments contain some new information, besides confirming some previously established facts. Tests on the enzyme and anti-enzyme preparations used will be deferred to a separate section (p. 209), following the thrombin-formation experiments.

(7,A) THROMBOPLASTIC ACTION OF PLATELETS. POTENTIATION BY (a) AHG and (b) TRYPSIN.

EXPERIMENT 35 (1956). TABLE XXXVIII.

PURPOSE. To study the thromboplastic action of platelets, and the modifying effects of added (a) antihemophilic globulin (AHG) and (b) trypsin.

METHOD. The two-stage procedure on dialysed eluate (see Table VI), as repeatedly used in previous experiments.

REAGENTS. Eluate (No. 127), dialysed, as previously described (p. 126).

AHG: Dr. R. H. Wagner's 488 'purified' preparation of May 9, 1956 described on p. 137.

Tryp.: 2% trypsin (Fairchild Bros. & Foster, N. Y.) in glycerol-borate buffer (equal vols.) prepared Jan. 5, 1945 (1) and stored in refrigerator at 4°C. Dil. 1:100, with imid. buff. sal., before use. Its proteolytic activity was tested in Expt. 44 (p. 209).

Plat.: 10x washed, frozen and thawed dog platelets prepared Oct. 29, 1952 (1) and frozen-stored at -20°C.

T. M.: 5 ml mixtures, in imidazole-buffered saline, containing 0.1 ml eluate, 0.1 ml AcG (1:5 dil.), + stated amounts of 'thromboplastic' additives, + 0.5 ml 0.02 M $CaCl_2$.

Clotting tests: 0.2 ml fibrinogen (dog) + 0.2 ml T. M., after successive incubation periods. 27°C.

RESULTS: These are given in Table XXXVIII, and show the following:

(1) with platelets as the only 'thromboplastic' additive, thrombin formation is weak but unequivocal and yields a 30.3" c.t. end-point in 30 min., corresponding to about 35% activation of the prothrombin.

(2) with AHG, a marked potentiation is seen in the 5 min. and 10 min. tests. The trace of fibrinogen in the AHF preparation caused a small clot in T.N. (2) in about 4 min. This was removed and the mixture remained clear thereafter. It is possibly the adsorption of a little thrombin on this fibrin (removed) which explains the somewhat longer c.t.'s in the later tests of (2). The end-point is at about 30 min., as in (1).

(3) a control, with AHG only (no platelets), is very poorly activated but does yield a 164.2" c.t. in 30 min.

(4) with trypsin, the potentiation is very marked. Even after 1 min. incubation, a 155.5" c.t. was obtained, whereas the other series (1), (2) did not give a clot in the 1 min. incubate in over 2 hrs. Moreover, the end-point (35.7") was definitely hastened to a 10 min. incubation period.

(5) trypsin, alone, is very feebly thromboplastic, with a trace of thrombin-formation still continuing after incubation for 1 hr. (445"→).

(6) trypsin enhanced the minor effect of AHG (in absence of platelets) in 10-20 min. incubations, but the 30 min. end-point (172.6") was, if anything, a very little inferior to (3). Here again a trace of fibrinogen (less than in (2)) required removal after 7 min.

CONCLUSION. Antihemophilic globulin (AHG) is clearly able to 'potentiate' the weak thromboplastic activity of an <u>old</u> (over 3½ years!) well-washed (x10), frozen-stored (-20°C) dog platelet suspension. A trypsin solution (from a stock 2% sol. in 50% glycerol + borate buffer), which had been

preserved in the ordinary refrigerator for over 11 years, was an even more striking 'potentiator' of the platelet activity. The actions of AHG, trypsin, individually and in combination, effect very little thrombin formation in our eluate mixtures containing prothrombin, proconvertin, PTC, and added AcG + Ca, but no added thromboplastin.

EXPERIMENT 36 (1956). TABLE XXXIX.

PURPOSE. To study the potentiation of platelet thromboplastin by (1) fibrinolysin and by (2) weak thrombin.

METHOD. This experiment was performed on the same day (24 May 1956) by the same technique as Expt. 35 (p. 194).

REAGENTS. The salts, including 0.02 M $CaCl_2$, eluate (dialysed), AcG (1:5), and platelet preparation were the same and used in the same amounts as in Expt. 35.

LYSIN: A 43 month old preparation of (dog) serum fibrinolysin, described on p. 211. The 1:40 borate buffer dilution of this lysin was taken from frozen storage (-20°C) thawed and used in the experiment, i.e. 0.1 ml per 5 ml T.M.

THR.: Bovine thrombin (Upjohn's, p. 96), 0.1 units/ml in buff. saline, freshly prepared, clotted an equal (0.2 ml) vol. of test fibrinogen (dog) in 6 min. At the 1:50 dilution in T.M. (7), therefore, there was not more than 0.002 of a thrombin unit per ml. This extremely small amount could not possibly add its own direct effect to the observed clotting-times. The control (9), without platelets or enzymes, serves for both Experiments 36 and 35.

RESULTS: are given in Table XXXIX, and show the following: Compared with (a) the negligible trace of activation (9) in the absence of platelets and enzymes (which are practically without effect, alone), and with

(b) the test (1), of Expt. 35, with platelets alone, both the aged fibrinolysin (8) and the very weak thrombin (7) give evidence of a significant potentiation of the platelet thromboplastic action. That this is not more marked (as in Expt. 37, p. 201, for instance) is probably due to our use of unnecessarily weak additives. Nevertheless, the considerably shorter c.t.'s in the 5 min. test are quite meaningful.

CONCLUSION. Fibrinolysin and thrombin, therefore, are two other agents with which we are able to demonstrate a 'potentiation' of the thromboplastic action of platelets. Considering the age (over $3\frac{1}{2}$ years) of the platelet preparation, a remarkable stability of the platelet thromboplastin is manifest.

OLDER EXPERIMENTS. In 1936, the author [126] performed some clotting experiments with platelets, extracts of the cornea and lens of the eye (chosen because of absence of a blood supply), and prothrombin, activated by Ca and cephalin. In retrospect, these data do not seem to merit inclusion among the much more definitive types of experimentation in the present thesis, except for mention of the fact that they led to the formulation of "a cephalin availability theory" (see later) and the suggestion that prothrombin (the "protein factor" of the coagulant) may occur in platelets, cornea, and lens ... "as the possible result of diffusion from the blood stream, via the lymph and tissue fluids in the case of the eye tissues".

In the next decade, only a few experiments were made with platelets (substituting for cephalin) in the activation of Howell-type prothrombin, in the presence of calcium. These did not seem to offer anything new. Eagle and Harris' [112] experiments (1937), which suggested that trypsin could coagulate blood by "the same effect as the physiological system

Ca plus platelets (or Ca plus tissue extracts)" inspired the present writer to an extensive series of studies with trypsin, and later, other proteolytic enzymes, including fibrinolysin (plasmin or tryptase, ref. [143]) in an endeavor to learn more about their possible relations in the blood clotting system.

In the period 1947-1949, and subsequently, many experimental testings of platelets in artificial clotting systems were made by the author and his graduate students, notably B. L. Travis. Some of the experiments, of this era [476] which sought to relate platelet functions in the thrombin-forming system with proteolytic enzymes and various antiproteases, will be reproduced in the following:

REAGENTS: Some special testings of the enzyme and anti-enzyme preparations will be deferred to a later section (p. 209).

Borate Buffer: 11.25g H_3BO_3 + 4g $Na_2B_4O_7$,12 H_2O + 2.25 g NaCl + dist. water to 1 liter, pH (glass electrode): 7.7 + 7.75; specific resistance: 170 ohms (at 21°C); effective ionic strength: 0.055.

Fibrinogen: B.F.: 1% borate buff. sol. of $BaSO_4$-adsorbed bovine fibrinogen (Armour's, p.95).

Thrombins: (a) Thr.: bovine thrombin (Upjohn's, p.96), courtesy of Dr. J. T. Correll. A 100 units/ml 'stock' sol. was prepared in borate buff. (b) Thr.* A special type of thrombin was prepared for one of the experiments and will be described in that connection (p. 201).

Prothrombins: Pro.: Several bovine prothrombins were made in our own laboratories, following the directions of Seegers et al. [431]. However, Dr. Seegers kindly supplied us with a number of his own highly 'purified' preparations. Our [156] extensive studies, using these products, were reported in 1948. Excellent as Dr. Seegers' preparations were, they did

show evidence of trace impurities, the control testing for which was an important part of our studies. One of these trace contaminants was active thrombin. In the tests to be cited, it should be mentioned that no pro-thrombin solution was used (with rare deliberate exceptions which are stated), unless a preliminary test (0.5 ml B. F. + 0.25 ml Pro.) yielded no trace of clotting in at least 30 min. Many of the experiments in the 1948 publication [156] were made before the establishment of our modern knowledge of the accelerator or co-factors (proaccelerin or AcG, syn. factor V, labile factor; and proconvertin or SPCA, syn. factor VII, stable factor, etc.). In the later experiments, particularly those about to be cited [476], AcG was always added to our thrombin-forming systems. The only exception was when studying the role of this particular factor.

AcG.: of bovine serum type, was prepared and kindly supplied by Drs. Ware and Seegers. A 0.1% sol. in borate buff. showed traces of pro-thrombin and very minute amounts of active thrombin, but these were considered to be non-significant in the experiments cited. It had no 'thromboplastic' effects (author's tests).

Thromboplastins: Tpln. A. was prepared by ourselves from acetone-dried dog brain, a borate buffer suspension of which (0.25%) showed a high degree of thromboplastic activity.

Tpln. B: Schieffelin & Co's 'Soluplastin' (p. 96), supplied through the courtesy of Dr. E. W. Blanchard, was used in some tests.

Ca: 0.05 M $CaCl_2$.

Fibrinolysin: Lysin: a 'purified' enzyme preparation from beef serum, [302] supplied through the courtesy of Dr. E. C. Loomis (Parke, Davis & Co., Detroit). 1% sol. in borate buffer was prepared immediately before use.

In later work, we used our own dog fibrinolysin [291].

Antifibrinolysin: Afln.: a 'purified' fraction from beef serum, also supplied by Dr. Loomis [303], in Nov. 1947. A 0.4% extract was made with borate buffer and centrifuged from a considerable residue of insoluble material.

Trypsin: Tryp. (A): Crystalline trypsin (from pancreas), prepared and supplied by Dr. M. Kunitz [323] (Rockefeller Institute, Princeton, N. J.) whose 'units' of activity are cited [353].

Tryp. B.: a very satisfactory commercial trypsin (Fairchild Bros. & Foster, N. Y.) of which a stock 2% solution was made in 50% glycerol and borate buff. (i.e. equal vols.).

Anti-proteases (Trypsin-Inhibitors): For antifibrinolysin, see above.

P. I.: pancreatic inhibitor (anti-trypsin), highly purified, (Lot P-A-97-1), Sharp & Dohme Labs., courtesy of Dr. L. A. Kazal. (cf. [353]).

S.B.I.: soybean inhibitor, a crystalline preparation, kindly supplied by Dr. M. Kunitz [272] (see above).

N.B.I.: a re-purified navybean inhibitor (see p. 209) kindly supplied by Dr. D. E. Bowman [59] (Indiana Univ.).

All these antiproteases were prepared in a 0.1% solution in borate buffer. The S.B.I. was diluted to 0.01%, before use. Special testings of the antiproteases will be cited later (p. 209).

Platelets. (Plat.), cf. p. 94. Well-washed platelets were obtained as follows: 100 ml dog blood was collected into 13 ml of 3.4% trisod. citrate. Repeated centrifugings for a few minutes at 1000 r.p.m., in the International Refrigerated Centrifuge, were performed until red cells were no longer visible in the sediment. The 'platelet-rich' plasma was then centrifuged for 30 min. at 5000 r.p.m. and the platelet

sediment resuspended and well washed at least three times, by thorough mixing with successive 50 ml lots of 0.9% NaCl containing 1/8 vol. 3.4% sod. citrate (we later used 'Triton', p. 94), and a final washing in plain saline. The final suspension, concentrated in 1-2 ml saline, was examined microscopically. Wright-stained smears revealed only granular platelet material, with no leukocytes and only a rare erythrocyte.

EXPERIMENT 37 (1949). TABLE XL.

PURPOSE (as in Expt. 36): To study the thromboplastic action of platelets and its potentiation by (A) weak thrombin and (B) fibrinolysin.

METHOD. A two-stage system, similar to previous testings of thrombin formation.

T.M.'s: 5 ml vol., containing (with borate buff.) Pro. + AcG. + Ca. + plat., with and without thr.* (p. 201) or lysin.

Clotting-tests, at 26°C: 0.5 B.F. + 0.25 T.M.

RESULTS: These are shown in Table XL.

DISCUSSION. This experiment is one which we have many times repeated to show the much better thrombin formation, in a system of purified Pro. + AcG + Ca + platelets, when a tiny trace of thrombin is also added (test 2). It must remain an open question whether a test system completely devoid of thrombin (even minutest traces) might someday be obtainable and fail (?) to activate with platelets and Ca (in the presence of proconvertin, AcG, etc.). Pending such a finding, it seems to be established that platelets are an 'incomplete' thromboplastin, which can be potentiated by thrombin. The Thr.* used in Expt. 37 was a special preparation. In fact, it consisted merely of the same prothrombin preparation, in borate buffer, which had been allowed to stand with $CaCl_2$ for several weeks in the refrigerator (4°C). Tests

showed that it was completely converted into thrombin, since incubation with thromboplastin and AcG gave no further enhancement of its clotting potency. Such a preparation seemed well chosen to avoid the introduction of any factors except the thrombin, unless, perchance, there are some unknown factors in the (recalcified) prothrombin itself. With these unsupported reservations, we must conclude that weak thrombin can act as a remarkable potentiator of platelet thromboplastin. Since AcG is added, this would not appear to be due to the known effect [492] of thrombin in converting proaccelerin to accelerin. Attempts were made to run controls, without platelets. These showed only insignificant shortening of the clotting-times in the parallel test (0), without activators (except for the Ca). This cited test (0) merely showed the trace of thrombin in the freshly prepared prothrombin solution and this could not be correlated with the weak thromboplastic action of the platelets, shown in (1). The added thrombin* (p. 201) (diluted) clotted a test fibrinogen in 750 sec., when freshly prepared, but rapidly lost potency on standing.

The fibrinolysin test (3) showed a potentiation of the platelet thromboplastic effect, qualitatively resembling that of thrombin, and even quantitatively similar, in this particular experiment. It is difficult to test a fibrinolysin for traces of thrombin, because the enzyme lyses the test fibrinogen before it has a chance to clot with a very weak thrombin. We did perform this control test, however, in the presence of antiproteases (esp. antifibrinolysin), which seemed to rule out this possibility. It is not possible, therefore, to deduce a common mode of action for these two dissimilar agents (thrombin and fibrinolysin) with any factual knowledge currently available.

CONCLUSION. We present the experimental facts, therefore, which, prima facie, indicate that (1) platelets have a weak thromboplastic action in systems such as those described, and that (2) weak thrombin and (3) serum fibrinolysin each have a significant 'potentiating' action on this platelet effect. Expt. 37 was even more satisfactory and controlled than the recent confirmatory Expt. 36.

(7,B) EFFECTS OF ANTI-PROTEASES ON VARIOUS 'THROMBOPLASTIC' ADDITIVES IN THROMBIN-FORMING SYSTEMS.

PRELIMINARY STUDIES: These will be cited in later sections. These clearly established the following facts needed for interpretation of the thrombin-forming experiments:

I. None of the antiproteases had any inhibitory action on the thrombin-fibrinogen reaction (see Expt. 43, p. 209, and Table XLVI).

II. In higher concentrations than those selected for the present experiments, trypsin was thrombinolytic and Loomis' fibrinolysin very feebly so, requiring several days incubation in order to demonstrate this.

III. Again in too high enzyme concentrations to be significant in the present experiments, a question of prothrombinolysis might arise [430,301]. However, Dr. Lewis, Ann C. Howe and the author [299] could not confirm this, but found instead a marked susceptibility of AcG to the proteolytic actions of fibrinolytic enzymes.

IV. When thrombin formation is very slow and weak, it is possible to encounter fibrinogenolysis by the enzymes, before the weak thrombin has a chance to form a clot. In fact, this is indeed one of the author's [137] methods for assaying trypsin and other fibrinolytic proteases, at high dilutions, as will be illustrated

in Expts. 44 and 45, pp.209 and 211, respectively.

V. There are quantitative relationships between enzymes and the respective enzyme inhibitors, which must be worked out and observed [291,292]. Furthermore, the enzyme inhibitors act in different ways [292] and some of them require a time-period, hence pre-incubation with the enzyme, in order to exert significant antiprotease effects. This pre-incubation is indicated by an asterisk (*) with designation of the anti-enzyme used.

All these considerations were carefully tested before using the previously described (pp.198-201) REAGENTS in the following groups of tests.

EXPERIMENT 38 (1948). TABLE XLI.

PURPOSE. To test antiproteases on platelet and trypsin additives in the thrombin-forming system.

REAGENTS. Plat.: 0.1 ml conc. suspension (p.200). Tryp.: 0.01% Kunitz's crystalline trypsin (p.200), 0.3 ml. P.I.: 0.1% pancreatic inhibitor (p.200), 0.1 ml. Afln.: 4% Loomis' anti-fibrinolysin (p.200), 0.3 ml. Pro.: 0.1% Seegers' prothrombin (p.198), 0.2 ml. AcG.: 0.1% Ware and Seegers' serum AcG (p.199) 0.2 ml. Ca: 0.5 ml 0.05 M $CaCl_2$.

METHOD AND RESULTS: These are shown in Table XLI. They demonstrate (1) weak thromboplastic action of platelets; (2) similarly weak thromboplastic action of trypsin alone; (3) marked potentiation of thromboplastic action, when trypsin is added with platelets; (4) inhibition of the last by P.I.; and (5) similar inhibition by Afln*.

CLOT-LYSIS: This was followed over 3 weeks in these experiments. It was noted in 3 days in (2), but was completely inhibited by the platelets

(3), as well as by the antiproteases (4; 5).

EXPERIMENT 39. (1948). TABLE XLII.

PURPOSE. To test antiproteases on platelet + fibrinolysin additives in the thrombin-forming system.

REAGENTS: Same as in Expt. 38, except for (a) use of 0.4 ml 0.5% Loomis fibrinolysin (p. 199), instead of trypsin, and (b) additional test with 0.2 ml 0.01% S.B.I. (soybean inhibitor, p. 200).

METHOD AND RESULTS: These are shown in Table XLII.

DISCUSSION. Test (1) of Expt. 38 (Table XLI) was also the control, with platelets alone, for the additional tests in Expt. 39 (Table XLII). (6) the fibrinolysin alone had a very weak thromboplastic effect, as compared with the control (11), in which Ca and AcG alone caused no detectable thrombin in tests watched for ½ hr. (7) the fibrinolysin, however, markedly potentiated the platelet thromboplastic action, apparently like trypsin did in tests (3) of Table XLI. (8) Unlike the trypsin expt. (4), however, P.I. did not inhibit this action of fibrinolysin. (9) Neither was AfIn* able to cause any significant inhibition of the fibrinolysin's potentiating action, again differing from the corresponding trypsin expt. (5). S.B.I. (10) seemed to inhibit in the earlier phases, although much less so later. It will be shown (p. 208) that this can be explained as due to a direct anti-thromboplastic action of the S.B.I. itself. In fact, it is somewhat significant that this was eventually largely overcome by the fibrinolysin preparation.

EXPERIMENT 40 (1956). TABLE XLIII.

PURPOSE. To test antiproteases in thrombin-forming systems, in which the thromboplastic additives are: (I) platelets only, (II) platelets +

antihemophilic globulin.

REAGENTS. Eluate, dialysed (p. 126): to provide prothrombin, proconvertin, PTC, etc. (0.2 ml, per 5 ml T.M.).

Plat: 0.2 ml of a new preparation of human platelets (normal), p. 94.

AHG: Dr. R. H. Wagner's antihemophilic globulin preparation, described on p. 137 (0.2 ml).

S.B.I.: 0.1 ml of a new 0.1% borate buffer solution of crystalline soybean inhibitor (Worthington Biochemical Lab., Freehold, N. J.).

P.I.: Dr. L. A. Kazal's pancreatic inhibitor preparation (p. 200), 0.1 ml. of 1% sol. in borate buffer.

N.B.I.*: Dr. D. E. Bowman's re-purified navybean inhibitor preparation (p. 209), 0.1 ml of 0.1% sol. in bor. buffer.

Afln*: Dr. E. C. Loomis' 1947 preparation (p. 200). A 0.4% two-day old extract (freed from insoluble material) in bor. buff., 0.3 ml. The last two inhibitors* were pre-incubated for 15 min., at 28°C., with the thromboplastic additives (I) or (II), respectively.

T.M's: Made up to 4.5 ml with borate buffer and then activated with 0.5 ml 0.04 M $CaCl_2$.

RESULTS: These are given in Table XLIII. They reveal:

I. (1) Platelets, alone, were significant activators.

(2) S.B.I. was markedly inhibitory (anti-thromboplastic), especially in the earlier phases of activation.

(3) P.I. and (4) N.B.I.* were not inhibitory. A very minor improvement was hardly significant.

(5) Afln.* was distinctly inhibitory, but whether this was due to the very old preparation could not be determined, as the last of this material was used up in this test.

II. (6) AHG caused the same important potentiation (? thromboplastin generation) of platelets as with cephalin (see test 7, Table XIII). Compare with test (1).

(7) S.B.I. was very antithromboplastic in this mixture also (cf. (2)).

(8) P.I. and (9) N.B.I.* were non-inhibitory.

EXPERIMENT 41 (1948). TABLE XLIV.

PURPOSE. To test antiproteases in thrombin-forming systems activated by Ca and tissue thromboplastin.

REAGENTS AND METHOD: Same as in Expts. 38 and 39, except for use of 0.5 ml tpln. A. (p.160) as the only thromboplastic additive.

RESULTS: These are given in Table XLIV. They show:

(1) Marked activation with this strong tissue thromboplastin.

(2) Its complete inhibition with S.B.I. -- an important control finding (cf. Expt. 39).

(3) No inhibition by P.I, and (4) none by Afln.*

EXPERIMENT 42 (1956). TABLE XLV.

PURPOSE. To test antiproteases in thrombin-forming systems activated by Ca and cephalin.

REAGENTS AND METHOD: Same as in Expt. 40 (I), namely using dog eluate (prothrombin, etc.), AcG, borate buffer, but substituting 0.1 ml 0.1% cephalin (p.127) for the platelets.

RESULTS: These are given in Table XLV. They reveal:

(1) Satisfactory activation by the cephalin.

(2) S.B.I. (p. 200 , 0.1 ml of 0.1% soln.): was again anti-thromboplastic (cf. tests (2) and (7), Table XLIII; and test (2), Table XLIV).

(3) P.I. (p. 200, 0.1 ml of 0.1% soln.): was not inhibitory: neither was

(4) N.B.I.* (p.209 , 0.1 ml of 0.1% soln.).

GENERAL DISCUSSION OF EXPTS. 38 - 42.

Of the various anti-proteases tested, the soybean inhibitor introduced complications because of its direct anti-thromboplastic effects, against platelets (Expt. 40 (I)) or platelets + AHG (Expt. 40 (II)), tissue thromboplastin (Expt. 41), or cephalin (Expt. 42). The others were reliable inhibitors of the enzymes trypsin and fibrinolysin in the systems studied, and had no significant antithromboplastic effects of their own. Antifibrinolysin in Expt. 40 (I) was a questionable exception. Inhibition by antiproteases, of the platelet-thromboplastin potentiating action of trypsin was good evidence that this phenomenon depended upon the proteolytic actions of the pancreatic enzyme. On the contrary, the apparently similar potentiating (to platelet thromboplastin) effects of fibrinolysin were not removed by antiproteases in amounts sufficient to suppress all proteolytic action. This unexpected difference could not be explained except by postulating some unknown factor in the enzyme preparations used. A suggestion that it might be the 'Hageman factor' [401] was noted (p. 43) in the introductory section, but further exploration of this must await the future. Qualitatively, at least, platelets seemed to be potentiated by trypsin in very much the same way as cephalin [149] (p. 187) and weak thromboplastin [156] (pp. 187-190) were. We concluded that there was an important common denominator in these comparisons. It could be a common thromboplastic phospholipid mobilised or made available to an enhanced degree by the 'disaggregating' (Pope [379], cited [138]) action of the proteolytic enzyme. Such an idea fits the author's "cephalin availability theory" and could explain the significance of 'thromboplastic enzymes' like trypsin (cf. p. 186).

(7,C) CONTROL TESTING OF ENZYMES AND ANTIPROTEASES.

EXPERIMENT 43 (1948). TABLE XLVI.

PURPOSE. To show that anti-proteases have no significant effect on the thrombin-fibrinogen reaction.

METHOD. Timing of the clotting of a test fibrinogen with pre-incubates of thrombin and the various antiprotease preparations. 2 ml thrombin (bovine, Parke, Davis & Co.'s 'thrombin topical', p. 96, 5 units/ml) + 2 ml antiprotease (or borate buff., in control) incubated, for periods of $\frac{1}{4}$ min. to 1 hr., and 0.5 ml samples of mixture tested on 0.5 ml B.F., Armour's, (1%), p. 95.

RESULTS: These are given in Table XLVI. They were negative with regard to any significant effects of : (1) P.I. (p.200), 0.1%; (2) S.B.I. (p. 200), 0.005%; (3) N.B.I. (p. 209), 0.01%; (4) Afln. (p. 200), 0.1%, even with an hour's pre-incubation with the thrombin.

EXPERIMENT 44 (1956). TABLE XLVII(A).

PURPOSE. To demonstrate proteolytic activity in a long preserved (>11 yr.) trypsin preparation and to show its inhibition by two types of trypsin-inhibitors.

METHOD. The fibrinogenolytic technique originally described by the author [137] in 1943.

REAGENTS. Borate buffer, pH 7.7 (p. 128). Thr.: bovine thrombin (Upjohn's, p. 96), 20 units/ml. B.F.: 1% bovine fibrinogen (Armour's, $BaSO_4$-treated, p. 95).

Tryp.: The >11 yr. old (commercial) trypsin preparation used in Expt. 35 (p. 194). The 2% stock solution was diluted 100x with borate buff.

N.B.I.: a highly purified (reprecipitated with $(NH_4)_2SO_4$) antiprotease from navy beans, prepared by Dr. D. E. Bowman [59] and kindly supplied

us in 1948. A fresh 0.1% solution of the dried preparation was made up in borate buff.

P.I.: A highly purified pancreatic trypsin-inhibitor prepared Jan. 22, 1948 (Lot No.: P-A-97-1) by Dr. L. A. Kazal (Sharp and Dohme Labs., Glenolden, Penna.). Fresh 0.1% soln. in borate buff.

L.M. (lysing mixture): 1 ml B.P. + 0.5 ml tryp. + 0.5 ml inhibitor, incubated at 28°C., with testing of 0.2 ml samples at intervals, on adding to 0.2 ml Thr. Successive prolongations of the clotting-time indicate the rate and extent of the fibrinogenolysis.

Note: The P.I. acts immediately and is added just before the trypsin. The N.B.I.*, on the other hand, resembles serum antifibrinolysin in requiring a period of time to inhibit the proteolytic enzyme [292]. Hence, 20 min. pre-incubation of N.B.I. with trypsin was allowed before adding the fibrinogen. Borate buff. was used to make up the 2 ml L.M. vol. as needed.

INCUBATES:

1) Control, without enzymes;

2) Trypsin, alone;

3) Tryp. + N.B.I.*;

4) Tryp. + P.I.

RESULTS: These are given in Table XLVII(A). They clearly show:

1) stability of fibrinogen in buffer (control);

2) marked fibrinogenolysis by the diluted old trypsin;

3) complete inhibition of lysis by the N.B.I.*;

4) almost complete inhibition, also, by the P.I.

CONCLUSIONS. This experiment affords evidence of the remarkable stability of trypsin at ordinary refrigerator temperatures (4°C.), in glycerol-borate

buffer (equal vols.) solution. It indicates suitability of this old enzyme preparation for the special platelet potentiation tests of Expt. 35 (p. 194), and anti-heparin Expt. 47 (p. 213). Similar tests were made on this preparation and on crystalline trypsin solutions preliminary to the 1947-49 experiments (pp. 198, et seq.).

EXPERIMENT 45 (1956). TABLE XLVII(B).

PURPOSE. To test the proteolytic activity of an old (>43 months) dog serum fibrinolysin preparation, and its inhibition by antiproteases.

METHOD. The fibrinogenolytic technique [137], as used in Expt. 44, also employing the same REAGENTS, except for: (a) replacing the trypsin by lysin: a 'purified' fibrinolytic enzyme (plasmin or tryptase) from dog serum, prepared by Dr. Jessica H. Lewis and the author [291] 29 Oct. 1952. A 1:40 dilution in borate buffer had been preserved, frozen, at -20°C.

(b) Afln. (0.4%, in borate buff.) was prepared by 2-day extraction, at 4°C., of a Nov. 1947 dried anti-enzyme preparation of Dr. E. C. Loomis' (see p. 200).

DATA: These are shown in Table XLVII(B). They demonstrate:

1) Stability of the control fibrinogen (buffer only);
2) Marked fibrinogenolysis by the old enzyme solution, complete in 10 min.
3) Inhibition of proteolysis when the enzyme was pre-incubated for 15 min. with 0.1% N.B.I.* (equal 0.5 ml vols.)
4) Similar inhibition by Afln.* (also pre-incubated 15 min. with the lysin).

CONCLUSION. This dilute enzyme preparation also preserved an excellent degree of activity and was judged suitable for the tests with platelets

in Expt. 36. The antifibrinolysin was also satisfactory. Similar tests were made with the enzyme systems used in the 1947-49 experiments, previously cited.

(7,D) FIBRINOLYTIC PHENOMENA AND PLATELETS.

The use of borate buffer, with its mild bacteriostatic effect, proved very valuable as a preservative in our earlier [156] prothrombin-activation experiments and enzyme studies (ref. [291]). In these systems, it was often possible to demonstrate weak fibrinolytic enzymes requiring days or weeks to produce clot lysis. We shall cite only those incidental observations which pointed to certain relationships of the platelets to proteolytic phenomena. Chief among these was the demonstration of an antiprotease in platelets, particularly noted in the inhibition or retardation of fibrinolysis by trypsin. This is evident in the clot-lysis data of Table XLI (Expt. 38, p. 204). References to platelet 'anti-fibrinolysin' are given on p. 89. We had observed it in Expt. 39, and other similar experiments in 1947-49.

(7,E) THE 'AcG-LIKE' FACTOR IN PLATELETS [476].

EXPERIMENT 46 (1950). TABLE XLVIII.

PURPOSE. To test for an 'accelerator' factor in washed platelets, using an AcG-poor prothrombin, activated by Ca and a strong tissue thromboplastin. Comparison with varying concentrations of Ware and Seegers' AcG preparation.

METHOD. The usual two-stage technique (1) without added AcG, (2) with platelets, and (3) and (4) with varying concentrations of added AcG.

REAGENTS. Pro.: One of Dr. Seegers' highly purified bovine prothrombins.

Tphn. B.: 'Soluplastin' (p.96).

Plat.: p. 200.

AcG.: p.199 .

T.M.'s: as described in Table XLVIII.

RESULTS: These are given in Table XLVIII.

DISCUSSION. The poor thrombin formation in (1) with tpln. only, indicated the accelerator deficiency in the chosen prothrombin. In (2) platelets were found to have only a little effect. Since there was already a strong thromboplastin present, this small effect could have been due to some 'AcG-like' factor in the platelet preparation. Comparisons with the two additions of 'purified' serum-type AcG (3,4) suggested that this platelet factor was roughly comparable to what amounted to a concentration of only 1:2,500,000 in the final thrombic mixture or 1: 100,000,equivalent vols. of platelets and AcG . Questions as to the lability of 'platelet AcG' during the manipulations of platelet preparation were difficult to answer. We have found significant amounts of platelet AcG in our more recent (1952-56) clinical routine tests, discussed later (see Table LII).

CONCLUSION. These early experiments led us [476] (see p. 88) to wonder whether the 'AcG-like' factor described by Ware, Fahey, and Seegers [490] was a germine platelet component, or might merely be adsorbed on to the platelets from the plasma [222]. Our tests, which will not be cited in detail, indicated the platelet accelerator to be of the plasma AcG-type or proaccelerin (see p. 43). More about platelet analyses for this factor will be given later in data on clinical cases. (p. 235).

(7,F) EFFECTS OF HEPARIN ON THROMBIN-FORMATION BY (I) PLATELETS; (II) PLATELETS + TRYPSIN; or (III) TISSUE THROMBOPLASTIN.

EXPERIMENT 47 (1956). TABLE XLIX.

PURPOSE. To study heparin inhibition of the thromboplastic action of

(I) platelets, and differences from (II) platelets + trypsin and (III) tissue thromboplastin.

METHOD. Essentially the usual method III two-stage technique of following thrombin formation, with slight modifications of volumes to control for the heparin carried over into the thrombin-fibrinogen (clotting-test) mixtures.

REAGENTS. Eluate (dialysed, No. 127), p.135 , was used to provide prothrombin, proconvertin, etc. 0.2 ml/5 ml T.M.

Plat.: Human platelets (p. 94), 0.2 ml.

Tryp.: 0.5 ml of a fresh 1:100 dil., with imid. buff. sol. (pH: 7.3), of the 11 yr. old Stock (2%) trypsin, described on p. 194 .

Tpln.: 0.2 ml 'Soluplastin' (p. 96).

Ca.: 0.04 M $CaCl_2$.

Hep.: Imid. buff. sal. dilutions of heparin ('Liquaemin', Organon Inc. (Hoffman-La Roche), Orange, N. J.). 0.5 ml of 2 units/ml hep. in T.M. (b series): 0.1 ml of 0.2 units/ml hep. in (c) series of fibr. + T.M. tests.

T.M.'s and clotting test mixtures (0.2 ml fibrinogen (dog) + 0.1 ml (a) 0.2 unit/ml hep. or (b) imid. buff. sol. + 0.1 ml T.M.): are described in the caption of Table XLIX.

RESULTS: (Table XLIX) show, in:

(2) practically complete inhibition of platelet thromboplastic activity by heparin, in concentration of 0.2 units/ml in the thrombin-forming mixture.

(1 a, c) was the appropriate control, supplying the same final concentration of heparin, namely, 2/4 of 0.2 = 0.1 unit/ml, as in (2) tests.

(1 a, d) with no heparin, were only a little better than (1 a, c), thus showing that the effects of heparin, in the concentrations tested,

were very minor on the thrombin-fibrinogen reaction.

(3) the significant trypsin potentiation of the thromboplastic action of platelets again confirms the data of Expts. 35, 38. The only difference was that, in Expt. 47, the final clotting tests were performed in the presence of heparin.

(4) The ability of trypsin to overcome the heparin inhibition, and hence restore the platelet (+ enzyme) thromboplastic activity, was very strikingly demonstrated by these tests.

(5) The trypsin, alone, was only weakly thromboplastic. This strongly suggested that the activity which was 'released' from the heparin inhibition by trypsin was not that of the enzyme itself, but must have come from the platelets.

(6) The strong tissue thromboplastin (Soluplastin) was also able to overcome the inhibitory effects of heparin in this thrombin-forming system. This again confirms our 1939 Expt. (29, p. 179, tests 5, 6).

DISCUSSION. Unlike our earlier experiments (29, 31), which used excessive amounts of heparin, as commented upon p. 185, the present study (Expt. 47) employed concentrations of heparin which might be compared favorably with those in the therapeutic or physiological range. Hence, it is particularly convincing that such amounts of heparin were able so completely to inhibit the thromboplastic action of platelets in our suitably chosen thrombin-forming system. Inability of this heparin, however, to antagonise tissue thromboplastin or trypsin + platelets was equally significant evidence of the much greater potency of these thromboplastins.

CONCLUSIONS. The foregoing data not only suggest (1) that the usual

anticoagulant effects of heparin are intimately associated with inhibition of the thromboplastic function of platelets, but also (2) that platelets are much more akin to a simple phosphatide (? cephalin), as in Expt. 29, than to ordinary preparations of tissue thromboplastic extracts. Quantitative interrelationships undoubtedly exist, and an extremely weak tissue thromboplastin would closely resemble platelets and cephalin in these experiments. However, we do believe the facts line up behind the postulates of our 'cephalin availability theory'. (p. 261).

(7,G) ANALYTICAL FRACTIONATION OF PLATELETS.

This line of investigation was not pursued in this thesis. The reader may be referred to current work in this field by Dr. W. H. Seegers, of Wayne University, Detroit, but the brief reference we have given to this on p. 86 will have to suffice. The one group of analytical data, with which the present writer had some connection, was that of Mrs. Betty Nims Erickson and collaborators, in the 1940's, cited on p. 76. From these we wish to reiterate the important finding (also credited to Chargaff [78], on p. 75) that acetone-insoluble phospholipid or cephalin is a major fraction of the platelet lipoids.

(7,H) QUESTION OF A 'FIBRINOPLASTIC' FACTOR IN PLATELETS.

The author's [134] term 'fibrinoplastic' (see p. 27) for factors which accelerate the thrombin-fibrinogen reaction, through some non-specific colloidal or surface effect, would seem to be appropriate for such action of platelets (fractions?) as that claimed by Ware, Fahey, and Seegers [490] and allegedly confirmed by others (see p. 89).

B. L. Travis and the present author, in 1947-49 (p. 115 of ref. [476]) were unable to demonstrate any significant effect of (dog) platelets

on the thrombin-fibrinogen reaction.

EXPERIMENT 48 (1956). TABLE L.

PURPOSE. To re-study effects of adding platelets to thrombin-fibrinogen mixtures.

REAGENTS. Plat.: 0.1 ml of normal human platelet suspension (pp. 94; 200)
Thr.: bovine thrombin (Upjohn's, p. 96), 0.1 ml of serial dilutions, with imid. buff. saline (p. 97) of 20 unit/ml stock solution.
Fibr.: 0.2 ml of (dog) $BaSO_4$-treated fibrinogen (p. 94).

METHOD. Clotting-times (sec.), at 26°C., of thr. + plat. (or buff. saline, in controls) + fibr.

RESULTS: are given in Table L. They show no significant effects of the platelets (1) as compared with the controls (2).

DISCUSSION. The tests were meticulously performed. The duplication of values in repetition of the last two measurements indicates the insignificant experimental error. Essentially similar results were obtained with the 3½ yr. old dog platelet preparation described on p. 169.

CONCLUSION. We are unable to confirm any 'fibrinoplastic' action of platelets and wonder whether such findings by other workers may not be the result of some alterative (? denaturation) phenomena. Denatured proteins often do have some effects on the thrombin-fibrinogen reaction [60].

(7,I) PLATELET UTILIZATION DURING CLOTTING.

EXPERIMENT 49 (1955). TABLE LI.

PURPOSE. To investigate the rate of disappearance of platelets during the clotting of (A) normal and (B) hemophilic bloods.

METHOD. Bloods were collected by clean puncture of the antecubital vein, using No. 20 new needle and siliconized syringe. (I) was immediately placed in a siliconized tube, at 37°C., and samples pipetted off at

the stated intervals (Table LI) into a series of siliconized red cell hemocytometry pipettes and the platelet counts [62] made as soon as practicable thereafter. (II) 4.5 ml of blood was mixed with 0.5 ml 0.1 M sod. citrate, in a siliconized pyrex bottle and held at 37° until the control period, when its platelets were counted, as usual.

RESULTS: are given in Table LI.

DATA: (A) The normal blood clotted soon after removal of the 6 min. sample. Averaging the 5 and 6 min. tests, i.e. just before clotting, the platelet count had dropped in this time to 104.5/320 = 32½% of the original (0') value. The rapid drop in the platelet counts thereafter probably represents chiefly the removal of platelets by entanglement in the fibrin clot.

(B) The hemophilic blood started clotting in 23 min. Averaging the 4, 5, and 7 min. tests, the platelet count had fallen in this time to 76% only (137.3/182) of the original (0') value. The pre-clotting 10-15 min. period, i.e. 3 observations, gave a similar value, viz. 87%, within the 'experimental error'. Only after clotting was there an abrupt fall in the platelet count suggesting removal of the formed elements entangled in the fibrin clot.

CONCLUSION. There seems to be a significant preservation (80 ± 4%) of platelets during the longer pre-clotting period in hemophilic as compared with normal (32%) human blood. This could reflect a diminished 'utilization' of platelets in the pre-clotting phase of hemophilic blood. Platelet counts after the onset of clotting are not significantly different and, in all probability, depend upon platelet removal by entanglement in the fibrin clot.

8. DARK-FIELD MICROSCOPIC OBSERVATIONS OF PHOSPHOLIPIDS, PLATELETS, AND OTHER FORMED ELEMENTS OF THE BLOOD AND BONE-MARROW.

(8,A) FOREWORD. Siedentopf and Zsigmondy[441], applying the optical principles worked out by Abbe and Helmholtz, first used the dark-field method of microscopy. It was quickly applied to the study of living cells and micro-organisms by numerous investigators in Germany, France, and elsewhere. The early history of its use for study of the blood was reviewed, in 1913, by Aynaud and Jeantet[27]. Aynaud[24,25,26] used it, as well as ordinary microscopy, in a monumental study of the alterations of the blood platelets, in a work which deserves far more recognition than it has received by occasional brief mention in the more modern reviews[411,415]. Stübel[459], in 1914, gave some good descriptions of the dark-field appearances of platelets, with excellent photomicrographs. The present author demonstrated cellular and clotting appearances in a drop of human blood preserved at body temperature under the dark-field microscope, before the British Association for the Advancement of Science, meeting at Cape Town, in 1929. This initiated an interest pursued from time to time over many years, leading to publications in South Africa[122] and in America[124,139]. The following account summarizes those observations relating to the role of phospholipids in the alterations of platelets and other formed elements, which occur after withdrawal from the body. It is original more particularly in the novel explanation which is arrived at from an analysis of the data reviewed.

(8,B) PLATELETS

1) Alteration during blood coagulation. (Figure 18).

METHOD: A simple small drop of finger prick blood is placed on a scrupulously clean slide and spread thinly with gentle pressure from a No. 0 coverslip. It is examined under the oil-immersion lens of a good dark-field microscope, with an adjustable diaphragm in the objective to permit best optical resolution of the minute details observed. A bright tungsten band or similar evenly focussed (e.g. Burton lamp) illuminant is adjusted for optimal results and the cardioid condenser (dark-field) is accurately centered. The heat of the lamp usually warms up the blood drop sufficiently without requiring the 37°C chamber (enclosing the whole microscope), which was used in our earlier observations.

OBSERVATIONS: There are no significant alterations in the chylomicrons ('hemoconia'), erythrocytes, and leucocytes[124] in the few minutes which elapse before the appearance of the fibrin 'needles', which indicate the clotting of the blood. The platelets, however, typically 'alter' so rapidly that it is rare to observe them in their normal circulating form, namely, that of a tiny ovoid disc, about $\frac{1}{4}$ - $\frac{1}{2}$ the diameter of an erythrocyte, and with a delicate feebly refractile contour, a clear translucent periphery, ("optisch leer", Stübel[459]) and a few central granules of varying refractility, which are devoid of Brownian or other movement. There is no trace of any nucleus. Viewed in profile, the platelet appears 'kennetjie' ('tip-cat' or 'batônnet', Aynaud[24]) -shaped, or fusiform

('spindelformig', Stübel[459], see p. 52. Altered forms of platelets occur in a matter of seconds after withdrawal of the blood. First there is a swelling into a spherule, the shape of which is quite evident when it happens to be rolling over in fluid currents in the plasma. This form has an even fainter outline, but more distinct inner granulation, consisting of the larger and brighter particles formerly observed, together with a background ('granulomere') of finer hazy granules only faintly visible. It attains to three or four times the size of the original platelet and evinces a 'stickiness' which causes it to adhere to the slide or coverslip. Once adherent, its outline may continue to expand, apparently from several foci, until a diameter of some 10μ is reached. We term this a spreader form. It is seen in only a small minority of the altering platelets, however. Much the commonest change is that which has been frequently described by earlier observers, namely, the stellate form (see plate 1 of Figure 18). This shows a number of processes or, as we prefer to call them, 'excrescenses' protruded from the margin of the platelet and taking up practically all the material of the outer layer or 'hyalomere'. The excrescences are of three kinds, viz. (1) rounded or 'vesicular' ('bosselures', Aynaud; 'kugelige Bläschen', Stübel), which are most frequent under these simple conditions (see plate 3 of Fig. 18); (2) club-like; and (3) filamentous (see Fig. 3 in ref. [124]).

The delicate vesicles of (1), above, seem to hover like a captive balloon at the surface of the platelet. After a certain

degree of swelling they have been observed to shrink temporarily with an adjacent eddying of hemoconia (chylomicrons), suggesting release of contents into the surrounding plasma. As they gradually swell again, there appear a few granules, some apparently entering in from the body of the platelet, and these granules show vigorous Brownian movement, suggesting that the contents of the vesicle have now become liquified. Several vesicles usually form from each platelet and their size varies up to 5 or 6 μ. Sometimes they rupture completely and disappear, but it is far more common for them to remain for hours, either anchored to the residue of the platelet body or, occasionally, breaking away and floating free. The clubs and filaments are essentially similar to the vesicles, except in shape and in the lack of particulate contents. They are very delicate and oscillate vigorously with the jerkiness of typical Brownian movement. They frequently become detached. The feebly refractile, double-contoured free-wriggling filaments are characteristic objects. They often adhere to red cells and other objects. We suggest, as did Graszberger[472] for the erythrocyte filaments (see p. 229), that a number of observers in the past have mistaken these for spirochaetal (or other) micro-organisms and erroneously tried to explain certain diseases (e.g. Hodgkin's disease, rheumatoid conditions, etc.) as 'infections' with such agents.

From the body of the platelet, we[124] have seen, on one occasion, during the earlier phases of alteration, a single bright particle jerking in and out of the parent element in a

manner resembling the agonal movements of the granules in dying leucocyte fragments, as described by the author[122] in 1930. This could be regarded as evidence that the platelet is a necrobiotic cellular (see megakaryocyte) fragment. Such outline of the body of the platelet as may remain visible after formation of the 'excrescences' exhibits delicate form changes for a short time, but soon ceases to be distinguishable, especially when several platelets adhere together. The ultimate result of platelet alteration is the formation of an adherent granular matrix of large and small particles, in which body outlines are no longer visible. Adhering to the mass are numerous vesicles, clubs, and filaments, and often several fibrin needles (see Fig. 1 of ref.[124]).

2) Agglutination of platelets. This characteristic phenomenon of normal platelet alteration was first described by Hayem[213,214], in 1877-1878, and especially studied in 1917 by J. H. Wright and G. R. Minot[511] under the name 'viscous metamorphosis' coined by Eberth and Schimmelbusch[113], in 1886.

Under the dark-field microscope, it is seen that the platelets which happen to be thrown together while acquiring the 'stickiness' previously noted during the period of swelling, readily adhere to form a clump, which is increased by the addition of other platelets that chance along. It is this removal by agglutination and adherence to glass and other wettable surfaces, rather than disintegration, which makes it so difficult to find platelets in serum from clotted blood, and also renders any

attempt at counting of the platelets invalid if even a trace of clotting has occurred. Aynaud[24] and others, including ourselves[124] used paraffined surfaces to minimize these clumping phenomena. The more recent introduction of non-wettable siliconed surfaces is an improvement over these earlier methods, which we have frequently used in more recent years and which has been commented on in some detail by Marjorie B. Zucker[518] as noted on p.65. The phenomena of platelet clumping and partial disintegration (or 'alteration') normally in vitro make it difficult to study specific platelet-agglutination and platelet-lysis, e.g. after sensitization to certain drugs or antiplatelet sera[1]. Such studies must be performed with extremely careful technique and the use of siliconed surfaces.

3) Platelets and fibrin. The often reported finding of fibrin needles or filaments, adhering to altering platelets is, in fact, less important than the observations: (1) platelet alterations commence long before fibrin appears, (2) many fibrin needles can be seen, under the dark field, to appear de novo out of the background of the clear plasma, by no means requiring the platelets (or other visible object) as foci for their formation.

4) PLATELET PRESERVATION. The mere use of a non-wettable surface, e.g. silicone, sans anticoagulant, has only a minor effect in delaying the typical platelet alterations, which soon show all the appearances which we have described. Of course, it is highly probable that complete maintenance of non-wettability is impossible under the conditions of observation, and that there is bound to be some air-liquid interface.

4a) Anticoagulants afford a much more practical means of controlling platelet alterations. Aynaud's studies, see p. 219, must be given full credit for the thorough investigation of very many added agents investigated for their effects on platelet alterations. We have merely used citrate, oxalate, and, more recently, sequestrene-Na_2 (EDTA, see p. 140) to confirm the ability of the decalcifying anticoagulants to preserve platelets. By preservation, we mean that the alterations, which do still occur, are much less disintegrative. All changes are greatly retarded and there are some definite differences from the sequence of events which we have described above. Agglutination of the platelets is particularly delayed and may be negligible even after days, especially with sequestrene combined with the use of siliconed surfaces.

When citrated platelet-rich plasma is placed on an ordinary glass slide for dark-field observation, many single platelets are observed floating in the plasma. Some, however, sediment and adhere to an extent sufficient to resist the small fluid currents always seen in these wet preparations. Both free-floating and adherent platelets are rather more refractile than in the simple blood drop. The tiny disc form, frequently well seen edgewise, preponderates at first and may be regarded as the normal form. Its outline is well defined and there is a clear hyalomere and a few central stationary granules (granulomere). It soon begins to show delicate short wavy filamentous excrescences from one or more points along the edge. Confirming Stübel[459], we have sometimes

seen these filaments form, withdraw, and reform, but they soon remain extended and irreversible. After a few minutes they appear to become more definitely rigid. They seem to extend further over the course of several hours and may reduce the body of the platelet to insignificance. Sometimes a single very long filament extends from the small granular remnant of the body, in what we have termed a sperm-like form[124]. A number of rounded excrescences, usually one (or two) to a platelet, may also be observed. Unlike the vesicles of unpreserved platelets, however, these can be seen edgewise to be disc-like. Hence we[124] use the term plaquette-form. Except with vigorous artificial manipulation, it is unusual for either the filiform or plate-like excrescences to become detached. Preserved platelets may be washed with saline (0.85% NaCl) in silicone and kept in the frozen stage ($-20^{\circ}C$).

4b) Recalcification results in a re-acceleration of the retarded alterative phenomena in the platelets and if the plasma is still present, fibrin formation occurs a few minutes later. The recalcified platelets swell, adhere, clump, and (partially) disintegrate in much the same manner as in unpreserved blood, and the phenomena are essentially similar in recent preparations and in long preserved platelets (in silicone at $4^{\circ}C$) recalcified as usual. See plate 2 of Figure 18. It is rather remarkable to see the rigid excrescences soften and swell and vesiculate, often involving the whole of the platelet, except for most of the granulomere. Granules evincing Brownian movement may appear in the vesiculated forms.

Effects of varying citrate concentrations were minutely studied in our 1934 publication[124], to which reference may be made for details. The important conclusion was that the concentrations of decalcifying agent which prevented clotting also 'preserved' the platelets to the extent of maintaining rigidity of excrescences, lessened clumping, and absence of 'fluidity' of platelet content. We regard this as significant evidence that <u>calcium</u> has a specific role in the special ('liquefactive') disintegration phenomena by which the platelets come to play a very important role in the blood clotting phenomena. <u>Osmotic imbibition</u> of water by platelets (1) in hypotonic solution, e.g. on diluting with distilled water or (2) on adding saponin (see plate 4 of Fig. 18), in the presence of citrate, do not result in plasma clotting.

The conclusion we[124] reached in 1934 was that the platelet 'alterations' consist fundamentally of a 'myelin figure' formation from their surface phospholipids (see later) and that calcium, by its influence on the phase-relations of the lipoid-water system, specifically directs the water into the fatty platelet material thereby swelling and disrupting it. This appears to be a significant contribution, ordinarily, to the platelet release of factors which the platelets contribute to the blood-clotting system.

(8,C) MEGAKARYOCYTES[139]. Bone-marrow, freshly obtained from the femur or tibia of anesthetized rabbits, was quickly mounted in autologous serum, previously obtained from heart-puncture blood of the same animal. Dark-field microscopic appearances are shown in Plates 5 and 6 of Fig. 19.

In the former, no antigoagulant was used, and vesicular excrescences, containing granules oscillating with Brownian movement, are seen at the periphery of the megakaryocyte (in a small proportion of the giant cells observed). The excrescences are very similar to ordinary platelet alterations and, like the latter, often become detached. In the second series of observations, the marrow preparation was citrated (plate 6). This suppresses the slow amoeboid movement of the megakaryocytes and causes them to show a well-defined outline and a fixed glassy appearance of their very granular cytoplasm (very similar to leukocyte appearances in citrated blood preparations). A very few vesicles (see plate 6) continue to appear, however, but are smaller, more refractile, devoid (or almost) of granules, and resemble the plaquette form of correspondingly treated platelets (p. 226).

We have never observed a living megakaryocyte fragmenting into platelets[510] and believe the phenomena described are absent of less marked in the less mature megakaryocytes. They were not observed in polykaryocytes (osteoblasts), which are unrelated to platelet origin.

(8,D) THROMBOCYTES[139]. The nucleated thrombocytes of amphibia (frog), reptiles (turtle), fish (fresh water sun-fish), and birds (fowl) have been studied under similar conditions. Plate 7 (Fig. 19) shows an altered frog thrombocyte, lying on top of a large fibrin filament. Note the vesicular bodies derived from the thrombocyte, some still attached and others released for some distance. As frequently noted in the earlier literature on

thrombocytes, their disintegration is often very sudden and explosive in character. This scatters excrescences, chiefly vesicles, and granular material to some distance from the parent thrombocyte, as well shown in plate 8 (Fig. 19) in the case of the sun-fish observation. It is noteworthy, however, that many vesicles persist in the region of the original adherence and disintegration of the thrombocyte. The dark-field observations confirm the essential similarity of the alterations of thrombocytes, megakaryocyte margins, and mammalian platelets.

(8,E) ERYTHROCYTE STROMATOLYSIS, ETC. Furchgott[182], in 1940, made dark-field observations of altering erythrocyte 'ghosts' (stroma of hemolyzed red cells) in contact with lyotropic salt solutions such as lithium perchlorate or potassium thiocyanate. He was apparently unaware of our earlier data on platelet alterations and, independently, suggested the 'myelin figure' explanation of the phenomena observed. His search of the red cell literature revealed several earlier observations (e.g. Oliver[355], Kite[267], Auer[23], and others) of essentially similar appearances from both hemolyzed and non-hemolyzed red cells. In some observations made by the present author in Cape Town in 1930 and recorded in the photomicrograph reproduced in Plate 9, Fig. 20, 'stromatolytic' filaments were observed extending from the margins of young erythroblasts in a drop of heart blood from a rabbit embryo. Plate 11, Fig. 20, photographed by an erstwhile graduate student (P. H. Ralph) working under the author's supervision at the University of Michigan in the early 1940's, shows apparently similar thread-like processes connecting

points at which normal adult human red cells have apparently touched and then withdrawn from one another. Plate 10, Fig. 20, also prepared by Dr. P. H. Ralph, is a striking example of the spiculated stromatolytic appearances occurring in vitro in slide (dark-field microscopy) observations of a nucleated red cell from a tadpole[139].

Conclusion. There is clearly a common phenomenon connected with surface 'alterations' of the several varieties of blood and bone-marrow formed elements collectively considered in the fore-going observations. The basis for attributing these phenomena to a myelin figure formation from the surface phospholipids of these formed elements, follows from the next series of observations.

(8,F) MYELIN FIGURE FORMATIONS FROM PHOSPHOLIPIDS.

Foreword. R. Virchow[483] first described myelin forms (figures) as microscopical appearances at the edges of nervous tissue observed in contact with watery solutions. He interpreted them in terms of surface (interphase) relations between the lipoid and aqueous phases. J. B. Leathes[284] studied them very extensively, in 1925. He photographed, at successive ½ minute intervals, the large myelin figures appearing at the edge of a film of lecithin, in contact with a wide variety of watery solutions, chosen to illustrate the effects of (a) specific ions, (b) osmotic concentration, and (c) adsorbed substances. The myelin forms were readily produced by distilled water. Leathes noted a suppressive or inhibitory effect of calcium ions, said to be overcome by 0.01 M NaOH and by cholesterol.

Other retarding and many favoring agents were recorded by this observer. The present author, in 1934[124] reproduced some of the lecithin experiments of Leathes. Plates 12A and 12B (Fig. 20) show the dark-field appearance (400x magnif.) of the edge of the phospholipid film: (A) at the start, in contact with air, showing only the linear highly refractile margin; and (B) 3 minutes after contact with distilled water, showing the typical large (initial) myelin forms flowing out into the watery phase (upper right part of photo). Clearly visible in the original, but poorly reproduced in the photomicrograph (and therefore retouched) is the 'translucent border', as described by Leathes[284].

Our observations established one or two new points which we believe to be of considerable significance.

Firstly, all watery solutions cause an initial myelin figure outflow (i.e. into the aqueous phase), which is followed by a further expansion after a 5-10 minute 'delay period'.

Secondly, the dark-field method permits interpretation of Leathes' translucent border as a reciprocal phenomenon, namely, a penetration of the water into the lipid phase. The secondary expansion phase, we observed to be very dependent upon the particular ions in the watery solution. If distilled water or sodium chloride (and similar salt) solutions were used, the 'secondary' myelin figure outflow went on with increasing speed and vigor, but the inflow of watery phase into the lipid was limited and showed little further penetration beyond that seen in the initial phase. With calcium salt solutions,

these phase relations were reversed. There was the same initial myelin figure outflow, but little extension after the delay period. Secondarily, however, the inflow of this solution into the lipid proceeded apace and without limit, extending the 'translucent border' throughout the lipid portion. Our important conclusion was that calcium is specific in directing water into the lipid phase, as is well-known from the work of physical chemists with phase-relations of calcium and sodium salts in soaps and other fat-water emulsions.

To Leathes' conclusion that "myelin forms are due to surface growth and localized imbibition of water", we would add that calcium ions specifically generalize the imbibition and cause a 'reverse' myelin figure appearance, as can be seen very clearly in dark-field observations of lecithin films.

Applying these ideas to platelet (etc.) alterations, we conclude that the calcium has the specific role, not of causing the excrescence formations (which can occur in citrate, etc.) but in the liquefaction of the excrescences. By directing water into the altering platelets or thrombocytes, calcium plays an important role in their peculiar disintegrative phenomena. What about the nature of the lipid itself? In our 1945 observations[139], we were able to include some dark-field observations on other phosphatides, namely, the purified preparations of Dr. J. Folch (of the Rockefeller Institute, N. Y.), which are described on p.174 .

The chief additional information obtained was that cephalin, of the several phospholipids studied, yielded

myelin forms which, in size and appearance, e.g. vesicles, were often so similar to the above-described platelet, megakarocyte, and thrombocyte alterations, that it seemed reasonable to regard them as identical.

Actually, the fundamental myelin figure phenomenon was common to most of these phosphatides in contact with watery solutions and calcium salts caused a granular deposition penetrating into the lipoid and interfering with its hydrotropic dispersion into the watery phase. The strength of the salt solutions had only minor effects suggestive of some membrane phenomenon in which water penetration rates are partly controlled by osmotic considerations.

The inositol-phosphatide (III) behaved peculiarly in that it went directly into colloidal suspension as 'chylomicra' instead of showing 'myelin forms'. This is evidently representative of the behavior of the bulk of lipoidal and lipoprotein materials of plasma.

Concerning the protein components of the surfaces of platelets, etc., our experiments have nothing to say.

(8,G) CONCLUSION. In summary, then, our comparisons of the dark-field microscopical appearances of (a) water-phospholipid films and (b) in vitro 'alterations' of platelets, megakaryocytes, thrombocytes and certain erythrocytes, indicates the fundamental importance of 'myelin forms (figures)' and related phenomena at water lipid interfaces. We believe these to be extremely primitive and essentially non-vital phenomena of lipoid-rich surface membranes. Not only is there an

hydrotropism of the lipid material, causing the appearance of surface 'excrescences' of the various types described, but there is also a specific role for the calcium ion. This reverses the phase relationships and directs water into the fatty phase, thus contributing to the peculiar disintegrative phenomena studied. Cholesterol, proteins, osmotic factors, pH, and perhaps other factors need additional study as further possible modifiers of the basic phenomena which we have described.

G. CLINICAL CASES WITH PLATELET PROBLEMS

FOREWORD. Our research program, 1950-55, with regard to the investigation of clinical cases with hemorrhagic and related disorders, was largely planned and executed by Dr. Jessica M. Lewis, Research Associate and Co-investigator, with advice, assistance, and some participation by the author. Invaluable technical assistance was rendered by Francis M. Morgan, Doris C. Ferguson, Iris Rudin, Ann C. Howe and Bertha G. Jackson. Since 1953, a graduate student, Mr. James W. Fresh, has made many able contributions especially to Group I studies (see below), and, in the past year, Miss Mary Jane Patch has been a most helpful collaborator. The staffs of the University's (N. C.) Memorial Hospital, Duke Hospital, the Veterans' Hospital, and ORINS Hospital have cooperated with the patient material, as have a number of private practitioners. We are indebted to Dr. Marjorie B. Zucker of the N. Y. U. College of Dentistry, Physiology Department, for the serum serotonin assays[519], used to assess the platelet vasoconstrictor factor (item 17, see Tables).

In preparing the data for this thesis, the author has proceeded independently and has not consulted Dr. Lewis, who now has her own Research Laboratory at the University of Pittsburgh. Gratefully acknowledging the leading role of Dr. Lewis and the individual contributions of each member of our group, the author, nevertheless, assumes sole personal responsibility for the following presentation and analysis of the data selected.

CASES: represent some 86 patients and 32 normal mothers and their infants. They are classified into 9 groups. No attempt will be made to analyse them from the clinical viewpoint. Rather, they represent merely a physiological contribution of analytical laboratory methods, with which to approach a rational diagnosis and understanding of the clinical hemostatic problems. Our 'groups' are chosen from this physiological viewpoint to serve the purpose of this thesis enquiry.

METHODS: for the most part, have been described on pp.91-108, and will not be further elaborated. An itemization (1-30) facilitates organization and reference. In the first Table (LII), the mean and range of test values are given for (A) normal healthy adults (randomized as to sex and age), (B) parturient mothers, and (C) their normal newborn infants (cord bloods).

0, ±,(i.e. trace or minor), + ... ++++ : indicate relative degrees of (7) bleeding tendency, and (12) clot-retraction (in glass). Retraction was also observed in silicone (cf. 11), but this will not be reported, since the differences from (12) were relatively minor. In group summaries, the number of cases was divided, for each test, into the following categories:

POS.: significant alteration of the test value.

NEG.: not significantly different from the normal (norm.).

?: doubtfully significant.

N.T.: no test performed.

RESULTS: are presented in Tables LII - LX.

Group I: (A) NORMAL ADULTS, (B) PARTURIENT MOTHER, (C) NORMAL NEWBORN INFANTS (CORD BLOODS), ref.[172]. TABLE LII.

(A). NORMALS: were from healthy adults, randomized as to sex and age. They were mostly medical students, staff, and hospital personnel, including a few colored helpers. The means of the various tests were statistically determined on significantly large groups, usually more than 50 cases.

(B). MOTHERS: the 32 cases studied permitted venepuncture during parturition. They all appeared healthy and well, and gave no history of recent infections, antibiotic or excessive salicylate therapy, dietary inadequacy, or gastrointestinal disturbance. The obstetrical anesthesia was non-contributory. Pregnancy, labor, and the puerperium were uncomplicated by toxemia, hemorrhage or thromboses.

(C). INFANTS: the 32 newborn babies yielded blood samples from the umbilical cord before it had stopped pulsating. All babies were delivered without trouble, were uninjured, and appeared normal. None developed any bleeding tendency. Some mothers and some babies received vitamin K., questions concerning which are answered in the cited references[172,173].

TEST RESULTS: See Table LII. For purposes of this thesis, comment will be limited to the noting of the absence of any platelet problems in (B) and (C). All tests, in this regard, fall within normal (A) limits. A single exception (10%) of low platelet accelerator (16) in (C) was of doubtful significance (possible lability).

The many interesting findings in other tests are fully dealt with in the cited publications, and include data on another

125 newborns, and most of their mothers, which are not included in the present more completely studied group.

GROUP II. CONGENITAL (FAMILIAL) THROMBOCYTOPENIA. (Cases 1-2). Table LIII-A.

Because of their unusual interest (ref.[453]) these cases will be reported individually, in Table LIII-A.

Case 1. (A.B.): was examined on two occasions, (a) 1 Feb. '54, before treatment, and (b) 20 Apr. 855, a few days after splenectomy. In tests (a): the plat. count (14) was very low (38,000) and assays for specific platelet factors (15 - 18) were not attempted. Clot. retr. (12) was almost absent, but prothr. consumption (25) was amazingly normal. The child had bleeding symptoms (7) at this time, dating back to shortly after birth. Bleed. time (9) was very prolonged, and the T. T. (tourniquet test) (8) was strongly POS.

Family histories (F. H.) (6) will not be detailed in the present studies, but were significantly POS. in Cases 1 and 2.

In tests (b), after splenectomy, all test values were restored to normal, with the questionable exception of a 50% plat. tpln. (15). The patient developed no further bleeding problems in a 12 month follow-up.

Case 2. (D.D.): was not a bleeder (7), despite the POS. Fam. Hist. (6). Nevertheless, his plat. count (14) was low (98,000) and tests 8, 12, 15, and 16 were significantly abnormal. He should, therefore, be regarded as a potential bleeder.

GROUP III: HEREDITARY THROMBOCYTOPATHIC PURPURAS. Case 3: GLANZMANN'S THROMBASTHENIA. Cases 4, 5. PSEUDOHEMOPHILIA, WITH PLATELET PROBLEMS. TABLE LIII-B.

Case 3. (N.P.): was a severe bleeder (7), with POS. Fam. Hist. (6). The plat. count (230,000) was normal, but clot retr. (12) was absent and the bleed. time (9) and T.T. (8) were very significantly POS. Plate. accel. (16) was not tested, and plasma proaccelerin (29) was a low normal (62%). Plat. tpln. (15) and ser. serotonin (17) were normal (100%). Test 19 was NEG., but serum antithr. (20) seemed to be increased. Prothr. consumption (25) was normal (95%).

Conclusion: This rare case seems to be one of the very few reported (ref. [383]) which conform to Glanzmann's[188] 'hereditary hemorrhagic thrombasthenia'.

Cases 4, 5: A.C. and S. C., mother and daughter, presented a familial (6) disorder, which corresponded most nearly to the classical descriptions[484,485,486,260,262] of PSEUDOHEMOPHILIA (ref.[383,453]).

Case 4: was a 52 yr. old white woman giving a + (+) history of bleeding tendency (7) since 19 yrs. of age. The chief manifestations were ecchymoses and a moderately severe post-operative hemorrhage. Bleeding time (9) and clotting-times (10, 11) were normal, as was clot retr. (12). Her plat. count (14) was normal (238,000), but the plat. tpln. (15) significantly reduced (50%) and plat. accel. (16) a low normal (50%). Serot. (17) N.T. The T.T. (8) was significantly +++. Prothr. consumption (25) was normal (>95%), as were AHF (22), PTC (23)

and other tests. Plasma proaccel.(29) and proconv. (28) N.T. Fibr. (21) was elevated.

Case 5: the daughter, 20 yrs. old, had also suffered +(+) ecchymotic bleedings. Her plat. count (14) was a low normal (104,000) and the plat. tpln. (15), 25%, and plat. accel. (16), 20% unequivocally reduced. Serot. (17) N. T. Plasma proaccel. (29) was normal (90%), AHF (22) likewise, and probably PTC (23), 60%. Prothr. consumption (25) was quite normal, as were other tests, incl. a slightly questionable (71%) (recip., see p. 99) prothr. time (26). Clot retr. (12) was a satisfactory +++. Fibr. (21) was elevated.

DISCUSSION. In these two cases of pseudohemophilia, the platelet problems were relatively minor and functional (thrombocytopathic). They must be regarded as of questionable clinical significance in a type of hereditary disorder which appears to be primarily a vascular dysfunction. We had 6 other cases of pseudohemophilia, 2 of hereditary telangiectasia, and 2 with bleeding hemangiomata, none of whom had any platelet problems, and whose tests, therefore, are irrelevant to the present enquiry.

ACQUIRED DEFICIENCIES OF PLATELET FUNCTIONS: will be presented in the other groups. The UREMIA cases (Group VIII) are especially interesting (p. 246).

GROUP IV: ACQUIRED THROMBOCYTOPENIA. (Cases 6 - 30). Table LIV. These 25 cases were clinically diagnosed as follows:

(A): 11 acute ITP (idiopathic thrombocytopenic purpura), of which 3 may have been due to drugs, and 3 to infections, namely, (1) respiratory infection in a 17 mo. old child, (2) diphtheria

in a 4½ yr. old child, and (3) infectious (? toxic) hepatitis in an 18 yr. old white girl.

(B): 7 chronic ITP, incl. one of 2-mths. duration, assoc. with rheumatoid arthritis in a 15 yr. old girl.

(C): 5 were associated with anemias namely, (1) two aplastic anemias, (2) two macrocytic anemias, one pernicious and the other occurring during pregnancy in a 22 yr. old woman, and (3) one with Banti's syndrome.

(D): 2 were associated with metastatic (bone) carcinomata in elderly males, with primary prostatic malignancy.

TEST RESULTS: are given in Table LIV, in summary.

DISCUSSION. Plat. counts (14) ranged from 5,000 to 84,000, except for the 174,000 (at this exam., Dec. 10, '53) in a 33 yr. old colored woman with pernicious anemia (v. 'C'). Many showed a severe bleed. tend. (7), one was doubtful, 4 N.T., and only one gave no bleeding history. The T.T. (8) was POS. in 19 and questionable in 2 others, who were negroes, whose dark skin interfered with the reading of this test. Bleed. time (9) was POS. in 17 and questionable in one. Clot.-time (glass, 10) was definitely prolonged in only one case, but the silicone c.t. (11) was pos. in ten cases. This indicates the superiority of test 11 in cases with borderline clotting-time increases. Clot. retr. (12) was POS. in 17 cases. None showed fibrinolysis (13). Platelet functions (15, 16, 17) were usually reduced, pari passu, with the lowered platelet count (14). For (15) plat. tpln., testing 20 cases, 19 were POS. and questionable in one. For (16) plat, accel., testing 19 cases, all 19 were POS.

For (17) ser. serotonin, testing 12 cases, 6 were POS.

Prothr. consumption (25) was defective in 16 and questionable in one. This is highly significant, but the quantitative correlation between platelet levels and degree of failure to utilize prothrombin showed a very poor correlation. It is suggested that the quality of the platelets, in terms of functional capacities, is more important than mere numbers, in controlling prothrombin utilization. The plasma components (22, 23) of this mechanism were normal except for one borderline (60%) and one low normal (65%) PTC assays, neither in the range which could be expected to influence the prothr. consumption test.

A small incidence (see Table) of other thrombin-factor deficiencies is unexplained. It is possible that liver function is occasionally disturbed, perhaps by hemorrhage. The elevated fibr. (21) in 9 cases may be evidence of a liver reaction. The low plasma proaccelerin (29) in 3 cases does not reflect the many more, 19 cases, low values for plat. accel. (16). Plasma-thr. c.t. (19) was definitely prolonged in 15 cases and slightly so in 8 more cases. We do not yet know the possible significance of this finding.

GROUP V: THROMBOCYTOSIS. (Cases 31 - 33). TABLE LV.

These three cases present different clinical disorders, but show, in common, very elevated platelet counts (14). Two (31, 32) had bleeding symptoms.

RESULTS: are detailed in Table LV.

DISCUSSION. Case 31: (R.G.) was a 63 yr. old colored man with primary adenocarcinoma of the stomach and generalized metastases.

At autopsy, the bone-marrow was found to be hyperplastic, with a greatly increased number of megakaryocytes. He was examined 4 or 5 times between June 23, '53 and his demise on 27 Aug. '54. The cited tests were on the first occasion, but the thrombocytosis persisted at similar levels through 29 June, '54. He developed a large hematoma in the upper right arm, inexplicably. Except for a low plat. accel. (16) on Jan. 6, '54, all tests of platelet function, incl. clot retr. (12), and prothr. consumption (25) were normal., Serotonin (17) was not tested, however. The fibrinogen (21) was a borderline (200 mg) on 6 Jan. '54 and plasma proaccel. (29) low (20%) on the same occasion. These findings could go with a fibrinolytic enzyme activation, which might have been overlooked in recording of a glass c.t. (10) of 15 min. and ++++ clot. retr. (12) on that occasion. We do not feel sure of the crude fibrinolysin test, however, unless all the clot dissolves within 24 hrs.

Case 32. (J.L.H.) was a 64 yr. old white male long known to suffer from polycythemia rubra vera. Gout was an interesting complication (ref.[503]). He had received P^{32} (red cell destructive) therapy at the Veterans' Hospital, and we examined him twice (30 Mar., '54 and 8 Apr., '55) after such courses of treatment, when he was showing some bleeding symptoms (cf. Group IX). Despite the extremely high platelet counts (14), the platelet function tests (15, 16), and esp. (17), serotonin, were significantly depressed. So were the prothrombin and related factors (26 - 29), but not the prothr. consumption (25), although this

might be misleadingly calculated because of the lowered level of the original plasma prothrombin.

Case 33. (E.M.), was a 23 yr. old white male leukemic, whom we have excluded from Group VI solely because of the unusual thrombocytosis. Serotonin (17) was not tested, but the other platelet function tests were normal. There was some hypoprothrombinemia (27) and prolonged prothr. time (26), which might have to do with liver function. This case showed no hemorrhagic symptoms.

GROUP VI: LEUKEMIAS, WITH THROMBOCYTOPENIA OR THROMBOCYTOPATHIA PROBLEMS. (Cases 34 - 67). TABLES LVI-A, LVI-B.

Besides the thrombocytosis case (No. 33, Group V), platelet problems of one kind or another presented in 34 more (i.e. all but 4) out of 39 cases of leukemias studied. The remainder of these test data will be separated into two groups (A, B) by arbitrarily dividing them at a 100,000/mm^3 level of the platelet count.

Table LVI-A: presents a summary of the thrombocytopenics (22 cases).

DISCUSSION: 12 of these cases recorded bleeding symptoms (7). Platelet counts (14) ranged between 17,000 and 100,000. Platelet function tests (15, 16, 17) were significantly reduced in all but one or two. Platelet agglutination (18) was demonstrated in 5, out of 11 tested, cases. The T.f. (8) was POS. in 5 cases, and bleed. time (9) prolonged in 7 cases. Silicone c.t. (11) was significantly prolonged in 15 cases. There were 16 cases, and one doubtful, with defective prothrombin consumption (25).

Antithrombin tests (19, 20) were significantly altered in many, especially if we include minor variations from the normal controls. 12, and 1 questionably, had a low plasma proaccelerin (29), but half again as many cases had low plat. accel. (16). 7 had prolonged prothr. time (26), but the specific thrombin-forming factors (except for the accel. noted) were usually normal, or nearly so.

Table LVI-B: summarizes leukemias with normal (or nearly normal) platelet counts, but with evidence of some or other deficiency of platelet functions, details of which can be noted in the Table. 5 of these 12 thrombocytopathia cases recorded bleeding problems (7).

In addition to the tests reflecting platelet functions (8, 9, 12, 14 - 18, 25), a prolonged silicone c.t. (11) might be significant in some instances. As compared with the A group's 17/22, the B group's 5/12 is a smaller proportion showing defective prothr. consumption (25).

CONCLUSION: It would appear that platelet defects, whether definite thrombocytopenia or functional platelet defects (thrombocytopathias), are commonly encountered in leukemias. This may very well play a leading role in the hemorrhagic tendency in leukemia. Cases with abnormal values in our tests, but no overt bleedings, may be regarded as potentially liable to episodes of bleeding.

Some less frequent reductions in other clotting factors (e.g. prothrombin, etc.) may reflect injury to liver, as well as bone-marrow, functions.

ADDENDUM. While both acute and chronic types of the common myeloid and lymphatic leukemias are included in both sub-groups, the (A) series contained relatively more of the acute types, while the (B) series had more chronics. Included in (A) also were (1) one case of coincident polycythemia, which is now a well-recognized[503] association, and (2) one case which was finally diagnosed as lymphosarcoma. Included in (B) was one case of monocytic leukemia.

GROUP VII: PLATELET PROBLEMS IN UREMIA (Cases 68 - 78).

TABLES LVII-A, LVII-B.

Out of 12 uremic cases studied because of bleeding problems, 11 showed various platelet anomalies. These are summarized in Table LVII.

(A) Cases 68 - 77: In this sub-group of 10 cases, 8 had definite bleeding problems, 1 denied such, and 1 lacked the necessary history (7). The findings itemized 8 - 12 were NEG. or infrequent. Platelet counts (14) were all within the normal range. Quite otherwise, however, were many of the platelet function tests. Thus, plat. tpln. (15) was low in 7 cases, and plat. accel. (16) in 7 and one doubtful. The two cases tested for serotonin (17) gave significantly low values. 4 cases, and one doubtful, showed defective prothrombin consumption (25), with insufficient lowering of the tested plasma components to account for this.

CONCLUSION. We believe these data offer a new explanation for the bleeding tendency in uremics, namely, that their platelets, while normal in number, are functionally defective (thrombocytopathic),

much as they are in many leukemics (Table LVI-B). The not-infrequent defect in prothrombin consumption could be a result of this also.

(B) Case 78: is of doubtful interest. The one borderline (50%) value for plat. accel. (16) on the first of the two examinations, is the only justification for its inclusion in Table LVIII. Platelet counts and other functional platelet tests were normal on the two occasions. The clinical diagnosis, of chronic pyelonephritis in polycystic kidneys, showed an infectious complication of an already toxemic nitrogen-retention. The low levels of PTC and thrombin-forming factors on 30 May, '55, the day before demise, could have been due to liver failure. We would just be guessing to blame her bleedings on some toxic disturbance to vascular integrity.

GROUP VIII. PLATELET PROBLEMS IN CASES WITH PLASMA CLOTTING DIFFICULTIES. (Cases 79 - 85). TABLE LVIII.

These 7 cases (including one questionable) represent a very small minority of our collected data on 52 hemophiliacs, 26 PTC-deficients, 24 acquired hypoprothrombinemics, 5 congenital hypoproconvertinemics, 4 congenital hypoproaccelerinemics, 8 cases with circulating inhibitors (anti-AHF or anti-PTC), and several others with obscure clotting problems. The data of the 7 cases are individually analysed in Table LVIII.

(A) Cases 79 - 83: might be sub-grouped as primarily severe liver disorders, with mild systemic bleeding problems. Two (80, 81) had marked jaundice, and four (80 - 83) showed

no response of their hypoprothrombinemia to vitamin K.

Plat. counts (14) in Nos. 79 - 82 were considerably reduced (31,000 - 92,000), and it was questionably low (128,000) in Case 83. Functional tests were N.T. on case 83, but were significantly low for (15) tpln and (16) plat. accel., in the four others. Serum serotonin (17) was quite normal in the same cases, however. The T.T. (8) was + in Case 80, but NEG. in 79, 81, 82. Clot Retr. (12) was only ++ in Case 82, but normal in 79. Lysis (see below) interfered in the other cases. The prothr. consumption (25) was quite normal in 79 - 82, and N.T. in 83. Plasma-thr. c.t. (19) was increased in the two specimens (79,82), in which there was no interfering fibrinolysis. Serum antithr. (20) tested 'normal' in 3 cases. The liver dysfunction could account for the low levels of PTC (23), prothrombin (26, 27), proconvertin (28), but not necessarily of the pro-accelerin (unless the liver has a 'storage function' for this component of the thrombin-forming system)[322,492].

Fibrinolysis was a complication, which could also be explained by the severe liver injury[453]. It interfered with determinations of clotting-times (10, 11), clot-retr. (12), and plasma-thr. c.t. (19). A borderline (200 mg) fibr. (21) in case 83 may have been due, in part (?) to the fibrin(ogen)olysin, but the fibrinogen was slightly above normal in Case 81, who also showed fibrinolysin.

(B) Case 84 (D.B.): was a 3 yr. old white female child, who was the most severe of the 4 congenital hypoproaccelerinemia cases whom we have studied, and was the only such case with unusual platelets. The absence of proaccelerin (29) sufficiently explains the bleeding tendency (7), the prolonged clotting (10, 11) and prothr. time (26), as well as the defect (43%) in prothr. consumption (25). True prothr. (27), proconv. (28), PTC (23), AHF (22), and fibr. (21) were all normal. There was no fibrinolysis (13), nor inhibitor (24), and clot retr. (12) was normal. The platelet count (14) was a normal 240,000 and plat. tpln. (15) normal (100%). However, the platelet accel. (16) was very low indeed, although it was measurable. The high value (250%) for serum serotonin is interesting, and the suggestion that this might be a physiological 'compensation' is an open question. It was this case that most strongly influenced us toward a tentative hypothesis that the platelet accelerator is very closely related to, if not identical with, plasma proaccelerin.

(C) Case 85. (C.R.): was a 70 yr. old white male with prostatic carcinoma and metastases. His bleeding problem was primarily associated, we believe, with a circulating fibrinolysin (ref.[453]). When first seen (10 Jan., '55), the plat. count (14) was low (36,000), as were plat. tpln. (15), only 6%, and plat. accel. (16), a barely assayable 1%. Plasma proaccel. (29) was also very low, and fibr. (21) seriously depleted. Our experience with cases of active fibrinolysin supports the thesis that fibrinogen and accelerator factor are the

two clotting factors most easily destroyed by the enzyme. At least in clinically encountered fibrinolysin concentrations, the other clotting factors are resistant. If our interpretation is correct, the enzyme, in Case 85, is also attacking plat. accel. and perhaps whole platelets, which could account for the anomalous platelet findings.

The thrombocytopenia could explain the defective prothr. consumption (25), and the lack of accelerator, the prolonged prothr. time (26). There was a significant increase in serum antifibrinolysin (24), which follows a pattern which we, and others[453], have observed in such cases.

The second examination, 7 weeks later, showed the enzyme still to be present, but in reduced amounts, and with the same high antifibrinolysin titer (24). Platelets (14) had increased to practically normal, and fibrinogen (21) and other tests (26, 27, 28, 29) were considerably improved, except for the important prothr. consumption (25), which registered an unaccountable zero. AHF (22) was not tested at this time, and it would have been interesting to have had this, perhaps vital, information.

GROUP IX. PLATELET PROBLEMS IN RADIATION SICKNESS. (Case 86).

TABLE LIX.

(A) In humans, our experience is confined to a single case, R.T. (No. 86), who was an elderly white male with a carcinoma of the colon, which had metastasised to the liver. With such a hopeless prognosis, he was being given radio-gold (Au^{198}) at the O.R.I.N.S. Hospital (Oak Ridge, Tennessee),

and the author, in consultation with the A.E.C., was permitted to obtain the test data given in Table LIX.

RESULTS: refer only to two tests, namely, prothr. consumption and plat. counts[153], but they are very interesting.

On the day (-1) before the radio-gold injection, the patient was in poor shape, and the prothr. consumption, at 67% (23% residual in 1 hr. serum), was definitely defective, although the plasma prothrombin was rather high, at 510 two-stage (p.103) units/ml, tested with a very reactive fibrinogen ('normal' = 400 - 450 units). It could have been evidence of benefit to the liver that the prothrombin consumption returned to normal for two weeks after the Au^{198} injection. The platelet counts were essentially normal throughout this period, as they were at the start.

The 19-day tests, however, showed the prothrombin consumption decreasing again (20% resid. = 80% cons.), while the platelets (158,000) were beginning to fall, although the level at this time (on a purely numerical basis) should not have been able to account for the POS. prothr. consumption test. The 22-day tests showed serious deficiencies of both platelets (48,000) and prothr. consumption (55% resid. = 45% cons.). On the following day, the patient died from a massive hemorrhage at the primary carcinoma site. This could, of course, have been a vascular erosion, but the defective hemostatic mechanisms were certainly a fatal handicap.

(B) DOG EXPERIMENTS (EXPT. 50). TABLE LX.

PURPOSE. To inject dogs with Au^{198} intravenously, in an

effort to localize the bulk of the radiation ('hard' γ and some β emissions) in the liver (which is known to sequester colloidal gold, as used for these experiments). Effects on the coagulation mechanisms were to be investigated, following such leads as appeared to be profitable and practicable under the rather restricted laboratory facilities made available to us, due to the exactions of protective measures against the very 'hot' dogs, who had to be isolated in outlying buildings devoid of the central laboratory's conveniences.

METHODS. The several test systems used were described in a publication[148] of these preliminary data, on (a) 2 controls, who received Au^{198} colloidal suspension, with an old preparation which had lost all but a trace of its radioactivity, and (b) 5 experimental animals, who received 20, 10, 10, 5, and a token 1.6 millicuries/kg. Au^{198}, fresh from the atomic pile, respectively.

Table LX summarized the day-by-day results, for the prothr consumption and plat. count tests, on 3 of the animals (I, II, III).

DISCUSSION: Lethalities were as follows:

(I): 20 mc/kg dose: death of animal on 9th day.

(II): 10 mc/kg dose: death of animal on 37th day.

(III): 5 mc/kg dose: animal surviving, and apparently recovered, 3 months later. Not illustrated are:

(IV): 10 mc/kg dose: apparent recovery after 4 weeks (according to pro. cons. test), but unexpected death on 83rd day.

(V): 1.6 mc/kg dose: minimal effects, and remained well until sacrifice on 106th day.

The prothr. consumption data and platelet counts, given in Table LX, showed some lack of correlation between the two tests. Thus, the prothr. consumption defect appeared in dogs II and III on the 6th day, at which time the plat. counts were still normal. Furthermore, the prothr. consumption test data were very different quantitatively on the 8th day, in these same two animals, although both platelet counts were moderately reduced and to about the same level.

AUTOPSY FINDINGS:

Dog (I): Liver was markedly icteric, hemorrhagic and necrotic. Duodenum showed a section, which nestled under the liver, to be swollen with a massive interstitial hemorrhage. Hemorrhages were widespread, and particularly noteworthy were (1) bleeding into neck from the venepuncture prick of the previous day; (2) pulmonary hemorrhages; (3) petchiae in serous membranes and in mucosa of alimentary canal; (5) lymph node hemorrhages.

Dog (II): Liver showed acute damage reported as "massive and complete hemorrhagic necrosis". Hemorrhages were widespread, incl. pulmonary.

Dog (IV): Liver showed marked chronic damage reported as "chronic cirrhosis, with atrophy of liver cords, engorged sinusoids, and considerable hemosiderin, apparently from old hemorrhage."

Dog (V): Liver and other organs were normal.

We are indebted to Dr. G. A. Andrews for the autopsy reports, and to Dr. Marshall Brucer, Head of the C.R.I.N.S. Medical Division, for making these studies possible.

DISCUSSION. The pathological data were supplemented with measurements of the residual radioactivity, which confirmed the major concentration of the Au^{198} in the liver. There were, however, significant amounts also in the spleen, lymph glands, and bone marrow. Hence, our preliminary attempt to localize internal irradiation for a particular organ (e.g. liver), by intravenous injection of a suitably chosen preparation, was not altogether successful. What was remarkable, however, was the extent of liver damage which radiations, thus achieved, could cause, without significant effects on prothrombin, fibrinogen, and other clotting factors.

No circulating (e.g. heparin-like) anticoagulant could be detected in our experiments.

The one test, which these preliminary experiments supported, as showing very significant results, was the prothrombin consumption test (whether by 2-stage of 1-stage methods). What is of relevance to the present thesis is the apparent lack, at times, of correlation with the well-known thrombocytopenic effect of radiations[14]. Jackson, Cronkite, et al.[96,97,245,246]. Penick et al.[374] and others[477] have reported on the post-irradiation thrombocytopenias and lack of effects on blood-clotting factors, incl. AHF[374], although causing

defects in prothrombin consumption. We accord great respect to these other workers, particularly to Dr. E. P. Cronkite for his work performed while a Commander in the U.S.N. (including participation in animal experiments both at the Naval Research Labs. (X-irradiation) and in the test atomic bombings at Bikini and Eniwetok). Cronkite also noted discrepancies of the two tests, especially during the recovery phase post-irradiation.

We believe the evidence for the significant role of platelets, as particularly established by Comdr. Cronkite's group of workers, to be most convincing. However, we wish to keep an open mind on the possibility for some role also of certain plasma factors. We have plans for studying the PTC and 'Factor X' levels, which we had hoped to carry out this year, but the writing of this thesis has necessitated a delay, which we hope to overcome in the near future.

CONCLUSION: Thrombocytopenia is a very significant correlate of the hemorrhagic problem of radiation sickness. However, the prothrombin consumption test appears to be a more sensitive index of the asscciated blood defect(s), and "would seem to reflect, with some accuracy, the severity and probable outcome of the radiation injury."[148] The possibility of discovering radiation damage to some plasma factor or factors, which work with the platelets in prothrombin utilization, is still an open question. Cats rendered thrombocytopenic by X-irradiation have been used to estimate platelet life-span, in crossed circulation experiments.[283].

PART III. INTEGRATION OF IDEAS

A. FORMULATION. An integration of ideas, based on the data of this thesis, permits detailed formulation of the following "working hypothesis":

1. Thromboplastic lipid. There is abundant evidence for a significant and essential role, in the physiological clotting process, of a factor or factors of a lipoidal nature, probably cephalin. Its chief mode of action may be termed thromboplastic. This signifies that it participates along with Ca^{++} ions, in the conversion of prothrombin to thrombin, in the presence of (pro)convertin, (pro)accelerin, and possibly other co-factors. It is probable that (a) the initiation of blood clotting is dependent upon the availability of the proper form of the postulated lipoidal thromboplastin, and (b) it may need some special preliminary reaction of 'thromboplastin generation' to present it in a suitable form to the thrombin-forming reaction. It is questionable that tissue or plasma thromboplastins are true lipoproteins[87]. Further (c) there are, in all likelihood, certain naturally protective inhibitory mechanisms to be overcome before thrombin formation can proceed. On these we may comment as follows:

2. Natural inhibitors. Heparin, with its co-factor, is a possible inhibitor which can be demonstrated in experimental systems. However, there is considerable question as to the artificiality of such test systems and the best modern evidence (p. 51) doubts that there is enough heparin in the normal circulating blood to prevent clotting. Nevertheless, such a mechanism could

prevail locally, where Mast-cells are abundant in and around blood-vessel walls (Jorpes[258]). Also, clinical[37,448] and experimental[498,255] conditions of true heparinemia have been shown to exist. Another possibility, which has been particularly advanced by Tocantins et al.[475] is that certain lipoidal anti-thromboplastins exist in the blood and are increased in certain states, e.g. hemophilia[472] and post-irradiation[471]. Tocantins is a very careful worker, but there have been criticisms of his techniques (e.g. the question of variable ionic strengths)[196] and the concensus of most American coagulationists is to minimize (though not to dismiss) the antithromboplastin idea, particularly in hemophilia, where the evidence for deficiency of a specific AHF or antihemophilic globulin (p. 42) is rather convincing.

3. Cephalin. A number of workers have endeavored to characterize the thromboplastic lipid and its complexes and also the lipid anti-thromboplastin(s). The present author has contributed in this area and current investigations are continuing, with collaboration of biochemical colleagues. The best evidence to date merely confirms the original discovery of Howell (1912) and Zak (1912). The data reproduced in this thesis are particularly strong support for this. The assay method, developed in the author's laboratory, is extremely sensitive and can detect the activity of thromboplastic (cephalin-like) materials at dilutions that run into the millions. This means that such activity may be picked up in purified materials in which the real factor is merely a

trace contaminant. We believe that this may explain the doubts[84,88,76] that the thromboplastic lipid is really cephalin. The chief basis of such doubts is the finding of activity in alcohol- or acetone-soluble lipid fractions. That some cephalin may find its way into such fractions as a contaminant is a very reasonable supposition and it may very well be true that lipid solubilities are not an exact method of fractionation when dealing with the crude mixtures extracted with alcohol and ether, benzene, chloroform, and other lipoidal solvents. Hence, we shall adopt as the current working hypothesis the conclusion that the essential thromboplastic lipoids are true cephalins or ethanolamine phosphatides. There is some old evidence (e.g. Gratia and Levene[200]) that the unsaturated fatty acids are necessary. Lysocephalins are inactive[221,53,300,79]. Certain synthetic cephalins, containing only saturated fatty acids were found to be inactive (thromboplastically) by ourselves, pp.175-178 , confirming earlier work [202,263]. A more recent synthetic cephalin, prepared by Baer et al.[28,29] and tested by our colleagues Dr. K. M. Brinkhous and other members of his Pathology Dept. (Univ. of N. Carolina)[282], was found to be very slightly active. Our tests (p. 178) show this to be negligible, suggesting a trace impurity. Other biochemical problems, noted on p. 178 , such as possible differences between α and β cephalins, etc., await further investigation.

4. Lipid antithromboplastin. Relatively crude cephalins extracted from brain and other tissues (see pp. 42, 127) are very satisfactory for blood clotting experiments[489]. They do contain

some anti-thromboplastic lipid, however, which limits their use at higher concentrations (p.157) and this may be the explanation (at least, in part) of the experimental finding of a cephalin optimum.

One interesting new possibility is opened up by the author's most recent data (p. 177), namely, that anti-thromboplastin may not be so much a single factor as a number of lipid fractions acting together, perhaps synergistically.

5. 'Complete'-ness of thromboplastin. A new way to demonstrate 'thromboplastin generation'. There is no doubt that aqueous or saline tissue extracts (thrombokinase or tissue thromboplastin), in most clotting test systems, have a definite superiority over any currently available phospholipid preparation. The author's Experiment 29, p. 179 , comparing Dr. Quick's rabbit brain thromboplastin with the analyzed equivalent of its extracted P-lipids, is a particularly striking example of this. Dr. Seegers, in personal communications, reported that his highly purified prothrombins were very poorly activated by cephalin, as compared with tissue thromboplastin. This the present author[156] could confirm (p. 160). However, there was some doubt whether Seegers' thrombin-forming systems were adequately provided with AcG and other necessary co-factors. It may be that the excellence of cephalin in our latest eluate (prothrombin + proconvertin) systems, with added AcG, was due to 'thromboplastin generation' in conjunction with the PTC and significant, though small, amount of AHF present (p. 191 and Table XXXVII). In our best experiments

(p.136) there were no differences in thrombin yield, but only a little difference in the rates of thrombin formation. Even this difference, however, disappeared (p.138) when more AHG (antihemophilic globulin) was added to the test system. These experiments represent a new way of demonstrating the phenomenon of 'thromboplastin generation'.

6. Intermediates. We are not at all sure whether a specific complex (? lipoprotein) type of complete thromboplastin is a necessary preliminary to thrombin formation. With so many factors known to participate, it would be difficult to avoid the surmise that a number of intermediates (see p. 84) occur during the thrombin-forming reaction. Exact identification of these and the sequence of their appearance offers a ripe field for future harvesting. Our own current conclusions favor one type of intermediary for which we believe the current experiments provide trustworthy evidence. We term this the Ca-cephalin-protein intermediary complex. The cited data indeed show: (1) thrombin-formation may be interrupted, during its early phases, by addition of decalcifying agents (oxalate, citrate, sequestrene, or, better, cationic exchange resins), resulting, not merely in arrest of the prothrombin activation, but in a progressive inactivation to what appears to be an inert or non-thrombin by-product (? autoprothrombin). However, when the thrombin is fully-formed, complete decalcification results in no significant effect on its coagulant potency. Unlike the postulated intermediary, therefore, true thrombin is not a calcium compound[265]. Of course, the decalcification may prevent prothrombin activation

when performed early enough, so that no Ca-ions are available for the activating processes.

(2) a new datum, easily demonstrated (p. 151) when using cephalin as the thromboplastic additive, is that a similar progressive inactivation during the intermediate phase can be effected by using the very mild lipoidal extractive benzene. Again, there is no such effect when the thrombin formation has been allowed to go on to completion. This we regard as evidence for participation in the intermediary of a free, available, mobile, and loosely-binding phospholipid (cephalin). We recall[149] the very poor activation by calcium alone, of Howell prothrombin, which our recent analyses (p.162) show not to be lacking in AcG, proconvertin, PTC, or AHG. Such prothrombin preparations, by actual lipid analyses, contained significant amounts of cephalin. If such amounts were added to the system in the free form, i.e. as purified cephalin suspension, they would give excellent thrombin formation.

7. The cephalin availability theory. This, then, is the basis of the author's 'cephalin availability theory', which postulates that the phospholipid thromboplastic factor must be liberated or made available in some way from its otherwise inert lipoprotein combinations. Protein-bound phosphatides are ubiquitous in both tissues and plasma, even hemophilic plasma[132]. How they become available to function as an essential thromboplastic factor in blood coagulation poses some very intriguing questions.

8. Thromboplastic enzymes. Since 1939, the author and his colleagues have performed a very large number of experiments designed to

explore the possibility that certain proteolytic enzymes may have some role in this availability of cephalin. In such action, we term them thromboplastic enzymes. Experimentally, the pancreatic protease trypsin (but not chymotrypsin) provides a considerable body of evidence, as follows:

1) trypsin addition facilitates the clotting of whole blood[107], decalcified plasmas (but not if there is too much oxalate or citrate[149], even hemophilic blood[480] or hemophilic plasma fractions.[132]

2) trypsin acts by promoting the conversion of prothrombin to thrombin[112], but again provided that there is not too great excess of oxalate or citrate[149].

3) in the thrombin-forming system, trypsin acts best if there is sufficient calcium and some (but sub-optimal) amount of cephalin or tissue thromboplastin. Our[156] most critical Experiment 32, p. 187 , indicates no effect of trypsin with adequate calcium unless a weak thromboplastin is also added.

Hence, we conclude that trypsin is not thromboplastic in its own right (controverting Eagle and Harris[112]) but merely makes the phospholipid (cephalin) and sometimes even the calcium more available for the thrombin-forming reactions. A possible basis for this action, and to explain minor activity in the absence of sufficient Ca and P-lipid, is a postulated ability of trypsin to 'disaggregate' Ca and lipid from their normally inert protein combinations.

4) the antiprotease experiments (pp. 203-208) clearly show loss

of the thromboplastic action of trypsin, parallel with removal of proteolytic activity by suitable treatment with these anti-trypsin preparations. Controls (pp. 209-212) show most of these antiproteases to be devoid of their own inhibitory effects on thrombin formation.

6) the trypsin clotting of completely platelet-free plasma (p. 119, Table V) cannot involve any platelet potentiation. This, therefore, calls for additional consideration of plasma sources of thromboplastic lipid, which will be considered on p.265 .

9. The fibrinolysin question. Cited evidence from the author's experience has been given to show essentially similar experimental phenomena for the natural plasma protease fibrinolysin (plasmin or tryptase[143]). However, there was a most significant exception (p.205), namely that these thromboplastic effects in platelet potentiation experiments (which system showed them most clearly), were not abolished by antiproteases. This must throw the gravest doubts[301] upon all the evidence concerning fibrinolysin and require a future investigation of some other unknown component of all our fibrinolysin preparations, which could explain the findings independently of the protease proper. Until this is definitively settled, we withhold any commitment as to a genuine clotting role for fibrinolysin, which was prematurely postulated by the author[136] in 1943.

10) Platelets. The occasional success in obtaining a completely platelet-free plasma which will not clot on simple recalcification, even in glass (p.118, Table IV), is the best evidence that platelets

are normally essential for blood coagulation. Three observations, however, still leave room for doubt. Thus:

(1) Why does this experiment so often fail, even when the technical skill does not seem to be less than in the few experiments which succeed[90,472]? Is it just a matter of a little fragmented platelet material eluding the experimental effort, or could it be that the plasma itself sometimes contains the trace amounts of thromboplastic factor needed? Tissue thromboplastin contaminants can certainly be ruled out in the careful performance of these experiments.

(2) Why is it that not only platelets and tissue thromboplastin additives work, but also, and to a very significant extent, cephalin (Table IV) and the proteolytic enzymes trypsin and fibrinolysin (Table V)?

A tentative answer could be that the common factor in all cases, is the presence of available thromboplastic P-lipid. Tissue thromboplastin must be adventitious, but undoubtedly occurs in many injuries to blood vessels. It appears to be quite the most effective. The next best, in our experiments, is not platelets (much concentrated as these were), but purified cephalin. Could not (experimental) trypsin 'disaggregate' some mobile cephalin from the plasma lipoproteins, as suggested on pp. 208,262? Let us defer any conclusions about fibrinolysin because of the doubts (see above) as to its purity in the preparations employed to date. Pancreatic trypsin does not occur in the blood, and we[293] believe the claims cited for such, e.g. in pancreatitis, are due to trypsin acting as a kinase activator of profibrinolysin.

(3) finally, just what is the effect of a wettable surface? It is true (p. 65) that wettable surfaces, and injured endothelium, p. 62 , have significance in the platelet alterations which ordinarily precede blood clotting. However, the effects noted in the platelet-free system of Expt. 4 (p.118) must be on some plasma component(s). Might there not be a considerable labilization of plasma lipoproteins, with release of some cephalin (thromboplastic P-lipid) on surface contact? Could this not, at times, be the cause of the experimental failure noted in (2), above, and such be an alternative explanation to that based upon postulated platelet fragments?

11. <u>The trigger mechanism</u>. Correlating these speculations with those of Erkelens[118], (p. 89), may it not indeed be possible naturally to initiate the clotting process with thromboplastic lipid (probably cephalin) from the plasma or plasma lipoproteins? This is a very intriguing question and upon its final answer will depend the elucidation of: (a) the still mysterious trigger mechanism, which physiologically initiates blood clotting, when needed for defensive hemostasis, and which is obviously not in operation (at least overtly) in the circulatory blood; and also (b) the true importance of the blood platelets.

12. <u>Clinical disorders of platelets</u>. The platelets show evidence of their important hemostatic functions in clinical bleeding disorders. Only part of these functions concerns the clotting mechanism. Other hemostatic roles of the blood platelets are related to (1) their ability to aggregate and adhere to injured

vascular endothelium to form cellular thrombi, and (2) their participation in the provision of a vasoconstrictor factor, which modern evidence (p. 67) suggests is serotonin or 5-hydroxy-tryptamine creatinine sulfate. Just how important these two mechanisms may be is best illustrated by congenital afibrinogenemia and some other serious clotting deficiencies. The rare congenital afibrinogenemic[296] lacks the essential ingredient (fibrinogen) for clot formation. That such cases survive, despite an overt hemorrhagic tendency, must point to the great physiological effectiveness of platelet (cellular) and vascular hemostatic mechanisms. Worthy of passing mention, also, is the evidence of Comparative Zoology that the lower, and phylogenetically older, animal species developed a thrombocyte function long before a plasma clotting-system evolved[189].

13. Myelin forms. In the platelet alterations (p. 65) after blood is shed and which, presumably, also occur intravascularly after vessel injury and in thrombotic conditions, the author's dark-field observations (pp. 219-227) provide very suggestive evidence for a significant role of the platelet lipids. In particular, it is concluded that the peculiar platelet excrescences are simply a variety of the long-known myelin figure formations. Of purified phospholipids examined with a similar microscopic technique, cephalin presented appearances which often simulated the platelet alterations in very minute detail. These alterative phenomena are not peculiar to platelets, but could be shown (pp. 227-230) also in examinations of megakaryocytes, thrombocytes (of lower animals) and occasional erythrocytes undergoing stromatolysis.

Furchgott[182], in 1940, also suggested the myelin figure interpretation, for the last, but apparently was unfamiliar with the author's 1934 platelet observations.

14. Preservation of platelet thromboplastic component. Of the several factors which platelets may contribute to the blood-clotting mechanism, it would seem reasonable to conclude that the chief is its thromboplastic component. This is, at least in part, extremely stable, as shown by Expt. 35 (p. 194). J. L. Tullis[479], who is directing the platelet work of the Harvard Plasma Fractionation Commission, notes (op. cit., p.148) . . . "During storage, platelets undergo a gradual aging process. A loss of fibrils (cf.[124] excrescences) is one of the first morphological changes to be seen. Moreover, there is a concurrent loss of clot retracting ability without loss of thromboplastic activity". . . . (Tullis' italics). Using, as test system, the ability of preserved platelets to restore coagulability to platelet-poor plasma, the Harvard scientists claim remarkable preservation of platelets on storage. The present author believes this needs to be re-considered in view of evidence that many other platelet functions may be physiologically needed and most of them are highly labile. Platelets, fractionated from human donors for clinical use, should be used almost immediately and, even then, there is evidence that their therapeutic benefits are short-lived.

15. Potentiation of platelet thromboplastin. The second major point about the thromboplastic action of platelets is its potentiation by antihemophilic factor, PTC, etc. (see p. 195). Current thinking concerns a postulated 'thromboplastin generation'

from some reaction (probably a series of reactions) between the platelets and the several plasma components. The evidence of workers with the thromboplastin generation test points to need not only for calcium but also for factor V (proaccelerin), factor VII (proconvertin), if not for (pro)thrombin itself, in thromboplastin generation. The types of experiment pursued in the present thesis have not explored the possibilities of thromboplastin generation as a separate phase, i.e. prior to the activation of prothrombin to thrombin. However, Expt. 40, (p. 205) does show the important potentiation of platelet thromboplastic action by purified AHG and Expt. 11 shows that cephalin can substitute for platelets in this phenomenon, whereas the complete thromboplastin (tissue tpln.) in sufficient amounts is not improved by the plasma component addition.

Again we conclude that the phospholipid cephalin most probably is the stable factor in platelets. This is a weak and incomplete thromboplastin in its own right, apparently, but yields a complete thromboplastin in the presence of sufficient AHG, PTC, etc. Our eluates (Table VI) contain these plasma components, especially PTC, but could probably be freed, if necessary, from the contaminant trace of AHF. This present AHF contaminant explains why our eluates and the older Howell prothrombin were so well activated by cephalin, as compared, say, with Seegers' more purified prothrombins.

Another potentiation of platelet thromboplastic action by a trace of thrombin (Expts. 36,37) is not easy to explain in our

systems, which are supplemented with AcG (accelerin) of the serum type i.e. not the precursor proaccelerin. Thrombin is known[492,290] to play a role in the activation of proaccelerin to accelerin. This removes a lag phase in our two-stage thrombin-formation tests. Contrary to the claim of Seegers et al., that platelet accelerator is like serum - AcG, Dr. Lewis and the author have noted many times, that it shows the lag phase, in two-stage clotting systems, which we regard as evidence for proaccelerin (Seegers' plasma-type AcG). Were it not for our added accelerin, therefore, thrombin could be effecting a change from the precursor form (proaccelerin) to the complete accelerator (accelerin) in the platelet component. It is still just possible that this is indeed the case and could account for the thrombin potentiation. Consistent with this possibility is the fact, which we[154] have several times demonstrated with our eluates, namely, increasing the AcG to an excess several times the equivalent of its strength in normal adult human plasma, enhances thrombin-formation, with no clear cut evidence of any AcG optimum. Pending more data on the exact quantities of AcG (and pro-AcG) in our systems, therefore, we conclude that the thrombin effect might be of the nature described. It is, however, conceivable that the mild proteolytic actions of thrombin could 'disaggregate' phospholipid from the platelet lipoproteins, as we postulate in the case of the much more powerful proteolytic enzyme trypsin. Lacking concrete evidence, at this time, such a concept is merely a tentative suggestion which could guide future enquiry.

B. FINAL SUMMARIZATION. The material in this thesis supports the idea that a major role in the natural clotting mechanism must be assigned to a group of factors which collectively form the 'thromboplastic' activator(s) which, in conjunction with ionized calcium, convert(s) the prothrombin (precursor) into the active thrombin, needed for catalyzing the conversion of fibrinogen to fibrin clot. We have advanced evidence for

1) the importance of certain lipoids (? cephalin);
2) the normal need, in plasma clotting, for platelets;
3) the particular significance of a platelet component, which has many analogies to cephalin, in the thromboplastic system;
4) potentiation of the thromboplastic actions of cephalin, of platelets, and of tissue thromboplastin (to some extent) by various experimental additives. Part of this may be explained as a 'thromboplastin generation' through co-participation of certain plasmatic components (anti-hemophilic globulin, PTC, ? etc.). Part, however, may be the result of certain proteolytic enzymes, particularly trypsin, 'disaggregating' bound forms of phospholipid from the normally unavailable lipoprotein combinations and thus rendering it 'available' for participation in the generation of thromboplastin and hence the conversion of prothrombin to thrombin;
5) possible Ca-containing and lipid-containing 'intermediates' in the thrombin-forming reaction(s);
6) myelin figure formation as an explanation of 'alterations' of platelets and certain other formed elements such as

thrombocytes, megakaryocytes, stromatolytic erythrocytes;

7) the multiplicity of factors which platelets may contribute to the blood clotting and hemostatic mechanism;

8) the occurrence of many clinical bleeding disorders due to deficiency of platelet functions. Thrombocytopenias denote deficiency of platelet numbers and hence of the total bulk of the platelet factors available in the body. Thrombocytopathias are deficiencies of specific platelet components and can be clinically significant even when the platelet count is normal. Bleeding in leukemias, uremias, etc., may often be accounted for in these terms;

9) the nature and modes of action of heparin, and other 'antithromboplastic' inhibitors, and of some anti-proteases, in relation to the mechanisms discussed;

10) the 'cephalin availability theory' of the author, as a useful hypothesis to explain the importance of the natural thromboplastic phospholipid. Lipid release from platelet, tissue, or possibly plasma (Expt. 4) sources may very well be the long-obscure 'trigger mechanism' which initiates blood coagulation.

APPENDIX I. FIGURES AND ILLUSTRATIONS

APPENDIX I. FIGURES AND ILLUSTRATIONS.

Figure 20. Dark-field microscopy of erythrocyte alterations and of myelin figure formation from lecithin.

9. Primitive erythroblasts from heart blood of rabbit embryo, showing stromatolytic filaments 229

10. Erythroblast of tadpole, showing numerous filamentous stromatolytic forms 230

11. Human erythrocytes, showing contact development of envelop (membrane) filaments 229

12A. Edge of lecithin film (lipid-air interface), showing smooth refractive edge 231

12B. Same edge showing 'myelin forms' produced by contact with distilled water 231

FIGURE 1.

A skeleton scheme of the clotting mechanism.

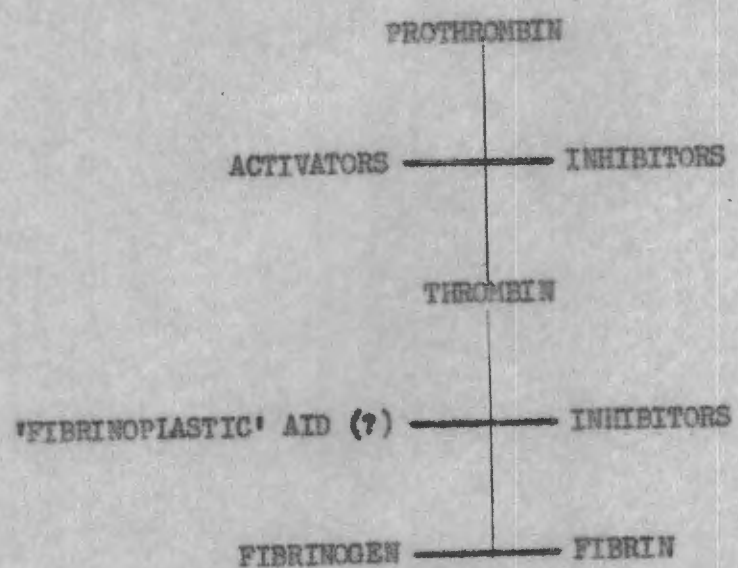
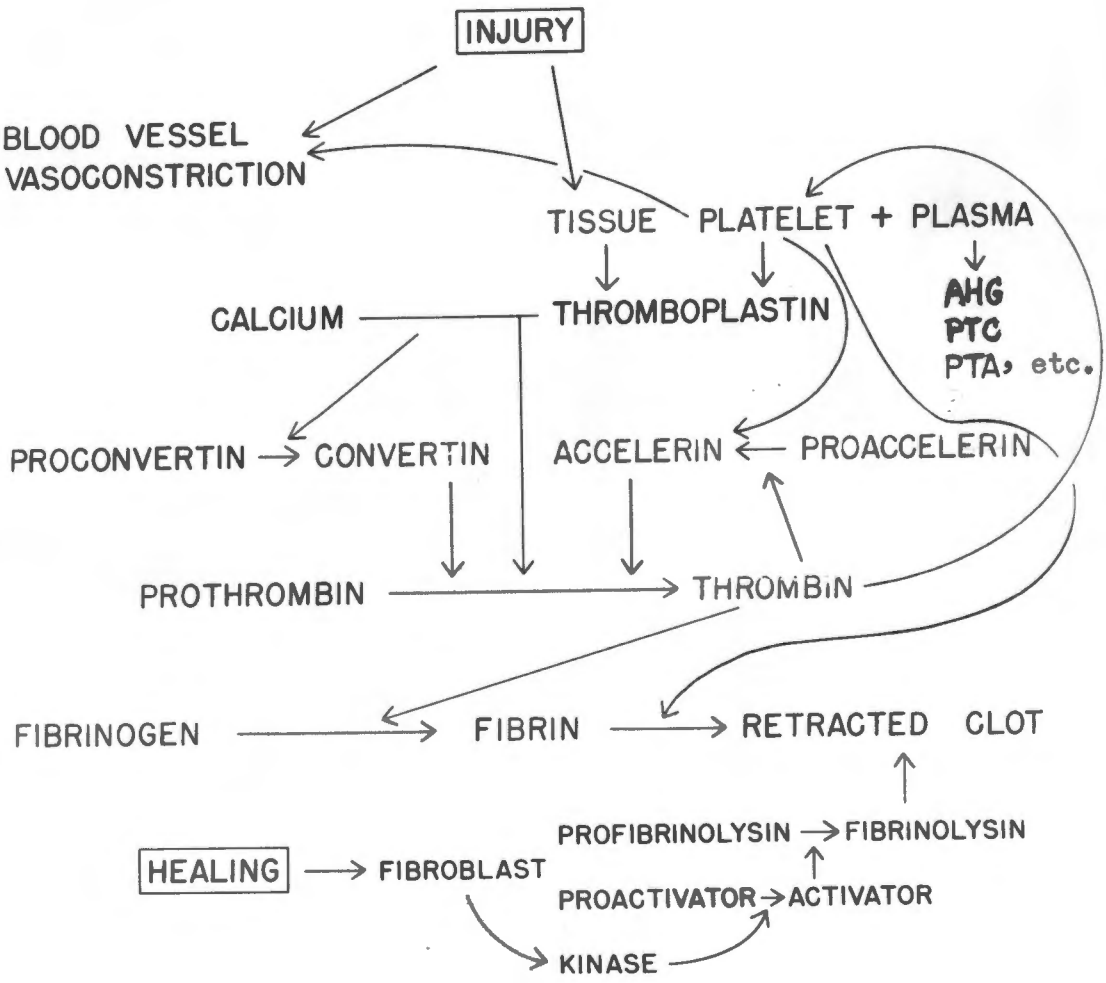


FIGURE 2


INJURY
BLOOD VESSEL
VASOCONSTRICTION
TISSUE
PLATELET + PLASMA
AHG
PTC
PTA, etc.
CALCIUM
THROMBOPLASTIN
PROCONVERTIN
CONVERTIN
ACCELERIN
PROACCELERIN
PROTHROMBIN
THROMBIN
FIBRINOGEN
FIBRIN
RETRACTED CLOT
PROFIBRINOLYSIN
FIBRINOLYSIN
HEALING
FIBROBLAST
PROACTIVATOR
ACTIVATOR
KINASE

EFFECTS OF CHANGE IN THROMBIN CONCENTRATION ON THE SECOND PHASE OF CLOTTING

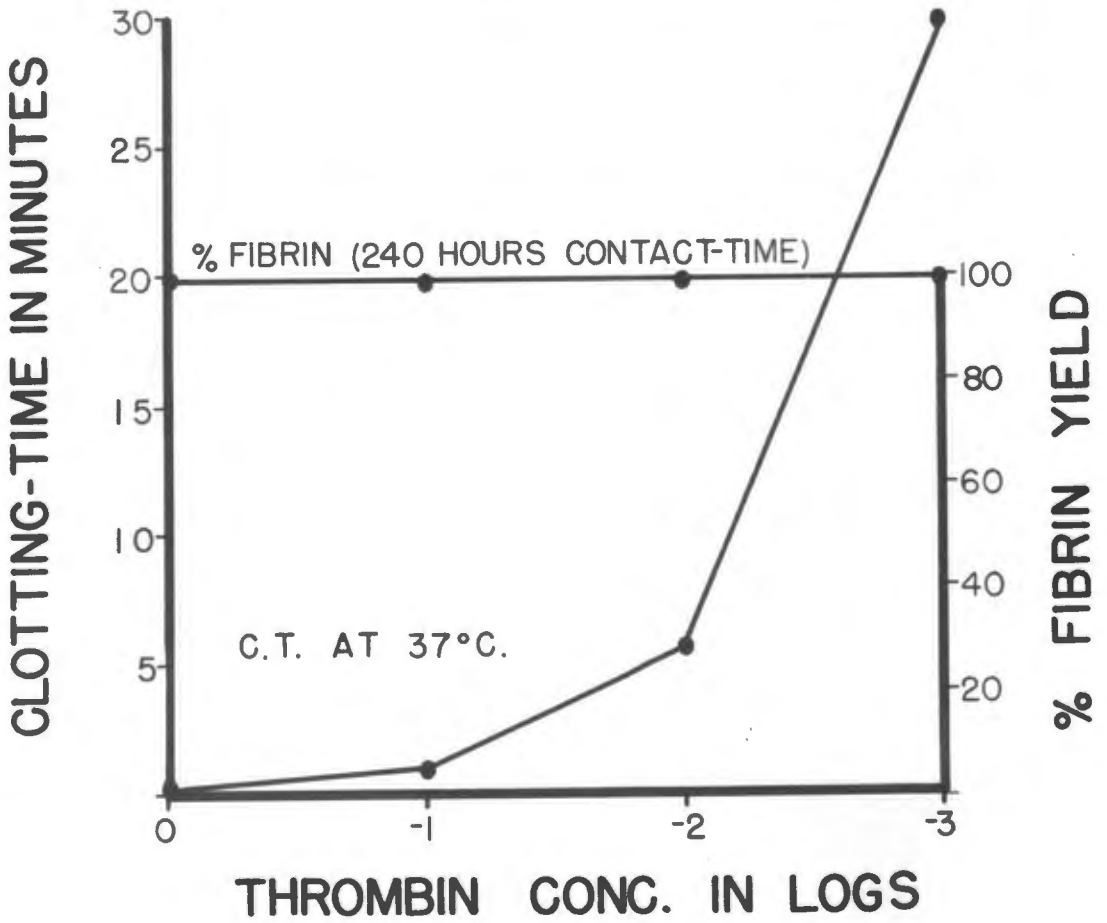


FIGURE 3

FIGURE 4

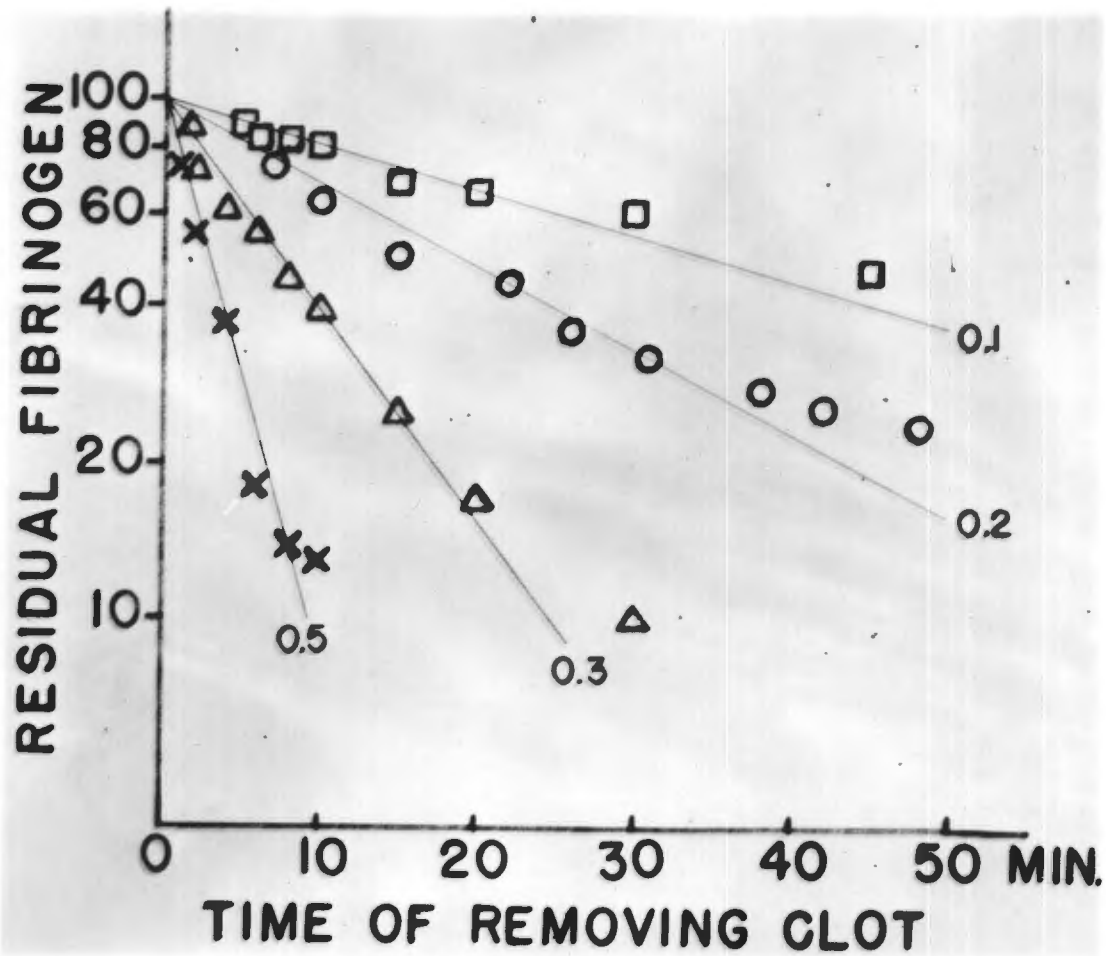

RESIDUAL FIBRINOGEN
100
80
60
40
20
10
0.1
0.2
0.3
0.5
0
10
20
30
40
50 MIN.
TIME OF REMOVING CLOT

FIGURE 5

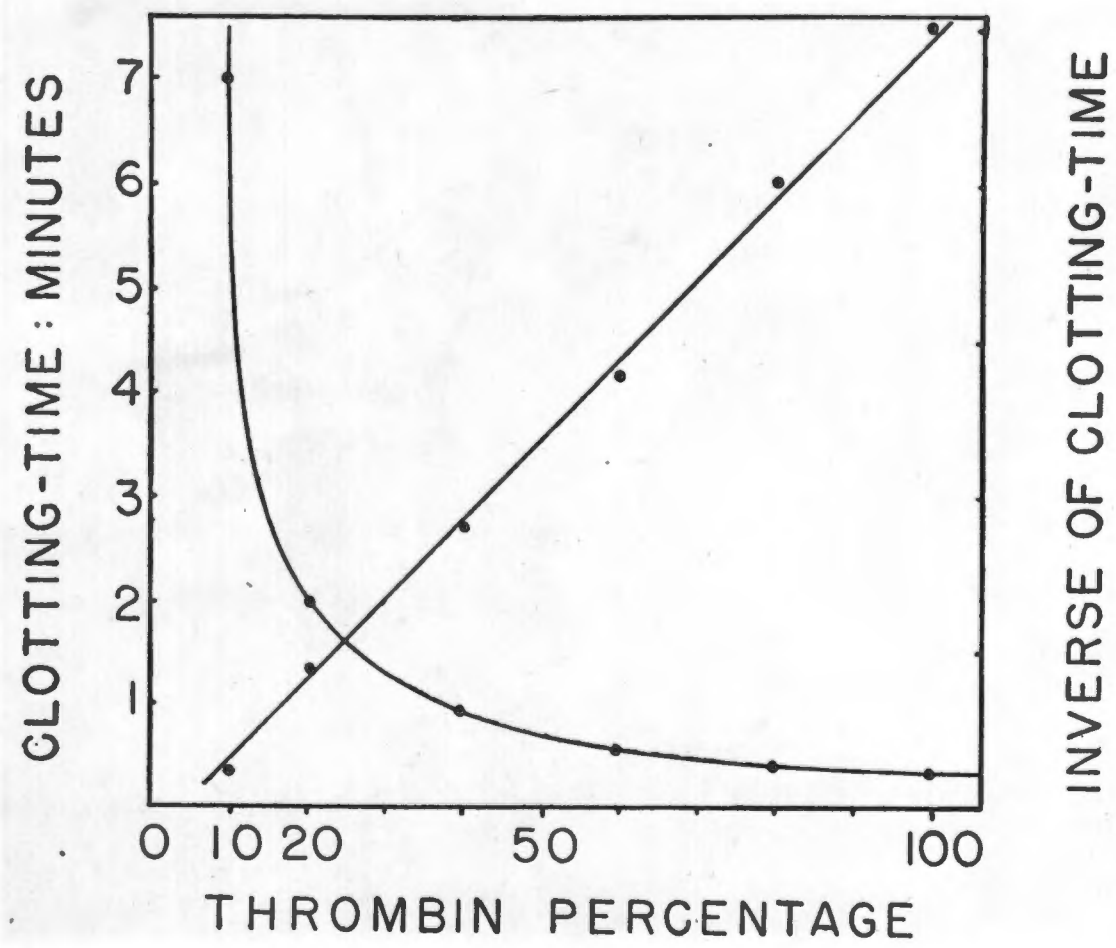

CLOTTING-TIME : MINUTES
7
6
5
4
3
2
1
0
10
20
50
100
THROMBIN PERCENTAGE
INVERSE OF CLOTTING-TIME

FIGURE 6

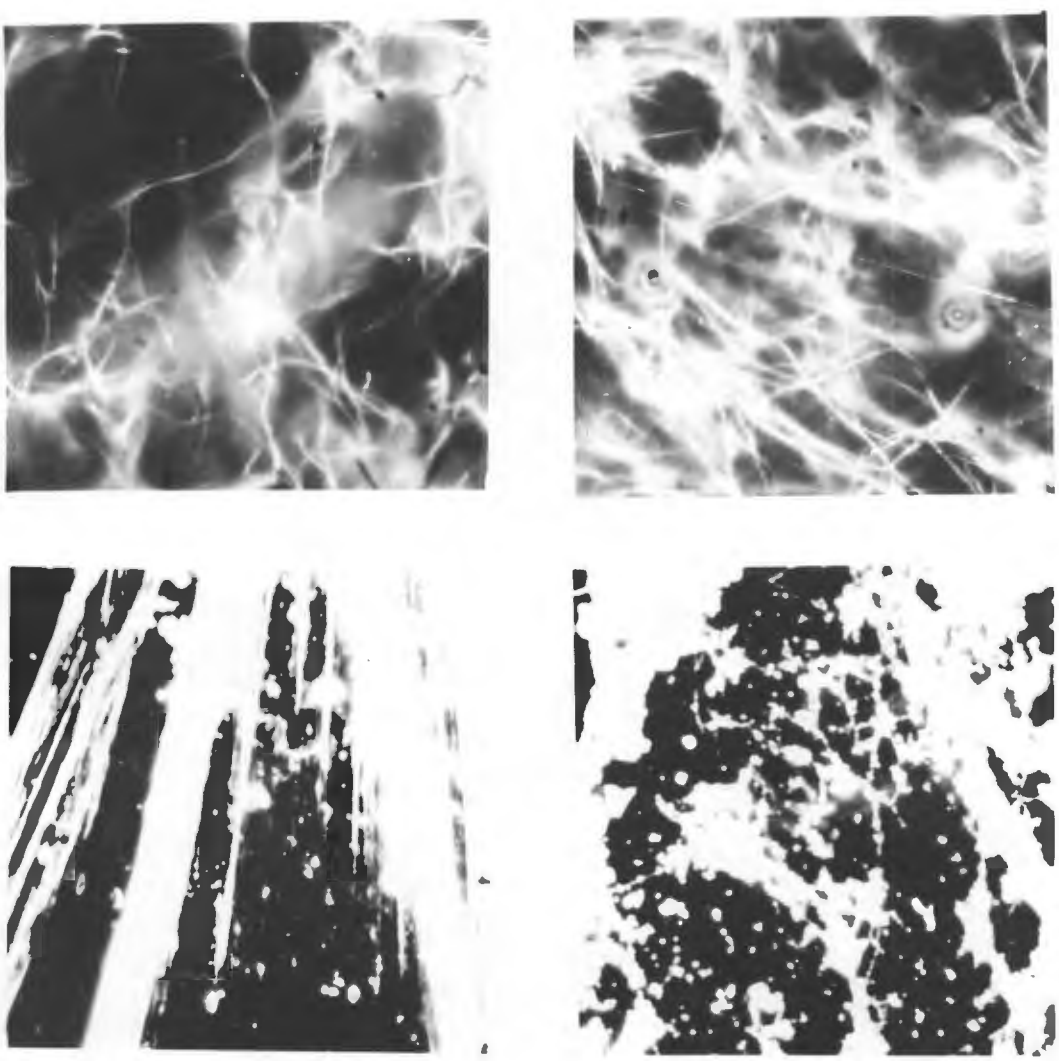


FIGURE 7

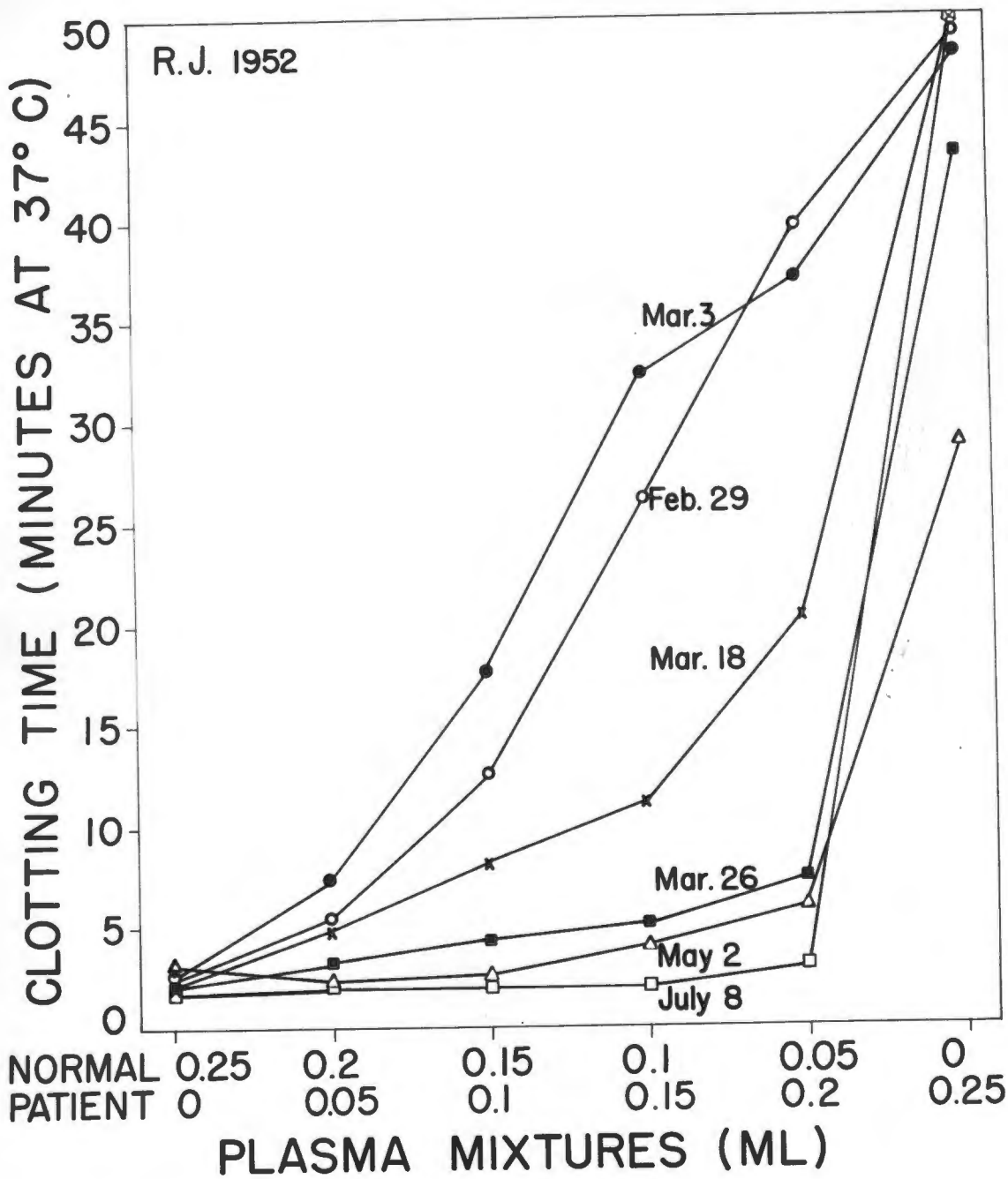

R.J. 1952
CLOTTING TIME (MINUTES AT 37° C)
50
45
40
35
30
25
20
15
10
5
0
Mar. 3
Feb. 29
Mar. 18
Mar. 26
May 2
July 8
NORMAL 0.25 0.2 0.15 0.1 0.05 0
PATIENT 0 0.05 0.1 0.15 0.2 0.25
PLASMA MIXTURES (ML)

FIGURE 8

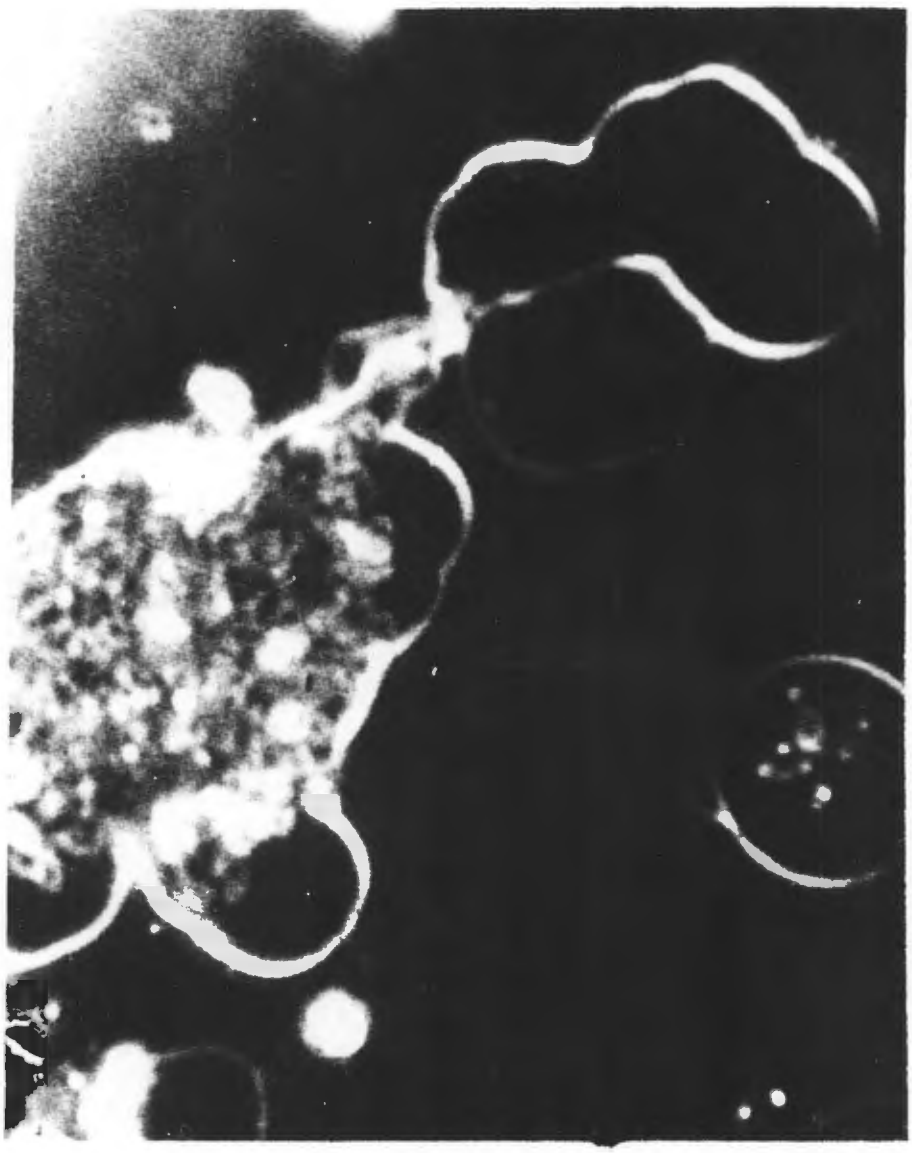


FIGURE 9

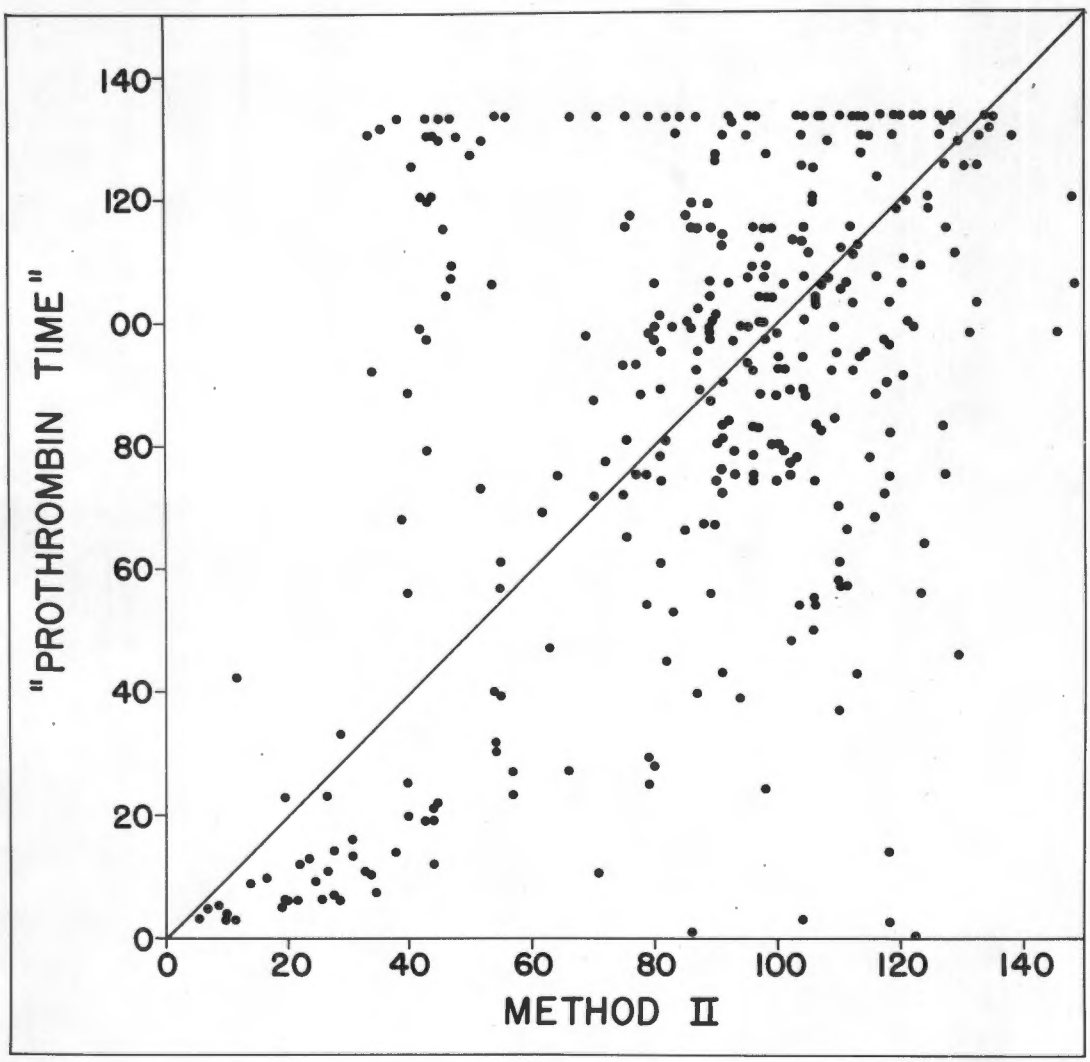

"PROTHROMBIN TIME"
140
120
00
80
60
40
20
0
0
20
40
60
80
100
120
140
METHOD II

FIGURE 10

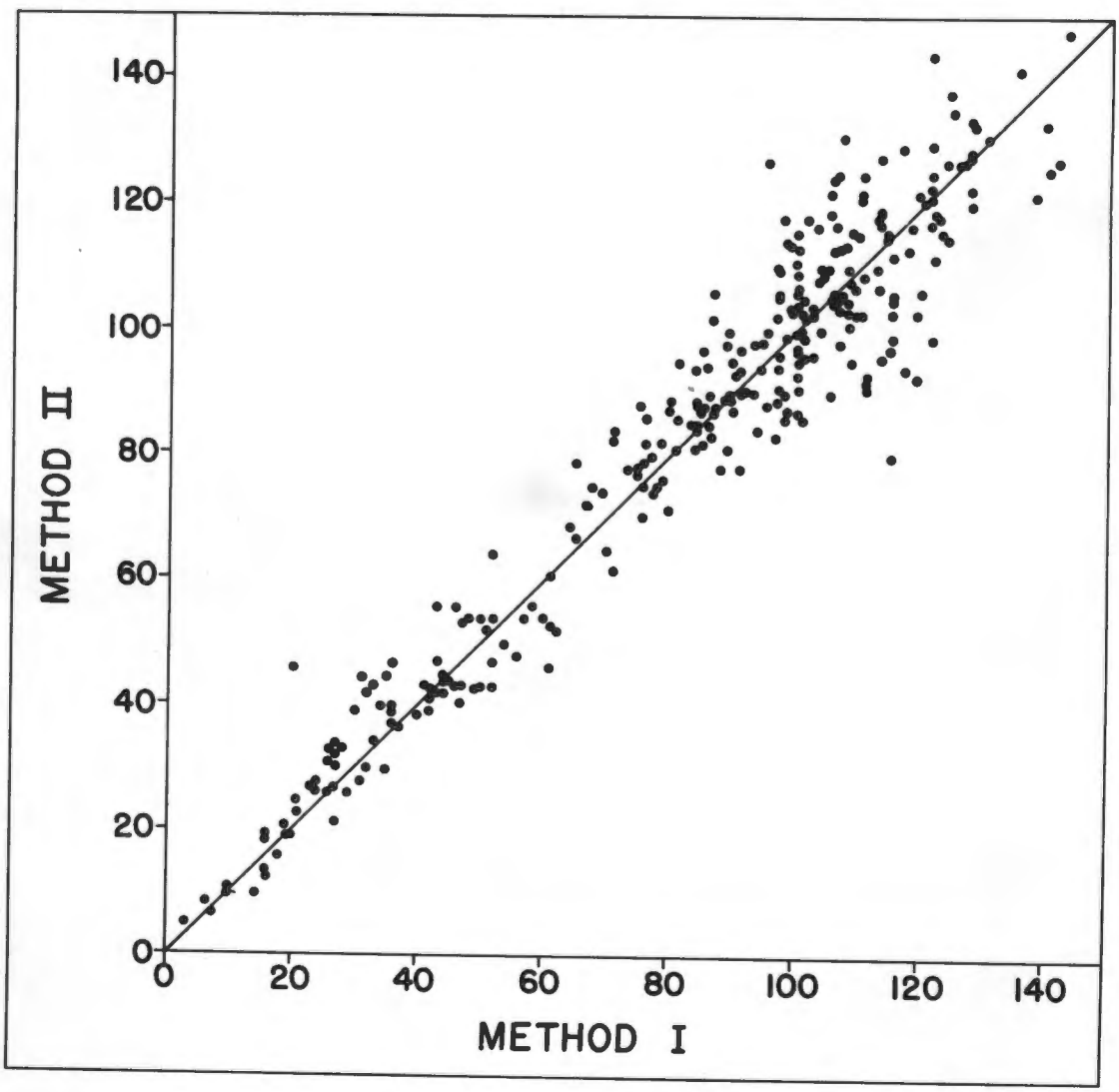

METHOD II
140
120
100
80
60
40
20
0
0
20
40
60
80
100
120
140
METHOD I

FIGURE 11

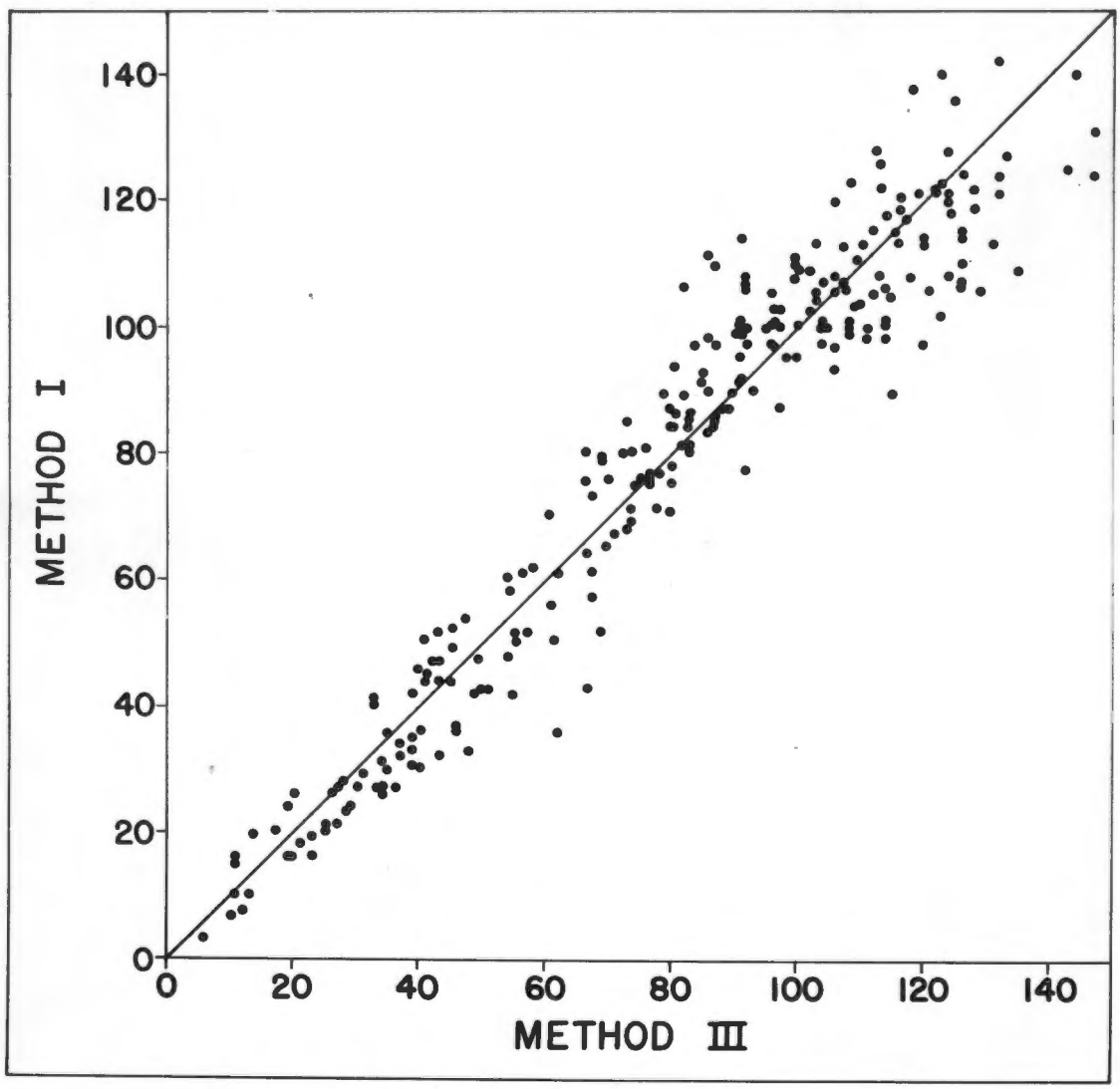


FIGURE 12

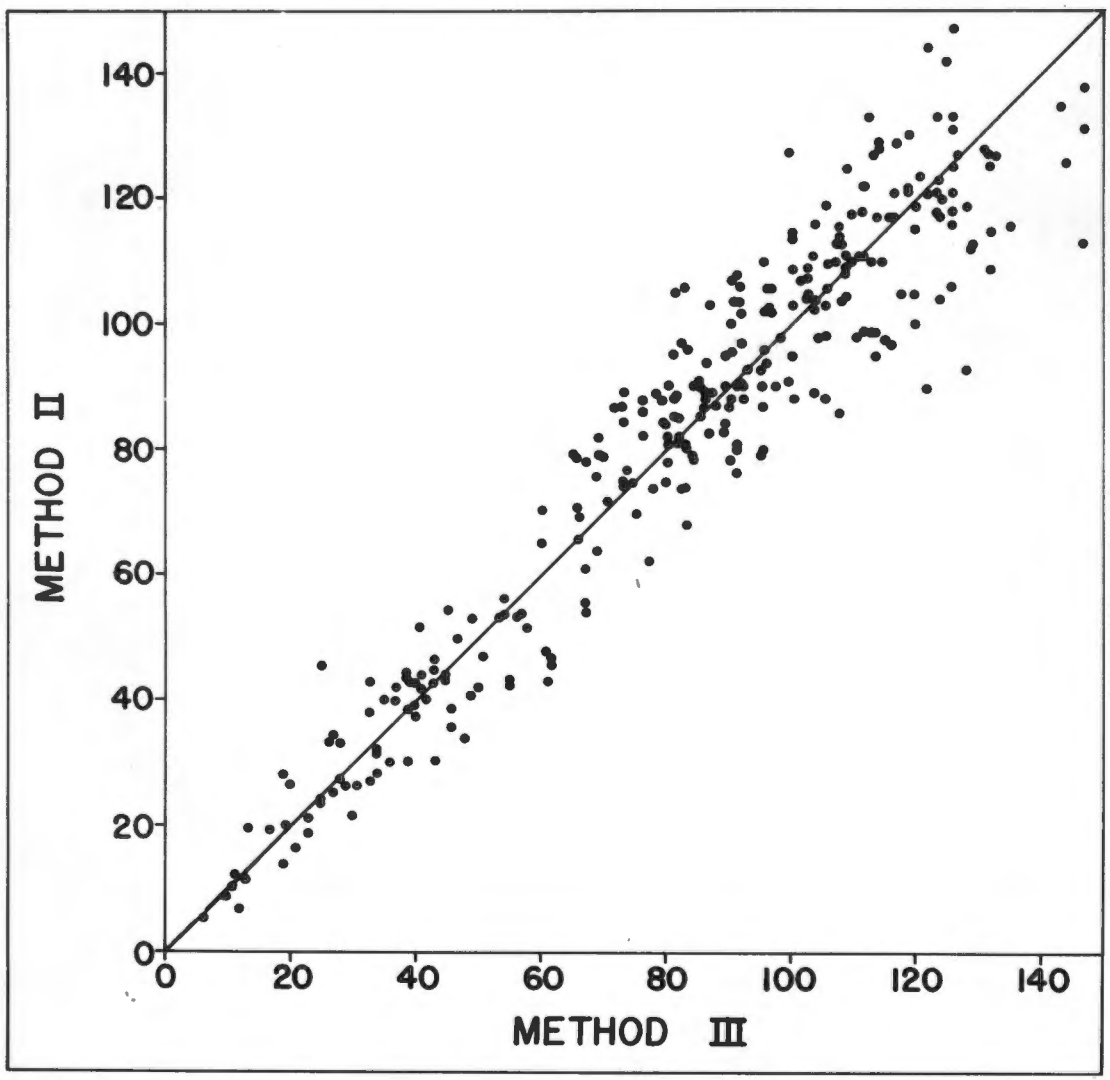


FIGURE 13

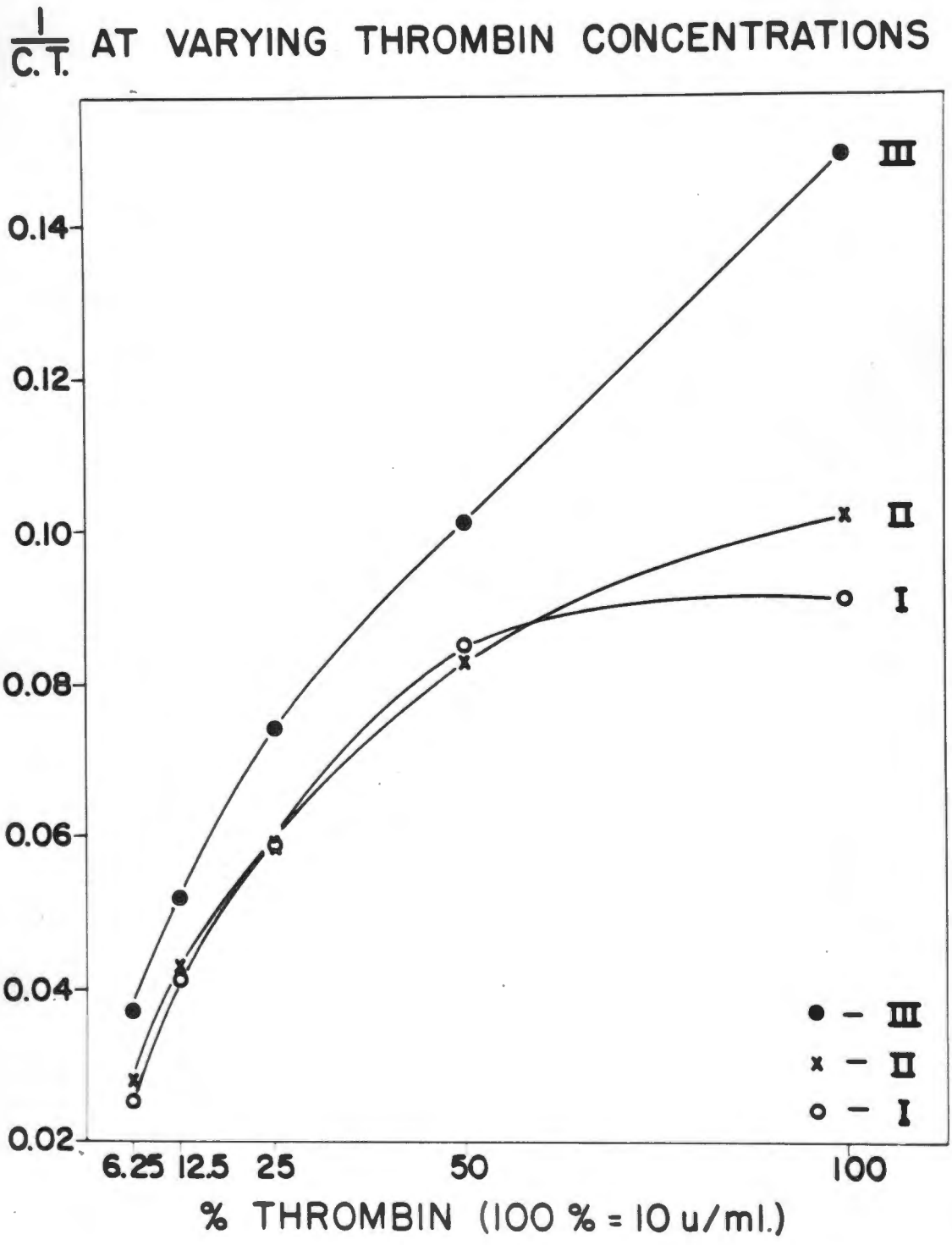
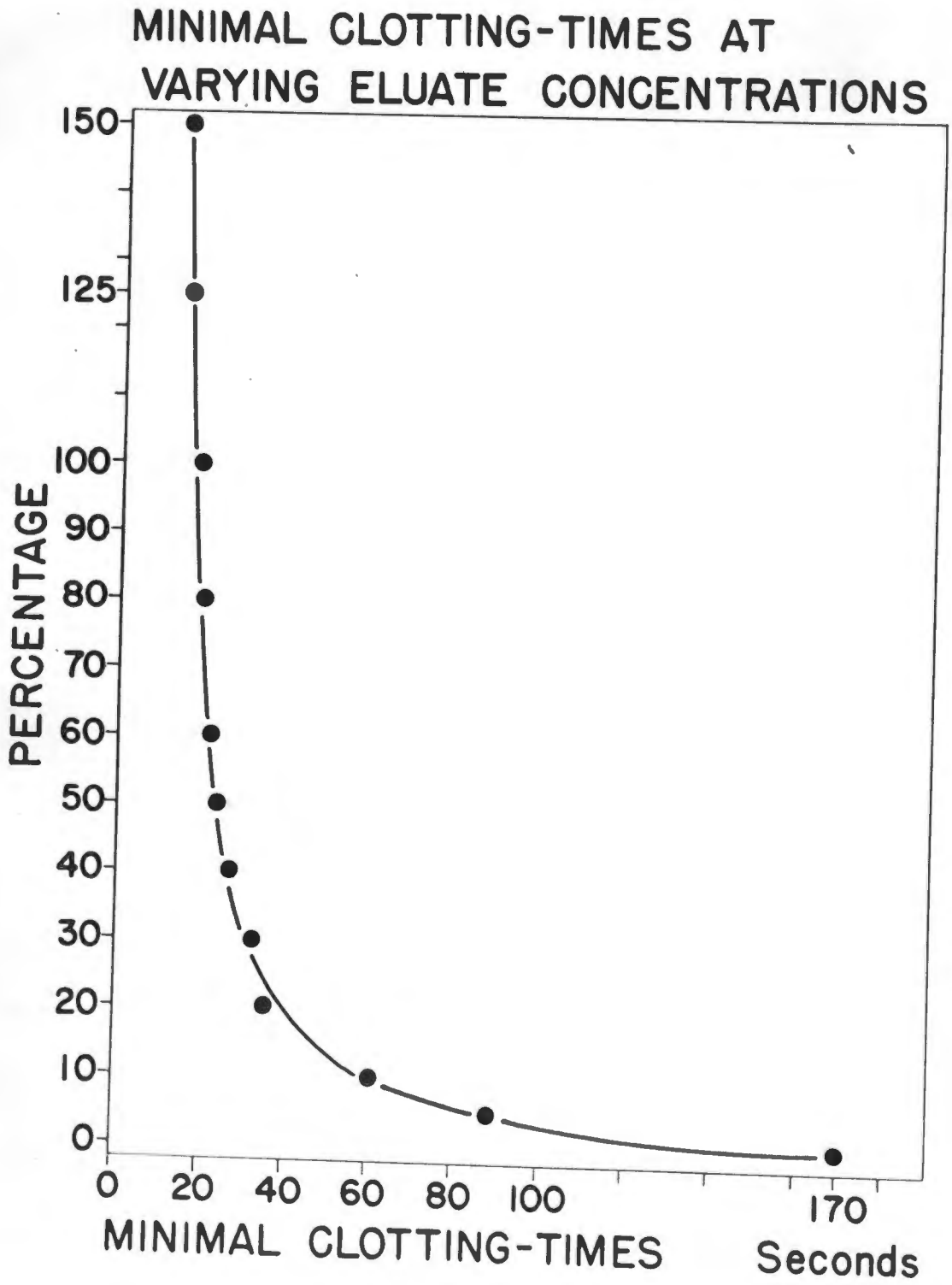

1/C.T. AT VARYING THROMBIN CONCENTRATIONS
0.14
0.12
0.10
0.08
0.06
0.04
0.02
III
II
I
● – III
x – II
o – I
6.25
12.5
25
50
100
% THROMBIN (100 % = 10 u/ml.)

FIGURE 14


MINIMAL CLOTTING-TIMES AT
VARYING ELUATE CONCENTRATIONS
PERCENTAGE
150
125
100
90
80
70
60
50
40
30
20
10
0
0
20
40
60
80
100
170
MINIMAL CLOTTING-TIMES
Seconds

09

FIGURE 15

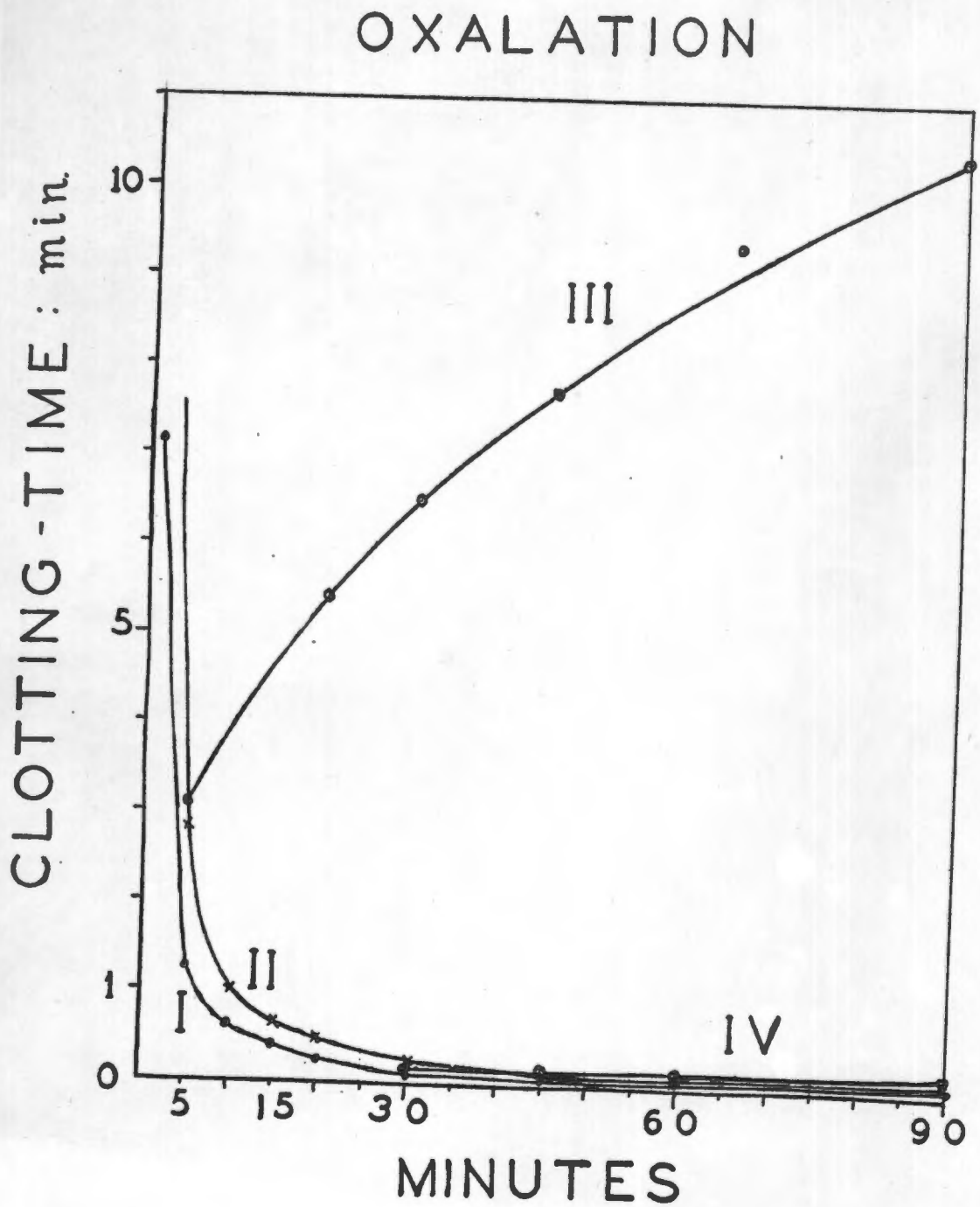

OXALATION
CLOTTING-TIME : min.
10
5
1
0
I
II
III
IV
5
15
30
60
90
MINUTES

FIGURE 16

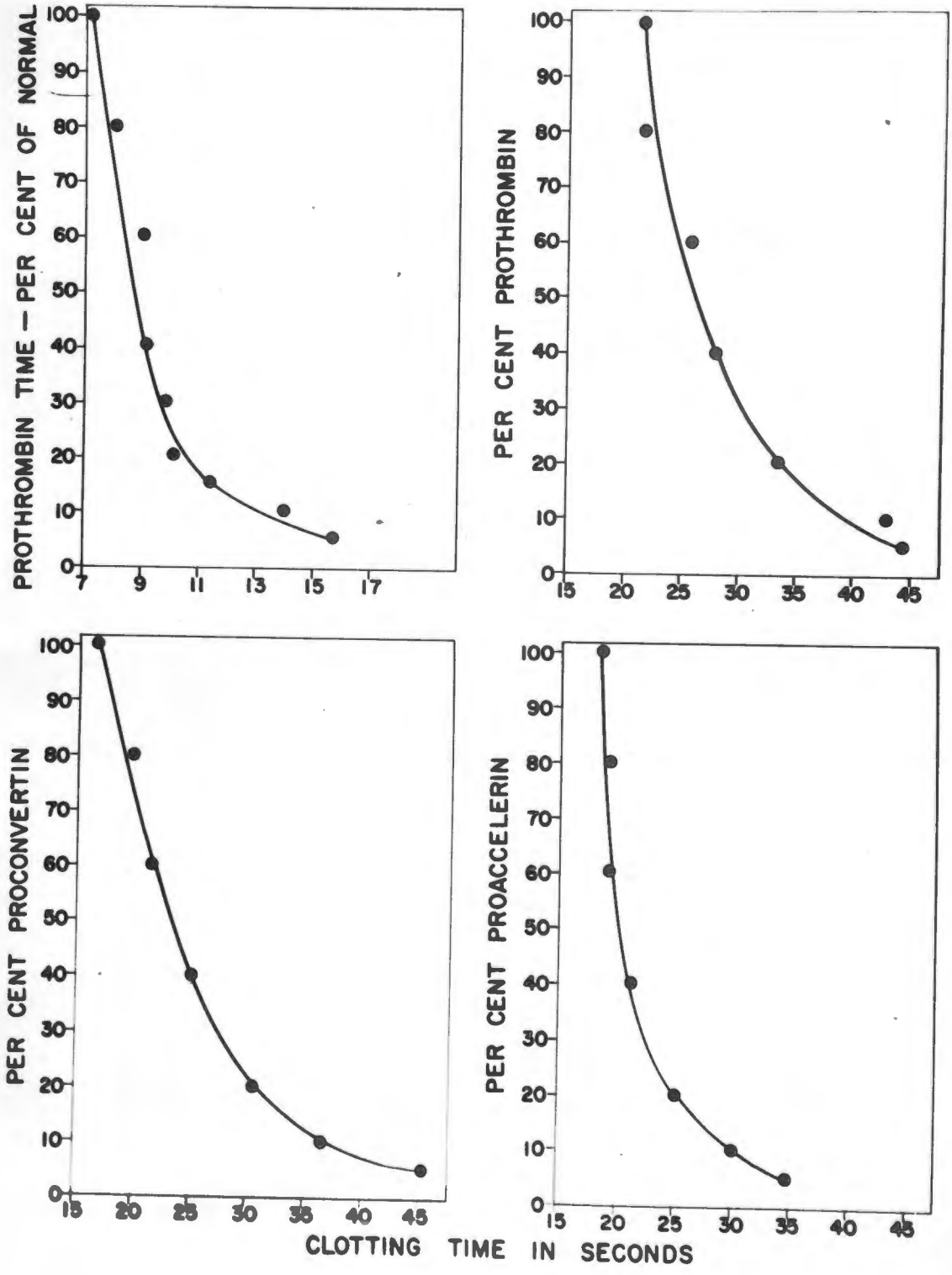
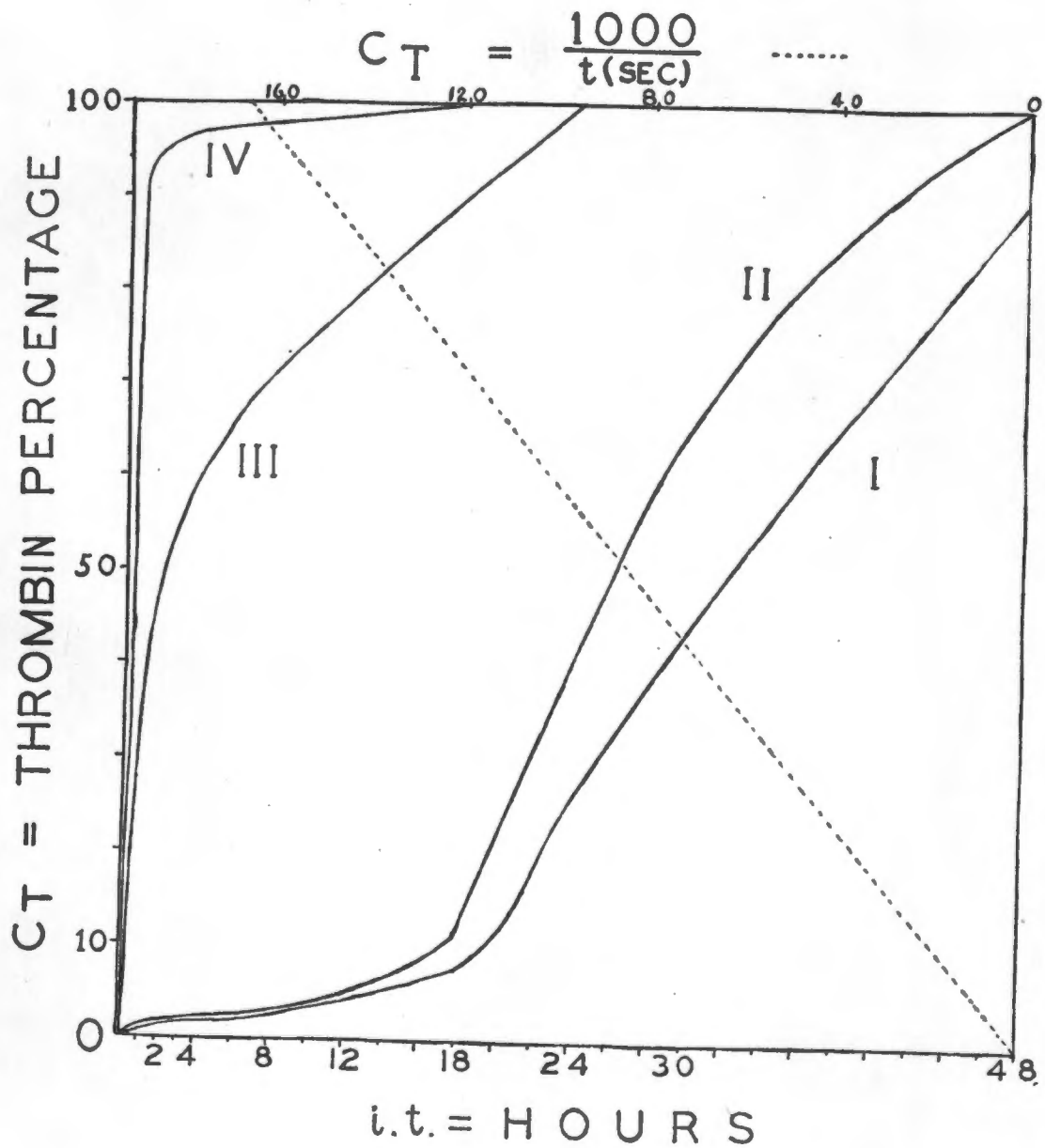

PROTHROMBIN TIME — PER CENT OF NORMAL
PER CENT PROTHROMBIN
PER CENT PROCONVERTIN
PER CENT PROACCELERIN
CLOTTING TIME IN SECONDS

FIGURE 17


$C_T = \frac{1000}{t(SEC)}$
16.0
12.0
8.0
4.0
0
100
50
10
0
IV
III
II
I
C_T = THROMBIN PERCENTAGE
2 4 8 12 18 24 30 48
i.t. = H O U R S

2

FIGURE 18

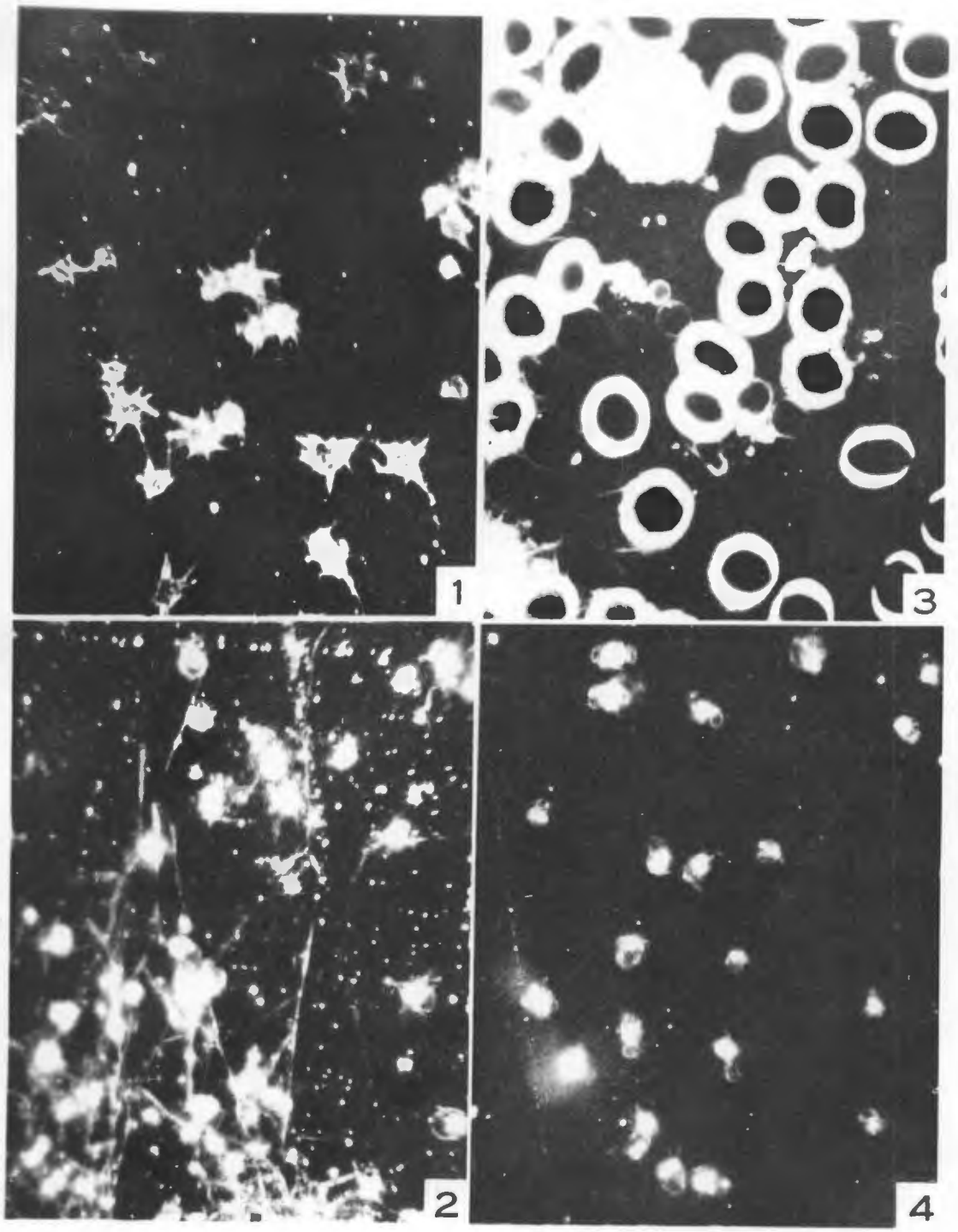


FIGURE 19

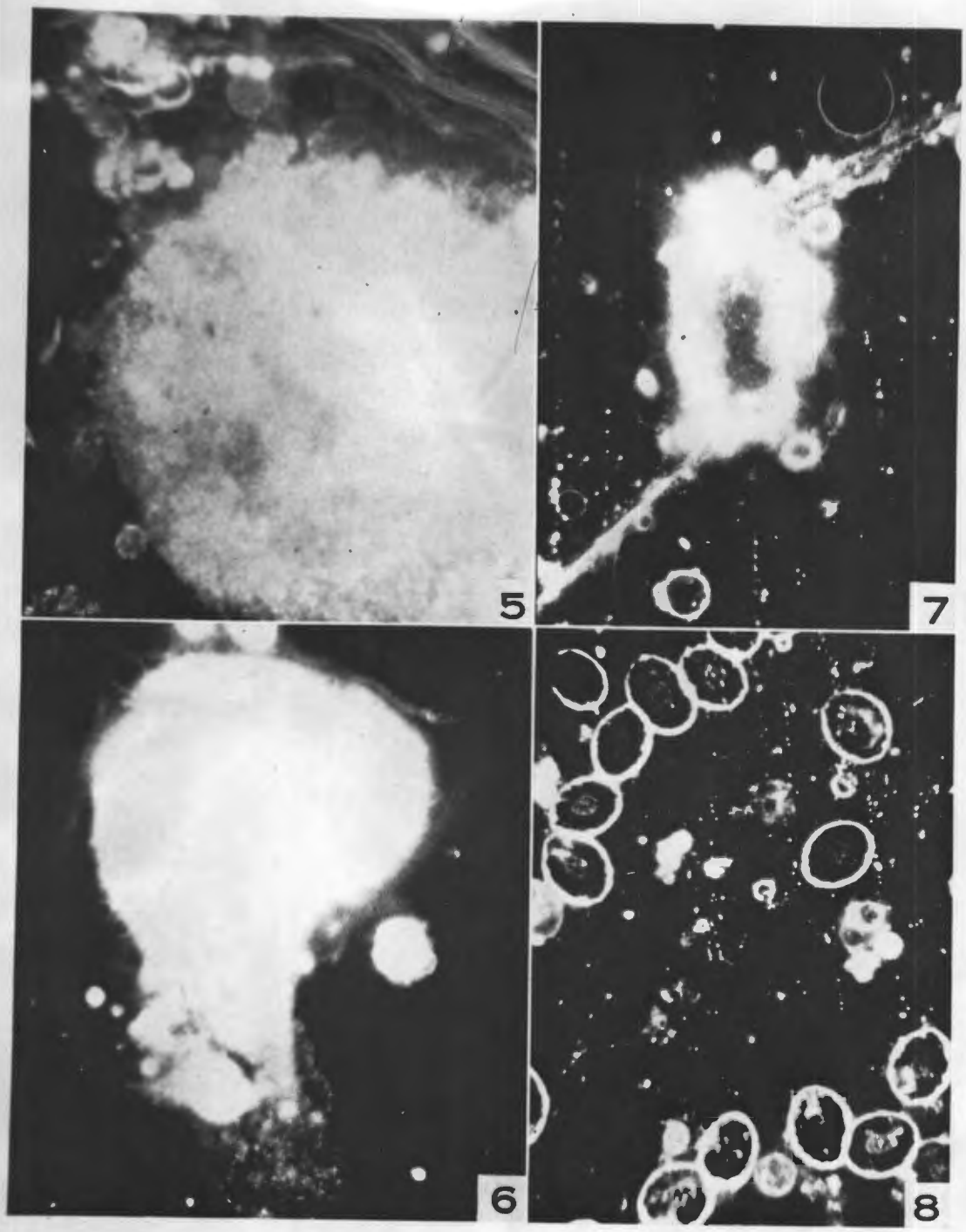
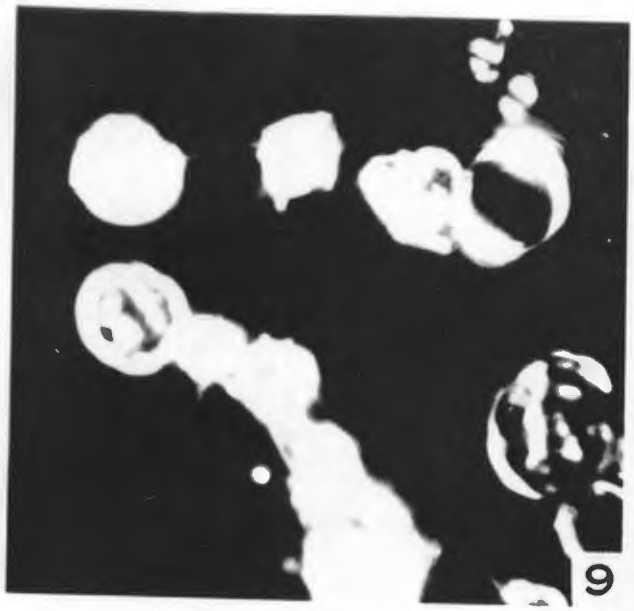
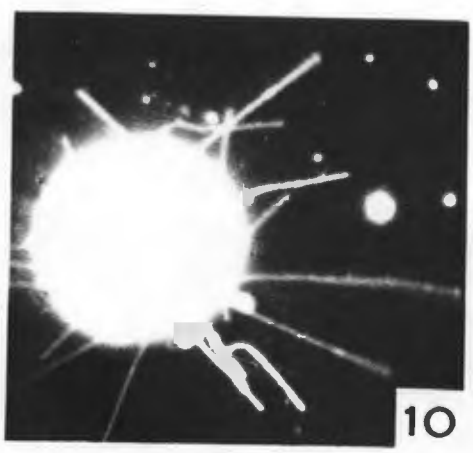
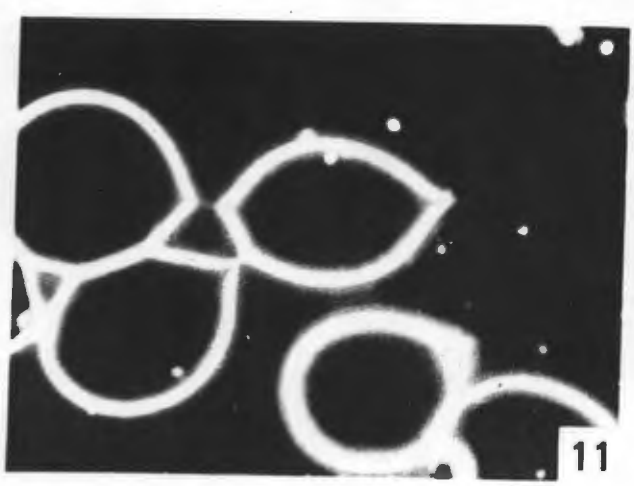


FIGURE 20







APPENDIX II. TABLES OF EXPERIMENTAL DATA

TABLE 1

CLOTTING OF SALT-TREATED MIXTURES ON DILUTION, AFTER STATED INCUBATION PERIODS. Seconds, 24°C.

	MIXTURE + 4.5% NaCl	ADDED with water	INCUBATION PERIOD (minutes): 1/2	5	10	15	20
1.*	Thr. + Fibr.	-	150"	85"	15"	5"	
2.	Thrombin	Fibrinogen	190"	200"	205"	215"	212"
3.	Fibrinogen	Thrombin	240"	245"	250"	300"	295"

*Mixture (1) clotted in 18 3/4 min.

TABLE II

CLOTTING-TIMES, WITH VARYING AMOUNTS OF (T) THROMBIN, OF (F) FIBRINOGENS (I, II, III - see text) CONTAINING DIFFERENT AMOUNTS OF SERUM FACTOR.

0.2 ml F + 0.2 ml T (bovine, Upjohn's at final unitage stated): seconds, at 25°C.

F.	10	5	2.5	1.25	0.625 (T units/ml)
I.	11.2"	11.8"	17.0"	24.4"	39.2"
II.	9.8"	12.1"	16.9"	23.3"	35.8"
III.	6.7"	9.9"	13.5"	19.1"	26.9"

TABLE III

CLOTTING-TIMES, WITH VARYING AMOUNTS OF CALCIUM, OF MIXTURES OF THROMBIN AND FIBRINOGEN, CONTAINING DIFFERENT AMOUNTS OF SERUM FACTOR.

0.2 ml F + 0.1 ml $CaCl_2$ (final molarity stated) + 0.1 T (5 units/ml): seconds, at 25°C.

F.	0.02	0.01	0.005	0.002	0.001	0(H_2O):	Ca(mol)
I.	18.2"	10.9"	9.7"	9.8"	10.5"	12.0"	
II.	22.2"	10.8"	9.6"	9.7"	10.3"	12.6"	
III.	15.5"	10.7"	9.0"	9.5"	9.8"	12.6"	

TABLE IV

CLOTTING OF PLATELET-FREE (DOG) PLASMA. EFFECTS OF GLASS(G) vs. SILICONE (S) SURFACES AND "THROMBOPLASTIC" AGENTS, viz. (1) PLATELETS (A) INTACT: (B) DISINTEGRATED AND (2) CEPHALIN. Clotting-times, seconds, at 38°C, after optimal recalcification.

ADDED:	SALINE		PLATELETS (A)		PLATELETS (B)		CEPHALIN	
SURFACE	S	G	S	G	S	G	S	G
S	∞	∞	435"	170"	465"	185"	155"	60"
*S	∞	∞	550"	195"	660"	235"	150"	60"
*G+	∞	∞	180"	170"	210"	205"	30"	30"

*Held at 34°C for 1 hr with silicone (S) or powdered glass (G+).

TABLE V

CLOTTING OF PLATELET-FREE (DOG) PLASMA, OPTIMALLY RECALCIFIED, WITH ADDITIVES NOTED. Seconds, 38°C.

	ADDITIVE	CLOT.-TIME
1.	Saline	∞
5.	"Soluplastin"	7"
6.	Cryst. trypsin (100)	118"
7.	Fibrinolysin (4 mg)	127"

TABLE VI

SPECIFIC ASSAYS OF ELUATES.

Unitage, per ml, expressed as percentage of standard normal human plasma values (100 per cent mean). PTC and AHF assays are given in Table XXXVII.

	ELUATE (modif.)	PROTHR. (2-stage)	PROCONV. (1-stage)	PROACCEL. (1-stage)	THR.	PTC	AHF
a.	Untreated	183	100	2	0		
b.	Dialysed	167	100	trace	0	+	+
c.	Dial.(b.e.)	171	115	trace	0		
d.	B.e.(only)	183	105	1	0		

TABLE VII

TEST FOR ANTITHROMBIN IN DIALYZED ELUATE AND IN SERUM-AcG PREPARATION: both reagents benzene-extracted.

	INCUBATION PERIOD (minutes):	1/4	5	10	15	20	30
1.	Sal. control:	26.1"	26.9"	24.6"	-	24."	23.9"
2.	Eluate (e.)	34.4"	34.7"	35.7"	-	36.2"	38.8"
3.	AcG (I)	23.3"	33"	128.4"	195"	267"	-
4.	AcG Ψ	22."	25.8"	29.9"	31.6"	35.1"	42.8"

Clotting-times (sec.) for 0.2 ml fibrinogen + 0.2 ml samples of thrombic mixture (T.M.), kept in siliconed tubes and tested (in glass) after the stated incubation periods. T.M. = 1.0 ml thrombin (bovine, Upjohn's, 20 units/ml) + 1.0 ml of (1) saline; (2) eluate; or (3) AcG (I): untreated; (4) AcG Ψ : partly purified (see Expt. 10, p. 134).

TABLE VIII

CLOTTING TEST MIXTURES (ml) OF FIBRINOGEN (b.e.) AND DIALYSED ELUATES (see text). EFFECTS OF BENZENE EXTRACTION.

Clotting-times, seconds, at temp. stated.

TEST	SAL.	ELUATE	AcO (b.e.)	CEPH. (0.5%)	FIBR. (b.e.)	Ca (0.02M)	CLOTTING TIME	
1.	0.2	-	0.1	-	0.4	0.3	475"	(26°C)
2.	0.1	-	0.1	0.1	0.4	0.3	450"	(")
3.	0.4	0.1(b.e.)	0.1	-	0.4	-	2250+"	(")
4.	0.3	0.1(b.e.)	0.1	0.1	0.4	-	2340+"	(")
5.	0.2	0.1(b.e.)	-	-	0.4	0.3	1005+"	(")
6.	0.1	0.1(b.e.)	-	0.1	0.4	0.3	221"	(")
7.	0.1	0.1(b.e.)	0.1	-	0.4	0.3	210"	(")
8.	-	0.1(b.e.)	0.1	0.1	0.4	0.3	69.6"	(")
9.	0.2	0.1(untr.)	-	-	0.4	0.3	354"	(")
10.	0.1	0.1(untr.)	-	0.1	0.4	0.3	151"	(")
11.	0.1	0.1(untr.)	0.1	-	0.4	0.3	107.5"	(")
12.	-	0.1(untr.)	0.1	0.1	0.4	0.3	46.3"	(")
SUPPLEMENTARY TESTS, substituting tissue thromboplastin* for the cephalin:								
13.	-	0.1(b.e.)	0.1	0.1*	0.4	0.3	16.7"	(37°C)
14.	-	0.1(untr.)	0.1	0.1*	0.4	0.3	15.7"	(37°C)

TABLE IX

A. CLOTTING-TIMES (SECONDS), AT 28°C, FOR MIXTURES CONTAINING DIALYSED ELUATE, WHICH HAD BEEN EXTRACTED WITH BENZENE BEFORE DIALYSIS 5 DAYS PREVIOUSLY.

	SAL.	ELUATE	AcG b.e.	EXTR.	CEPH. 0.5%	FIBR. b.e.	Ca 0.02M	CLOTTING-TIME
1.	0.1	0.1	0.1	-	-	0.4	0.3	207.6"
2.	0.2	0.1	-	-	-	0.4	0.3	340."
3.	0.1	0.1	-	0.1	-	0.4	0.3	280.6"
4.	0.1	0.1	-	-	0.1	0.4	0.3	218.6"
5.	-	0.1	0.1	-	0.1	0.4	0.3	57.6"

B. CLOTTING-TIMES OF ABOVE ELUATE AT VARIOUS PERIODS AFTER RE-EXTRACTION WITH BENZENE.

								5-15m.	3 hr.
1.	0.1	0.1	0.1	-	-	0.4	0.3	243.4"	(443.5")
2.	0.2	0.1	-	-	-	0.4	0.3	721"	509.6"
3.	0.1	0.1	-	0.1	-	0.4	0.3	483.1"	273.5"
4.	0.1	0.1	-	-	0.1	0.4	0.3	230.3"	180"
5.	-	0.1	0.1	-	0.1	0.4	0.3	69.9"	36"(6 hr.)

TABLE X

THROMBIN FORMATION FROM BENZENE-EXTRACTED, DIALYSED ELUATE.

A. COMPONENTS OF THROMBIC MIXTURES: ml.

THR. MIXT.	IMID. BUFF. SALINE	ELUATE b.e.,dial.	AcG($BaSO_4$ ads.) b.e.(x2) 1:8	EXTRACTIVE from plasma	$CaCl_2$ (0.1M)
1.	4.6	0.1	-	-	0.3
2.	4.5	0.1	-	0.1	0.3
3.	4.4	0.1	0.1	0.1	0.3

B. CLOTTING-TIMES (sec.), 1st. visible fibrin, for 0.2 ml fibrinogen + 0.2 ml Thr. Mixt., tested after incubation periods stated. 28°C.

TEST	1 min.	5 min.	10 min.	20 min.	30 min.	1 hr.	2 hr.	3 hr.	48 hr.
1.	1170"	-	1230"	745"	720"	720"	710"	700"	745"
2.	676"	-	488"	503"	435"	395"	334"	335"	365"
3.	480"	347"	336"	259"	259"	243"	249"	262"	560"

TABLE XI

THROMBIN FORMATION FROM UNDIALYSED ELUATES, WITH VARYING ACTIVATORS.

A. COMPONENTS OF THROMBIC MIXTURES: ml.

THR. MIXT.	SAL.	IMID. BUFF.	ELUATE	PRO-C.	AcG 1:8	CEPH. 0.5%	TPLN.	$CaCl_2$ 0.15M
4.	3.9	0.5	-	-	0.1(b.e.)	-	-	0.5
5.	3.8	0.5	0.1(b.e.)	-	0.1(b.e.)	-	-	0.5
6.	3.7	0.5	0.1(b.e.)	-	0.1(b.e.)	0.1	-	0.5
7.	3.8	0.5	0.1(b.e.)	-	0.1	-	-	0.5
8.	3.7	0.5	0.1(b.e.)	-	0.1	-	0.1	0.5
9.	3.6	0.5	0.1(b.e.)	0.1	0.1	-	0.1	0.5
10.	3.7	0.5	0.1	-	0.1	-	0.1	0.5
11.	3.6	0.5	0.1	0.1	0.1	-	0.1	0.5

B. CLOTTING TIMES (seconds), for 0.2 ml fibrinogen + 0.2 ml thr. mixt., tested after stated incubation periods. 28°C.

TEST	MINUTES 1	2	3	5	7	10	30	60	90
4.	1830"	-	-	-	-	1346"	932"	750"	733"
5.	538"	-	-	-	-	480"	-	290"	259"
6.	84"	43.8"	34.2"	21.4"	18.3"	21.7"	-	-	-
7.	895"	-	-	450"	-	216"	177"	181"	-
8.	21"	16.3"	19"	20"	-	-	-	-	-
9.	23.3"	18.8"	-	20.8"	-	-	-	-	-
10.	21.5"	20.8"	18.3"	21.3"	-	-	-	-	-
11.	19.8"	17.6"	21.6"	21.7"	-	-	-	-	-

TABLE XII

REFERENCE ASSAYS (Method III prothrombin 2-stage) OF STANDARD (CANINE) ELUATE, AT VARYING DILUTIONS.

Thrombic Mixture (T.M.): 3.7 ml saline, 0.5 ml imidazole buffer (pH: 7.3), 0.1 ml soluplastin, 0.1 ml AcG (1:5), 0.1 ml eluate (serial dilutions), 0.5 ml $CaCl_2$ (0.15 M).

Clotting-times, seconds, for 0.2 ml std. (dog) fibrinogen + 0.2 ml T.M., after stated incubation periods. 27°C.

T.M.	Conc. ELUATE	INCUBATION PERIODS (Minutes, at 27°C): 1	2	3	4	5	7	8	9	11	15	20
1	150%	18.3"	15.1"	14.6"	16.7"	-						
2	125%	19.8"	15.1"	15.7"	-	-						
3	100%	24.5"	19.7"	18.5"	19.9"	-						
4	80%	26.1"	20.1"	20.1"	19.5"	19.8"						
5	60%	32.1"	23.3"	21.5"	21.6"	23.1"						
6	50%	39.7"	26.3"	23.6"	24.9"	-						
7	40%	45.4"	-	26.9"	27.1"	28.1"						
8	30%	64.7"	-	33.6"	32.3"	37.8"						
9	20%	81.4"	-	37.5"	35.7"	38.5"						
10	10%	200.2"	-	73.8"	-	62.4"	60.9"	62.2"				
11	5%	317.3"	-	-	-	97.3"	88.4"	-	92.4"			
12	1%	1065"	-	-	-	447"	342"	-	200.2"	180"	169.7"	177.5"

0.15 ml and 0.125 ml of eluate, with corresponding reduction of the saline, were used for T.M. (1) and (2), respectively. The 0.1 ml (undiluted) is the chosen "standard" = 100 per cent.

TABLE XIII

EFFECTS OF PURIFIED ANTIHEMOPHILIC GLOBULIN (AHG) ON THROMBIN FORMATION FROM ELUATE.

The eluate (see text) provides prothrombin, proconvertin, PTC (etc. ?). AcG, Ca, and the stated THROMBOPLASTIC AGENTS are added, (+) with, or (0) without, AHG. Method III procedure, at 27°C.

Clotting-times (seconds) for 0.2 ml fibrinogen + 0.2 ml thrombic mixture (T.M.) after stated incubation periods.

T.M.	AHG	THROMBOPL. AGENT	INCUBATION PERIOD (minutes): 1	2	3	5	7	10	15	20	30	60	1080
1.	+	0	506"	-	-	309"	-	202"	-	175"	110"	100"	111"
2.	+	Solupl.1/1	19.9"	16.8"	15.9"	16.6"	-	-	16.9"	-	-	-	-
3.	0	"	22.2"	17.6"	15.9"	15.9"	15.9"	16.9"	-	-	-	-	-
4.	+	Solupl.1/5	81.6"	31.7"	22.7"	19.6"	17.5"	17.1"	18.1"	-	-	-	-
5.	0	"	72.4"	41.5"	25.1"	18"	17.3"	15.4"	17.6"	-	-	-	-
6.	+	Ceph.0.1%	233"	32.3"	16.8"	15.8"	15.7"	16"	-	16.2"	18.6"	-	-
7.	0	"	141"	104"	69.6"	40.1"	34"	28"	25.5"	26.9"	-	-	-

TABLE XIV

EFFECTS OF SEQUESTRENE-DECALCIFICATION ON THROMBIC ACTIVITY OF PROTHROMBIN + ACTIVATOR MIXTURES, TESTED AT DIFFERENT TIMES FROM START OF THROMBIN FORMATION.

Two-stage method, at 28°C, as described in text. Clotting-times (seconds) for tests at stated periods, timed from start of experiment:

(A) T.M. incubated alone, and EDTA added to fibrinogen.
(B) T.M. incubated with EDTA, from 5th minute.
(C) T.M. incubated with EDTA, from 68th minute.

TEST SERIES	TIME OF TEST (minutes) from start of incubation of original thrombic mixture (A): 2	4	5	10	15	30	60	68	78	128
A.	3600+"	609"	297"	86.9"	78.2"	85"	97"			
B.			312"	397"	-	620"	1440+"			
C.								74"	73.5"	73"

TABLE XV

SEQUESTRENE - 'REVERSAL' OF THROMBIN FORMATION.

Two-stage method, at 28°C, as described in text. Clotting-times (seconds) for tests at stated periods, from start of experiment (A; B) or from start of incubation (C; D).

MIXTURE	INCUBATION PERIOD (minutes): 1/4	1	2	3	5	10	30	60	90	185
(A) T.M. (alone)	-	-	80.3"	-	44.6"	47.8"	46.7"	45.1"	46.4"	47"
(B) T.M. + EDTA	-	-	108.4"	-	117"	135"	152.4"	174"	195"	209"
(C) Dialysed (B)	189"	184"	186"	181"	183"	192"	211"	-	-	-
(D) T.M. (alone)	-	146"	34.6"	25.6"	25.5"	26"	-	-	-	-

TABLE XVI

EFFECTS OF INCUBATION WITH OXALATE UPON THE CLOTTING-TIMES OF VARIOUS THROMBIC MIXTURES (see text).

Clotting-times (seconds), at 38°C, for 1 ml test mixture + 1 ml fibrinogen, after stated periods of incubation, subsequent to dilution with agent stated.

TEST	AGE of T.M.	ADDED AGENT	INCUBATION PERIOD (minutes): 1/4	5	15	30	60	90	120
A.	11 min.	water	15"	15"	15"	15"	15"	15"	20"
B.	12 min.	oxalate	55"	95"	160"	390"	1380"	2700"	4500"
C.	4 hr.	oxalate	40"	40"	45"	40"	60"	50"	50"

TABLE XVII

EFFECTS OF INCUBATION WITH CITRATE UPON THE CLOTTING-TIMES OF VARIOUS THROMBIC MIXTURES (see text).

Clotting-times (seconds) at 38°C, for 1 ml test mixture + 1 ml fibrinogen, after stated periods of incubation, subsequent to dilution with agent stated.

TEST SERIES	AGE OF T.M.	ADDED AGENT	INCUBATION PERIOD (minutes): 1/4	5	15	30	60	90	120
D.	9½ m.	water	15"	10"	15"	15"	15"	15"	20"
E.	10 m.	citrate	105"	195"	210"	270"	765"	840"	
F.	3½ hr.	citrate	215"	150"	140"	145"	145"	-	145"

TABLE XVIII

EFFECTS OF TREATMENT WITH DOWEX '50' UPON THE CLOTTING TIMES OF VARIOUS THROMBIC MIXTURES (see text).

Clotting-times (seconds), at 28°C, for 0.2 ml (or equivalent) test mixture + 0.2 ml 1% Bovine Fibrinogen, after stated periods subsequent to adding agent cited.

TEST SERIES	AGE OF T.M.	ADDED AGENT	INCUBATION PERIOD (minutes): 1	3	5	10	15	20	30	60
A.	-	Activ. Mix.	+	1320"	217.5"	33.5"	26.5"	29"		37"
B.	10 min. (A)	Dowex '50'	44.9"	-	150.6"	380"	-	496"	510"	-
C.	60 min. (A)	Dowex '50'	56.2"	-	98.1"	125.3"	-	141.8"	152.3"	-
D.	20 min. (B)	Activ. Mix.	213"	-	203.6"	254.5"	-	321.6"	-	-
*E.	-	Activ. Mix.	-	-	26"	25"	22.8"	23.2"	26.2"	-

* Original eluate treated for 1 hr. with Dowex '50' (next day).

TABLE XIX

EFFECT OF BENEZENE-EXTRACTION DURING CONVERSION OF PROTHROMBIN TO THROMBIN IN CEPH.-Ca-AcG ACTIVATED ELUATE.

Clotting-times (seconds), at 27°C, for 0.2 ml (or equivalent) test mixture + 0.2 ml 1% bovine fibrinogen, after stated periods subsequent to adding agent cited.

TEST SERIES	AGE OF MIXTURE	ADDED AGENT	INCUBATION PERIOD (minutes): 1	5	10	20	30	45
1.	-	Activ. mix.	-	31"	28.8"	30.3"	32.7"	-
2.	(1) 5 m.	Benzene extr.	30.7"	47.8"	79"	150"	169"	172"
3.	(2) 70 m.	Re-act. mix.	-	174.5"	185"	242.4"	-	-

TABLE XX

TREATMENT OF THROMBIN AND FIBRINOGEN WITH
(A) DECALCIFYING (ION-EXCHANGE) RESIN:
(B) BENZENE

Clotting-times (sec.) before and at stated periods after the treatment noted: 0.2 ml Upjohn's bovine thrombin + 0.2 ml fibrinogen.

	Before treatment	Period after treatment: 10 min.	20 min.	30 min.	60 min.	5 hr.
A.	13.7"	15.7"	-	-	20"	(12.9")*
B.	10.9"	13.5"	14.3"	11.2"	-	-

*Treated thrombin tested on untreated fibrinogen.

TABLE XXI

RATES AND YIELDS (MINIMAL CLOTTING-TIMES) OF THROMBIN IN STANDARD THROMBIC MIXTURES (T.M.), WITH VARYING CONCENTRATIONS OF CEPHALIN.

Ceph. conc. (final) in micrograms (γ) per ml of T.M. Clotting-times (seconds), at 28°C, for 0.2 ml fibrinogen + 0.2 ml T.M., tested at the stated incubation periods.
: denotes tests not carried to completion.

T.M.	CEPH. CONC. γ	INCUBATION PERIOD (minutes): 5	10	15	20	30	35	40	45	FINAL Φ THROMBIN
0.	0	502"	462"	362"	334"	315"	-	-	245"	trace
1.	100	77.2"	38.3"	28.1"	31.3"	31.5"	-	-	-	38%
2.	50	52.2"	26.3"	25"	22"	23.3"	27"	-	-	58%
3.	25	54"	44"	30"	26.9"	40"	-	-	-	40%
4.	12.5	67.3"	46.9"	41.6"	38.5"	31"	-	30"	-	?
5.	6.25	80.5"	47.3"	34.3"	29.7"	29.3"	-	31.5"	-	35%
6.	3.13	117.1"	77.6"	43.6"	37.8"	31.3"	-	31.5"	-	31%
7.	1.57	135"	55.4"	47.7"	44"	37.7"	-	-	34.1"	25%
8.	0.78	203.7"	-	108.8"	77"	43.8"	-	-	38.1"	19%

Φ computed as percentage of STANDARDS given in Fig. 14 and Table XII.

TABLE XXII

EFFECTS OF VARYING CEPHALIN CONCENTRATIONS ON THE CLOTTING-POWER DEVELOPED IN THROMBIC (i.e. PROTHROMBIN + Ca) MIXTURES.

Clotting-times (seconds), at 38°C, for 1 ml fibrinogen + 0.5 ml T.M., tested at stated incubation periods (see text).

	FINAL DIL. OF CEPH.	INCUBATION PERIOD (minutes): 5	30	60	120	180	RETESTED (4 hr.) 1:1000 Ceph. added
1.	1×10^{-3}	20"	7"	7"	7"	7"	12"
2.	1×10^{-4}	23"	10"	9"	9"	10"	12"
3.	1×10^{-5}	90"	23"	18"	15"	15"	12"
4.	1×10^{-6}	720"	140"	50"	30"	30"	12"
5.	1×10^{-7}	2160"	680"	330"	73"	65"	12"

TABLE XXIII

EFFECTS OF VARYING THROMBOPLASTIN CONCENTRATION ON ACTIVATION OF PROTHROMBIN, WITH SPECIAL REFERENCE TO THROMBIN YIELD.

"End-point" Clotting-times (sec.), at 25±2°C, after stated incubation periods (see text).

	FINAL CONC. OF THROMBOPLASTIN	END-POINT CLOT.-TIME	REQUIRED INCUB. PERIOD
1.	1 : 500	4"	1 hr.
2.	1 : 1000	4"	2 hrs.
3.	1 : 4000	6"	18 hrs.
4.	1 : 40,000	8"	3 days
5.	1 : 4000,000	10"	4 days

TABLE XXIV

A. ASSAYS OF CLOTTING FACTORS IN (HOWELL-TYPE) PROTHROMBIN AND PRECURSOR PLASMAS (DOG).

For methods, see text. Percentage units refer to standards (Fig. 16) obtained with normal (100%) dog oxalated plasma.
"Recovery" is computed (%) from (4) and (1), allowing for two-fold dilution in (4).

	PREPARATION	FIBR.	THR.	PROTHROMBIN 2-stage u/ml	PROTHROMBIN 1-stage	PROCONV. 1-stage	PROACCEL. 1-stage	PTC	AHF
1.	Citr. Plasma	+ (9.3")	0	254	100%	96%	80%	++	++
2.	Berkefeld Pl.	+ (10")	0	100	56%	68%	25%	N.T.	N.T.
3.	Defibr. Pl.	trace	0	144	64%	48%	22%	N.T.	N.T.
4.	Prothrombin	0	tr. 45'	37	10%	20%	25%	++	+
	Recovery	-	-	29%	20%	40%	50%	?	?

B. ANTITHROMBIN TEST ON HOWELL-TYPE PROTHROMBIN (Pro).

Clotting-times (seconds) for 0.2 ml fibrinogen + 0.2 ml sample of mixture of equal (1 ml) vols. Pro + thrombin (bovine, 20 units/ml), tested after stated incubation periods. 28°C.

INCUBATION PERIOD (minutes):	1/4	5	10	15	20	30
Test Clotting-Times:	17.6"	18.8"	17.9"	18"	17.3"	17.1"

TABLE XXV

EFFECTS OF DECREASING AMOUNTS OF CALCIUM ON ACTIVATION OF PROTHROMBIN- (AND PROCONVERTIN-) CONTAINING ELUATE, IN THE PRESENCE OF ADDED AcG AND TISSUE THROMBOPLASTIN.

T.M.'s: 10 ml (total) volume, in imidazole buffered saline (pH:7.3), containing 0.2 ml eluate, 0.1 ml AcGΨ (1:1), 0.5 ml "Soluplastin", and $CaCl_2$ to concentration (milli-Molar) stated.
Clotting tests: 0.4 ml fibrinogen + 0.2 ml (adjusted) Ca* + 0.2 ml T.M., at stated incubation periods.
Clotting-times (sec.), at 26°C.

T. M.	Ml 0.02 M Ca added/10 ml T. M.	Conc. Ca in T. M. (mM)	Conc. Ca* in T. M.+F. (mM)	INCUBATION PERIOD: 1 min.	5 min.	10 min.	15 min.	20 min.	30 min.	60 min.
1.	4 (ml)	8 (mM)	5 (mM)	47.9"	43.7"	42.3"	-	44.2"	47.9"	53.7"
2.	2 (ml)	4 (mM)	5 (mM)	58.6"	44.7"	42.6"	-	42.9"	45.9"	43.3"
3.	1 (ml)	2 (mM)	5 (mM)	75.8"	64."	42.2"	-	-	43.4"	45.2"
4.	0.5 (ml)	1 (mM)	5 (mM)	105.1"	46.1"	44.5"	42.4"	-	46.8"	46.5"
5.	0.25 (ml)	0.5 (mM)	5 (mM)	185.7"	89.8"	57.4"	50.2"	47.7"	42.7"	46.6"
6.	0 (buff. only)	0	5 (mM)	311"	254"	272"	-	256"	319"	280"

TABLE XXVI

EFFECTS OF INCREASING AMOUNTS OF CALCIUM ON THROMBIN FORMATION.

Same test conditions as in Table XXV (Expt. 23), except for the increased concentrations of $CaCl_2$.

T.M.	Ca CONC. IN T.M.	Ca CONC. IN T.M. + F	INCUBATION PERIOD (minutes): 1	5	10	20	30	60
I.	8 mM	25 mM	39.8"	38.2"	32"	35.3"	35.8"	36.1"
II.	10 mM	25 mM	43.3"	40.7"	40"	45.9"	41.7"	44.5"
III.	15 mM	25 mM	50.6"	37.4"	37.4"	37.5"	40.5"	41.1"
IV.	20 mM	25 mM	55.3"	35.9"	38.1"	39.3"	41.6"	40.8"
V.	25 mM	25 mM	81.9"	45.4"	47.3"	48.2"	46.1"	45.5"

TABLE XXVII

EFFECTS OF VARYING AMOUNTS OF Ca^{++} DURING PROTHROMBIN ACTIVATION.

T.M.: 5 ml total vol. containing, in borate buffer (pH: 7.7): 1 ml 0.1% Prothrombin H (ref.156) + 0.25 ml 0.5% Difco's (commercial) rabbit brain thromboplastin + 0.25 ml $CaCl_2$ (final molarity cited).

Clotting-times (seconds) at 29°C, for 0.25 ml T.M. + 0.5 ml B.F. (0.5%) + 0.25 diluent (containing same amount of tpln. as in T.M. and exactly enough $CaCl_2$ to bring the final concentration of calcium to 0.0125 M in each (T.M. + B.F.) final clotting test mixture.

T.M.	Ca IN T.M.	INCUBATION PERIOD (minutes): 1/4	5	10	15	30	60	120
1.	0	127"	126"	125"	125"	129"	139"	145"
2.	0.002 M	14"	12"	4.5"	4"	4"	4"	4"
3.	0.005 M	99"	6"	4.5"	4"	4"	4"	4"
4.	0.025 M	98"	6"	5"	4.5"	4"	4"	4"
5.	0.05 M	139"	75"	52"	21"	7.5"	5"	4"

TABLE XXVIII

"SPONTANEOUSLY" FORMED THROMBIN (II) COMPARED WITH RAPIDLY (Ca+tpln.) ACTIVATED PROTHROMBIN (I).

Clotting-times (sec.), at 25°C, for serial dilutions of the two thrombins, on the same fibrinogen, under the same test conditions.

MIXT.	RELATIVE STRENGTHS (by dilution): 100%	50%	25%	10%	5%	1%
I.	4"	8"	11"	22"	40"	84"
II.	4"	8"	11"	22"	42"	102"

TABLE XXIX

STABILITY OF PURIFIED PROTHROMBIN SOLUTION. ACTIVATION BY (1) Ca ALONE, (2) Ca + THROMBOPLASTIN (see text).

Clotting-times (seconds), at 25°C, of 0.5 ml fibrinogen + 0.25 T.M., after stated incubation periods.

T.M.	DATE OF EXPERIMENT	ACTIVATOR ADDED	INCUBATION PERIOD: 1 m.	10 m.	30 m.	1 hr.	2 hr.	6 hr.	1 d.	2 d.	4 d.	7 d.
1.	Oct. 8,'46	Ca (only)	112"	87"	66"	48"	38"	16"	9"	7"	3"	3"
2.	Oct. 8,'46	Ca+tpln.A	68"	14"	6"	5"	3"	3"	3"	3"	3"	3"
3.	Dec. 9,'46	Ca+tpln.D	20"	12"	8"	4"	3"	3"	3"	3"	3"	3"

TABLE XXX

STABILITY OF PROTHROMBIN AND THE THROMBIN FORMED FROM IT BY SIMPLE RECALCIFICATION.

For details, see text.
Clotting-times (sec.) of 0.5 ml fibrinogen + 0.25 T.M. (dil.), at stated periods.
Series (A): T. M. (2 ml) diluted with 0.25 ml buffer, before test.
Series (B): T. M. (2 ml) maximally activated with 0.25 ml tissue thromboplastin (within 1 hr.) before testing with B. F. "End-point" clotting-times cited. 23± 2°C.

TEST SERIES	AGE OF T. M. BEFORE TESTING: 1 hr.	1 day	2 days	3 days	8 days
A.	250"	85"	43"	25"	4"
B.	4"	4"	4"	4"	4"

TABLE XXXI

THROMBIN FORMATION IN PRESENCE OF VARIOUS THROMBOPLASTIC AGENTS, FROM BRAIN TISSUE

Thrombic mixtures (T) = 4 cc prothrombin + 0.5 cc borate buffer + 0.25 cc N/10 $CaCl_2$ + 0.25 cc cited agents, incubated for periods indicated. Clotting-times (sec.)*, at 25°C, pH = 7.75, for 0.5 cc T + 1.0 cc prothrombin-free fibrinogen. The P-lipids are 1:1000 suspensions in borate buffer.

T	THROMBOPLASTIC AGENT	INCUBATION TIME (min., at 25°C., pH= 7.75) 10'	20'	30'	60'	90'	120'	150'
0.	(Calcium only)	±	±	±	±	+	+	+
1.	Brain Susp.	45"	28"	27"	27"	27"	28"	30"
2.	P-lipid V	+	320"	180"	68"	53"	48"	49"
3.	P-lipid III	+	760"	340"	108"	78"	76"	78"
4.	P-lipid I	+	570"	380"	140"	90"	82"	82"
5.	P-lipid IV	245"	120"	70"	38"	39"	40"	42"

* + : clot starting in ½ -- 1 hr. and becoming solid later.

± : weak clot starting in several hours and incomplete in 24 hours.

TABLE XXXII

THROMBOPLASTIC AND ANTI-THROMBOPLASTIC ASSAYS

ON VARIOUS LIPOIDS AND THEIR CONTAMINANTS

Synthetic phosphatides are prepared as sodium salts.
Testing methods are described in the test.
Results are indicated by the following symbols:
+...++++ = varying degrees of thromboplastic (TPL-IC) activity,
+ = slightly enhances action of standard cephalin,
−...—— = varying degrees of inhibition (ANTI-TP) of cephalin,
N. T. = not tested.

No.	DESCRIPTION OF PREPARATION	TPL-IC	ANTI-TP
1.	Standard Cephalin (from dog brain), a crude mixture of phosphatides, etc.	++++	− (>50γ/ml.)
2.	1,2 - distearoyl α-cephalin, synthetic, >95% pure.	0	0
3.	1,2 - dipalmitoyl α-cephalin, synthetic, >95% pure.	0	0
4.	1,3 - dimyristoyl β-cephalin, synthetic, >95% pure.	0	0
5.	stearal-acetal-phosphatidyl ethanolamine, synthetic, >95% pure.	0	0
6.	myristal-acetal-phosphatidyl ethanolamine, synthetic, 80-95% pure.	——	——
7.	myristal-acetal-phosphatidyl ethanolamine, repurified from 6, >99% pure.	N. T.	0
8.	palmital-acetal-phosphatidyl ethanolamine, synthetic, >99% pure (group analyses)	±	0
9.	trace impurity (A_1) from preparation G.	N. T.	−
10.	trace impurity (A_2) from preparation G.	N. T.	−
11.	trace impurity (B_1) from preparation G.	N. T.	−
12.	trace impurity (B_2) from preparation G.	N. T.	0
13.	mixture of 7 + 9.	N. T.	—
14.	mixture of 7 + 11.	N. T.	0
15.	mixture of 7 + 9 + 11.	N. T.	——
16.	mixture of 9 + 11.	N. T.	——
17.	trace impurity (I) from preparation 8.	0	—
18.	trace impurity (II) from preparation 8.	±	0

TABLE XXXIII

EFFECTS OF HEPARIN ON FORMATION OF THROMBIN FROM RECALCIFIED PROTHROMBIN, IN PRESENCE OF VARIOUS THROMBOPLASTIC AGENTS.

T.M. (see text) incubated at 15°C, for periods stated. Clotting-times (seconds), at 38°C, for 1 ml fibrinogen + 0.5 ml T.M. Amounts of additives per 5 ml T.M.

T.M.	THROMBOPLASTIC AGENT	INHIBITOR	INCUBATION PERIOD (minutes): 1	5	10	20	30	60
1.	Cephalin (0.1 mg)	-	-	50"	20"	11"	9"	9"
2.	"	Heparin(0.5 mg)	-	∞	∞	∞	∞	∞
3.	Brain P-lipid (0.025 mg)	-	50"	20"	10"	8"	6"	6"
4.	"	Heparin(0.5 mg)	∞	∞	∞	∞	1800+"	360+"
5.	Tpln.Q (0.025 mg lipids)	-	35"	16"	9"	7"	7"	7"
6.	"	Heparin(0.5 mg)	720"	16"	10"	7"	7"	7"
7.	Trypsin (0.125 mg)	-	-	30"	15"	12"	11"	8"
8.	"	Heparin(0.5 mg)	-	97"	32"	13"	11"	9"

TABLE XXXIV

EFFECTS OF COFACTOR ON ACTION OF HEPARIN DURING AND AFTER THROMBIN FORMATION.

Details of experiments are given in the text.
Clotting-times (seconds), at 38°C, for appropriately diluted fibrinogen (see text) after incubation of T.M.'s for the stated periods, at 7.5°C.

T.M.	INHIBITOR	INCUBATION PERIOD (minutes): 5	15	30	60	90	120	TESTS ON T.M.(1) at 1 hr.	No.
1.	-	95"	22"	18"	18"	18"	18"	18"	5.
2.	Heparin	∞	+	900"	30"	23"	23"	19"	6.
3.	Cofactor	40"	20"	19"	20"	25"	29"	28"	7.
4.	Hep.+Cof.	∞	∞	∞	∞	∞	∞	23"	8.

TABLE XXXV

INHIBITORY EFFECTS OF HEPARIN DURING PROTHROMBIN ACTIVATION BY TISSUE THROMBOPLASTIN.

See text for details of experiment.
Clotting-times (seconds), at 25 ± 2°C, for incubated mixtures and the fibrinogens noted in the text.

T.M.	AND TEST	INCUBATION PERIOD: 5 min.	15 min.	30 min.	1 hr.	2 hr.	8 hr.	1 d.	2 d.	3 d.	7 d.
1a.	(without heparin)	63"	42"	20"	9"	6"	4"	4"	4"	4"	4"
1b.	(hep. in fibrinogen)	840"	60"	29"	12"	8"	5"	4½"	4½"	4½"	4½"
2.	(with heparin)	2 hr.	375"	60"	19"	13½"	10"	9"	8"	7½"	4½"

TABLE XXXVI

EFFECTS OF TRYPSIN ON ACTIVATION OF PROTHROMBIN, IN PRESENCE OF VARIOUS ADDED ACTIVATORS.

Experimental details given in text.
Percentages of prothrombin activated, after stated incubation periods. 25°C.
T.M.'s: 5 ml total vol., containing 4 ml Pro. D (0.2%) + stated additives.

T.M.	ACTIVATORS ADDED (ml)			INCUBATION PERIOD:								
	$CaCl_2$ 0.1 M	tpln.C 0.1%	trypsin 40 u/ml	5 min.	15 min.	30 min.	1 hr.	3 hr.	6 hr.	24 hr.	30 hr.	48 hr.
I.	0.25	-	-	-	±	±	+	1%	4%	40%	80%	100%
*II.	-	-	0.5	-	+	1%	1%	8%	20%	2%	+	-
*III.	0.25	-	0.5	6%	25%	35%	60%	100%	80%	4%	1%	-
IV.	0.25	0.75	-	40%	80%	100%	100%	100%				
V.	0.25	0.25	-	1%	5%	9%	20%	30%	35%	70%	80%	100%
*VI.	0.25	0.25	0.5	35%	42%	60%	75%	100%	50%	3%	1%	-

*NOTE: Clot lysis occurred in 3 days, in tubes containing trypsin.

TABLE XXXVII

PTC and AHF ASSAYS.

Agents:
- 0. Saline (substrate control)
- I. Dialysed eluate (p.126)
- II. Howell-type Prothrombin (p.161)
- III. Purified AHG (Antihemophilic globulin (p.137)
- IV. Normal human plasma (factor control)

Methods are described in the text.
The substrates (SUBS.) are plasmas from cases deficient in PTC or AHF, respectively.

AGENT	SUBS.	ORIGINAL MIXTURES (a) additive	(b) c.t.	(c) % pro.	AGE OF SERUM	SERUM TESTS (a) c.t.	(b) % pro.	PROT. CONS.
0.	PTC	sal.(control)	21.3"	126	½ hr.	23.9"	86	32%
0.	AHF	"	22"	115	1 hr.	22.7"	103	10%
I.	PTC	eluate (1:1)	22.3"	112	½ hr.	27.8"	46	59%
I.	AHF	"	20.9"	131	1 hr.	31.3"	35	73%
II.	PTC	Howell Pro.(1:1)	20.5"	137	½ hr.	29.7"	40	71%
II.	AHF	"	21.3"	126	1 hr.	26.5"	54	57%
III.	PTC	AHG (1%)	22.4"	109	½ hr.	51.7"	8	96%
III.	AHF	AHG (0.001%)	21.1"	129	1 hr.	36.9"	22	83%
IV.	PTC	Plasma (1:5)	21.9"	117	½ hr.	25.5"	65	44½%
IV.	AHF	Plasma (1:10)	22.7"	105	1 hr.	63"	3	97%

TABLE XXXVIII

POTENTIATION OF THROMBOPLASTIC ACTION OF PLATELETS BY (A) ANTIHEMOPHILIC GLOBULIN and (B) TRYPSIN.

Experimental details given in text. All T.M.'s contain 0.1 ml dialysed eluate, 0.1 ml AcG (1:5), and 0.5 ml 0.02 M $CaCl_2$, with imidazole-buffered saline, to 5 ml total volume.
Clotting-times (seconds), at 28°C, for 0.2 ml fibrinogen + 0.2 ml T.M., after stated incubation periods.

T.M.	(ml) OTHER ADDITIVES	INCUBATION PERIOD (minutes): 1	5	10	20	30	60
(A) 1.	(0.2) platelets	2 hr.	495.2"	65"	31.9"	30.3"	32.3"
2.	(0.2) platelets (0.2) AHG (1%)	2 hr.	92.6"	44.9"	42.7"	40.1"	45"
3.	(0.2) AHG (1%)	-	1 hr.	582.5"	349.6"	164.2"	246.1"
(B) 4.	(0.2) platelets (0.1) trypsin	155.5"	42.1"	35.7"	39.8"	37.5"	38.8"
5.	(0.1) trypsin	-	2 hr.	1609+"	791"	570"	445"
6.	(0.1) trypsin (0.2) AHG (1%)	-	732.6"	242.2"	195.4"	172.6"	174.2"

TABLE XXXIX

POTENTIATION OF THROMBOPLASTIC ACTION OF PLATELETS BY (C) WEAK THROMBIN and (D) FIBRINOLYSIN.

Experimental details given in text. All T.M.'s contain 0.1 ml dialysed eluate, 0.1 ml AcG , and 0.5 ml 0.02 M $CaCl_2$, with imidazole-buffered saline, to 5 ml total volume.
Clotting-times (seconds) at 26°C, for 0.2 ml fibrinogen + 0.2 ml T.M., after stated incubation periods.

T.M.	(ml) OTHER ADDITIVES	INCUBATION PERIOD (minutes): 5	10	20	30	60	90
(C) 7.	(0.2) plat. (0.1) thr.	191"	53.5"	29.5"	30.3"	26.9"	27.7"
(D) 8.	(0.2) plat. (0.1) lysin	194"	66.6"	39"	34.4"	31.3"	29.5"
9.	None (Control)	2 hr.	2 hr.	2 hr.	1560"	820"	-

TABLE XL

EFFECTS OF (A) WEAK THROMBIN AND (B) FIBRINOLYSIN UPON THE THROMBOPLASTIC ACTION OF PLATELETS.

Experimental details given in text.
T.M.: 0.2 ml Pro., 0.1% + 0.2 ml AcG, 0.1% + 0.5 ml $CaCl_2$ (0.05 M), the calcium being added after the other activators cited, making, with the borate buffer (pH: 7.7) diluent, a total 5 ml vol.
Clotting-times (seconds), at 26°C, for 0.5 B.F. (1%) + 0.25 T.M., at the stated incubation periods.

T.M.	ACTIVATORS (ml/5 ml T.M.)	INCUBATION PERIOD (minutes): 5	10	20	40	60
0.	0 (control)	885"	913"	920"	970"	1010"
1.	Platelets (0.2)	810"	620"	330"	190"	97"
2.(A)	Plat.(0.2)+Thr.*(0.5)	145"	52"	22"	12"	9"
3.(B)	Plat.(0.2)+Lysin(0.6)	60"	46"	15"	12"	9"

* Special thrombin, see text.

TABLE XLI

EFFECTS OF ANTI-PROTEASES ON THROMBOPLASTIC ACTION OF PLATELET-TRYPSIN ADDITIONS TO THROMBIN-FORMING SYSTEMS.

T.M.'s: 5 ml, in borate buff. (pH: 7.7), containing 0.2 ml Pro.(0.1%) + 0.2 ml AcG (0.1%) + 0.5 ml $CaCl_2$ (0.05 M), added after the various other activators and inhibitors cited. The asterisk (*) with the inhibitor denotes a 15 min. (room temp.) pre-incubation with the activator system.
Clotting-times (seconds), at 25°C, for 0.5 ml B.F. (1%) + 0.25 ml T.M., at successive incubation periods. Clot-lysis followed over 3 weeks.

T.M.	ACTIVATORS	INHIBITORS	INCUBATION PERIOD (minutes): 5	10	20	40	60	CLOT LYSIS
1.	platelets	0	720"	540"	270"	100"	65"	0
2.	trypsin	0	182"	140"	95"	58"	51"	3 d.
3.	plat.+tryp.	0	47"	29.5"	26.5"	23.5"	23.2"	0
4.	plat.+tryp.	P.I.	660"	240"	175"	113"	96"	0
5.	plat.+tryp.	Aftn.*	710"	490"	205"	103"	71"	0

TABLE XLII

EFFECTS OF ANTI-PROTEASES ON THROMBOPLASTIC ACTION OF PLATELET-FIBRINOLYSIN ADDITIONS TO THROMBIN-FORMING SYSTEMS.

T.M.'s: as in Table XLI, except for the substitutions noted in the text.
Clotting-times (seconds), at 25°C, in the usual tests.

T.M.	ACTIVATORS	INHIBITORS	INCUBATION PERIOD (minutes): 5	10	20	40	60
6.	fibrinolysin	0	390"	380"	368"	335"	320"
7.	plat. + lysin	0	29.1"	23"	22"	22"	22"
8.	plat. + lysin	P.I.	28"	22.5"	19.9"	20"	20"
9.	plat. + lysin	Aflin.*	37.6"	24.9"	22.4"	22.2"	20"
10.	plat. + lysin	S.B.I.	630"	135"	42"	38.5"	35.4"
11.	0 (Ca, AcG only)	0	∞	∞	∞	∞	∞

∞: No clots in 1/2 hour.
* : Pre-incub. 15 min.

TABLE XLIII

EFFECTS OF ANTIPROTEASES ON THE ACTION OF PLATELETS, (I) WITHOUT AHG; (II) WITH AHG (ANTIHEMOPHILIC GLOBULIN), IN THROMBIN-FORMING SYSTEMS.

T.M.'s: 5 ml, in borate buff. (pH: 7.7), contain 0.2 ml eluate (dialysed) + 0.2 ml platelets + 0.2 ml 1% AHG (in II) + 0.1 ml AcG (1:8) + 0.1 ml 0.1% inhibitor (0.3 ml of 0.4%, in case of the anti-fibrinolysin, test 5) + 0.5 ml $CaCl_2$ (0.04 M).
In tests 4, 5, and 9, the respective inhibitor* was pre-incubated with the activator (I or II) for 15 min., at 28°C, before adding the other components of the T.M.
Clotting-times (seconds), at 28°C, for 0.2 ml fibrinogen + 0.2 ml T.M., at the stated incubation periods.

T.M.	AHG(1%)	INHIBITOR	INCUBATION PERIOD (minutes): 1	3	5	10	20	30
(I) 1.	0	0	319"		69.8"	37.8"	29.9"	29.9"
2.	0	S.B.I.	-	-	951"	165"	88.5"	77.4"
3.	0	P.I.	-	62.7"	36.7"	28"	24.5"	26.9"
4.	0	N.B.I.*	-	-	36.2"	26	25.3"	23.4"
5.	0	Afln.*	-	-	833"	534"	410"	292"
(II) 6.	0.2 ml	0	67"	-	39.6"	35.3"	36.1"	39.5"
7.	0.2 ml	S.B.I.	-	-	621"	316"	264"	251"
8.	0.2 ml	P.I.	-	34"	31.5"	37.2"	39"	37.4"
9.	0.2 ml	N.B.I.*	-	-	35.3"	32.2"	24.7"	34.8"

TABLE XLIV

EFFECTS OF ANTIPROTEASES ON THE ACTION OF TISSUE THROMBOPLASTIN IN THROMBIN-FORMING SYSTEMS.

T. M.'s: 5 ml, in borate buff. (pH: 7.7), contain 0.2 ml Pro. (0.1%) + 0.5 ml tpln. A. + 0.2 ml AcG (0.1%) + 0.5 ml $CaCl_2$ (0.05 M), added after the various inhibitors cited. In (4), the thromboplastin was pre-incubated for 15 min., at 25°C, with the anti-fibrinolysin.*
Clotting-times (sec.), at 25°C, for 0.5 ml B. F. (1%) + 0.25 ml T. M., at successive incubation periods.

T. M.	INHIBITOR ml (conc.)	INCUBATION PERIOD: 5 min.	10 min.	20 min.	40 min.	60 min.
1.	0	22.5"	17.1"	15.2"	15"	14.2"
2.	S.B.I., 0.2(0.01%)	450"	420"	450"	450"	380"
3.	P.I., 0.2(0.1%)	28.8"	23.3"	21.8"	21.5"	20"
4.	Afln.* 0.3(0.5%)	28.5"	22.4"	20.6"	21.2"	21"

TABLE XLV

EFFECTS OF ANTIPROTEASES ON THE ACTION OF CEPHALIN IN THROMBIN-FORMING SYSTEMS.

T. M.'s: 5 ml, in borate buff (pH: 7.7), contain 0.2 ml eluate (dialysed) + 0.1 ml ceph. (0.1%) + 0.2 ml AcG (1:8) + 0.5 ml $CaCl_2$ (0.04 M), added after inhibitors stated. In test (4), the cephalin was pre-incubated for 15 min. at 28°C, with the N.B.I.*
Clotting-times (sec.), at 28°C, for 0.2 ml (dog) fibrinogen + 0.2 ml T. M., at successive incubation periods.

T. M.	INHIBITORS ml (conc.%)	INCUBATION PERIOD: 5 min.	10 min.	20 min.	30 min.
1.	0	56.5"	52"	50.8"	56.4"
2.	S.B.I., 1.0(0.001)	222"	156.5"	149.1"	163"
3.	P. I., 0.1(0.1)	48.8"	50.3"	57"	59.8"
4.	N.B.I., 0.1(0.1)	46.2"	44.2"	49.4"	53.6"

TABLE XLVI

LACK OF EFFECTS OF ANTIPROTEASES ON THE THROMBIN-FIBRINOGEN REACTION.

Clotting-times (sec.), at 25°C, for 0.5 ml fibrinogen + 0.25 ml thrombin (5 units/ml) + 0.25 antiprotease (or buffer, in control), the last two* being pre-incubated with thrombin for the periods noted.

	ADDITIVE	PRE-INCUBATION PERIOD: 1/4 min.	10 min.	20 min.	30 min.	60 min.
0.	Buffer	14.8"	15"	15"	14.6"	15.3"
1.	P. I.	17"	18.5"	18.5"	19.5"	20.5"
2.	S.B.I.	14.2"	16"	17"	17.1"	17"
3.	N.B.I.*	15.2"	16"	16.5"	16.7"	16.8"
4.	Afln.*	15"	16"	16"	15.5"	16.4"

TABLE XLVII

PROTEOLYTIC ACTIVITIES OF OLD ENZYME PREPARATIONS AND THEIR INHIBITION BY ANTIPROTEASES

Experimental details and descriptions of the enzyme preparations are given in the text.

The pancreatic inhibitor (P.I.) acts "immediately", whereas the navybean inhibitor (N.B.I.*) and antifibrinolysin (Afln.*) require 15-20 min. pre-incubation with the enzyme, before adding the fibrinogen to make the L. M.
Clotting-times (sec.), at 28°C, for 0.2 ml thrombin (20 units/ml) + 0.2 ml L. M., after incubation periods stated.

	L. M.	ADDITIVES	INCUBATION PERIOD: 10 min.	20 min.	30 min.
(A)	1.	0 (buffer)	14.2"	14"	14.3"
	2.	trypsin	540"	840"	∞
	3.	tryp. + N.B.I.*	14"	15"	14"
	4.	tryp. + P. I.	15.7"	22"	28"
(B)	5.	fibrinolysin			
	6.	lysin + N.B.I.*	21"	22"	18"
	7.	lysin + Afln.*	13"	13.8"	14.8"

TABLE XLVIII

EFFECT OF WASHED PLATELETS AS AN "ACCELERATOR" OF THE ACTIVATION OF AcG-POOR PROTHROMBIN.

Reagents and experimental details are described in the text.
T. M.: 5 ml, in borate buff. (pH: 7.75), containing 0.2 PRO. (0.1%) + 0.5 ml $CaCl_2$ (0.05 M) + the various activators noted.
Clotting-times (sec.), at 26°C, of 0.5 ml B. P. (1%) + 0.25 ml T. M., at successive incubation periods.

T. M.	ACTIVATORS ml (dilution)	INCUBATION PERIOD: 5 min.	10 min.	20 min.	40 min.	60 min.
1.	Tpln. B., 0.5	420"	300"	202"	141"	110"
2.	Tpln., 0.5 + Plat., 0.2	390"	240"	80"	22.4"	15"
3.	Tpln., 0.5 + AcG, 0.1 (0.001%)	300"	140"	52"	25.8"	20.3"
4.	Tpln., 0.5 + AcG, 0.1 (0.1%)	11.4"	9.4"	8.0"	7.8"	7.6"

TABLE XLIX

EFFECTS OF HEPARIN ON THROMBIN-FORMING MIXTURES ACTIVATED BY (I) PLATELETS; (II) PLATELETS + TRYPSIN; OR (III) TISSUE THROMBOPLASTIN. (See text.)

T.M.'s: 5 ml, in imid. buff. sal. (pH: 7.3), containing 0.2 ml eluate (dialysed) + 0.1 ml AcG (1:8) + 0.2 ml (I) plat., or (II) plat. + 0.5 ml tryp. (0.02%), or (III) Soluplastin (0.2 ml) + 0.5 ml (a) buff. sal., or (b) heparin (2 units/ml) + 0.5 ml $CaCl_2$ (0.04 M).
Clotting-times (seconds) at 28°C, for 0.2 ml fibrinogen + 0.1 ml (c) heparin (0.2 units/ml) or (d) buff. sal. + 0.1 ml T.M., after stated incubation periods.

T.M.		ACTIVATORS	HEPARIN in T.M.	INCUBATION PERIODS (minutes): 3	5	8	10	15	20	25	30	35
I. 1.	(a)	(d) Plat.	0	-	64"	-	29.9"		29.2"		29.5"	-
1.	(a)	(c) Plat.	0	98.6"	-	24.1"	-	23.8"	-	23.6"	-	24"
2.	(b)	(d) Plat.	+	-	-	-		-		-	1000+"	-
II. 3.	(a)	(c) Plat. +tryp.	0	61.4"	40.6"	-	36.4"	-	-	-	35.8"	34"
4.	(b)	(d) Plat. + tryp.	+	172.5"	64.5"	-	47.6"	-	35.4"	-	37.2"	-
5.	(a)	(d) Tryp.	0	570"	495"	-	392"	352"	407"	-	420"	-
III. 6.	(b)	(d) Solupl.	+	37.1"	35.8"	-	31.7"	-	-	-	30.1"	-

TABLE L

EFFECTS OF PLATELETS ON THE THROMBIN-FIBRINOGEN REACTION.

Method: 0.2 ml thrombin (conc. cited) + (1) 0.1 ml platelets or (2) 0.1 ml sal. + 0.2 ml fibrinogen.
Clotting-times (sec.), at 26°C.

ADDED THR. (units/ml) :	20	10	5	2.5	1.25
(1) with platelets :	10.5"	17.1"	31.5"	59.0"	129"(130")
(2) control, with sal.:	10.5"	17.8"	31.0"	59.5"	105"(105.8")

TABLE LI

PLATELET UTILIZATION DURING CLOTTING OF (A) NORMAL AND (B) HEMOPHILIC BLOOD

Platelet counts ($x1000/mm^3$) after stated times, in siliconed tubes, at 37°C, (I) in clotting bloods, (II) in sequestrene-plasma ('aging' control).

TIME (min.)	(A) NORMAL BLOOD (I)	(B) HEMOPHILIC BLOOD (I)	(II) CONTROL
0	320	182	
1	290	154	
2	265	192	
3	196	132	
4	238	150	
5	103	125	
6	106 (clotting)	-	
7	34	137	
8	31	-	
9	19	-	
10	31	143	
12.5	15	136	
15	18	160 (clotting)	
30	3.3	51	
45	4.2	14	
60	3.7	2.4	(A) 252
75	-	2	
90	-	1.7	
120	-	4	(B) 174

TABLE LII

GROUP I: (A) Normal adults; (B) Healthy parturient mothers; (C) Normal newborn infants (cord bloods).

1.	No. (Sub-group)	>50 (A)	32 (B)	32 (C)
2.	CASES	NORMAL ADULTS	MOTHERS	NEWBORNS
3.	AGE	19-55	15-43	0
	SEX	F, M	F	F, M
	RACE	W, C	23W, 9C	23W, 9C
4.	DATE(S), EXAM.	1950-55	1953-55	1953-55
5.	DURATION BLEEDING	neg.	neg.	neg.
6.	FAM. HIST. of BLEED.	neg.	neg.	neg.
7.	BLEED. TENDENCY	neg.	neg.	neg.
8.	TOURNIQUET TEST	neg.	N.T.	N.T.
9.	BLEED. TIME (min.)	6(2-10)		
	CLOTTING-TIME:			
10.	glass (min.)	9(5-13)	7(2-12)	5(1-10)
11.	silicone (min.)	33(15-50)	24(6-60)	12(3-33)
12.	CLOT RETRACTION	+++(+)	+++(+)	++++.....+
13.	FIBRINOLYSIS	neg.	neg.	neg.(+,2 cases)
14.	PLATELET COUNT x 1000/mm^3	249(143-356)	241(105-446)	271(136-523)
15.	PLATELET TPLN.*	100(65-150)	80-100+	80-100+
16.	" ACCEL.*	100(40-150)	60-100+	50-100+(one 10)
17.	SERUM SEROTONIN*	100(50-150)	N.T.	N.T.
18.	PLATELET AGGLUT.*	neg.	N.T.	N.T.
19.	PLASMA-THR. C.T.	15(13-17)*	16.5* (av.)	25.5* (aver.)
20.	SERUM ANTITHROMBIN	variable	normal	normal
21.	FIBR., mg/100 ml	364(200-540)	652(340-1020)	344(192-800)
22.	AHF*	100(60-135)	60-100+	60-100+
23.	PTC*	100(60-135)	110(83-143)	59(15-90)
24.	INHIBITORS, etc.	neg.	neg.	neg.
25.	PROTHR. CONS., %	95(90-99)	93(80-96)	83(55-95)
26.	PROTHR. TIME*	100(74-133)	119(68-133)	119(56-133)
27.	PROTHROMBIN*	100(70-136)	115(81-150)	43(30-59)
28.	PROCONVERTIN*	100(64-138)	135(100-216)	51(22-80)
29.	PROACCELERIN*	100(60-150)	70-100+	100-300
30.	REMARKS	Med. School personnel	Parity: 1-11	125 others excluded

* per cent, of standard normal, used throughout Tables LII - LVIII.

TABLE LIII-A.

GROUP II: CONGENITAL (FAMILIAL) THROMBOCYTOPENIAS.

1. 2.	No. CASE	Case 1 A. B.		Case 2 D. D.
3.	AGE	2	3	9
	SEX	M	M	M
	RACE	C	C	C
4.	DATE(S), EXAM.	1 Feb. '54	20 Apr. '55	20 Apr. '55
5.	DURATION BLEEDING	1-16 mo.	-	-
6.	FAM. HIST. of BLEED.	+	+	+
7.	BLEED. TENDENCY	+++	0	0
8.	TOURNIQUET TEST	++++	neg.	+(+)
9.	BLEED. TIME (min.)	60	2½	6½
	CLOTTING-TIME:			
10.	glass (min.)	6½	6½	5 3/4
11.	silicone (min.)	6½	7½	29½
12.	CLOT RETRACTION	0...±	+++	++
13.	FIBRINOLYSIS	0	0	0
14.	PLATELET COUNT x 1000/mm^3	38	320	98
15.	PLATELET TPLN.*	N.T.	50	20
16.	" ACCEL.*	N.T.	80	10
17.	SERUM SEROTONIN*	N.T.	N.T.	N.T.
18.	PLATELET AGGLUT.	N.T.	N.T.	N.T.
19.	PLASMA-THR. C.T.	±	±	±
20.	SERUM ANTITHROMBIN	+	N.T.	N.T.
21.	FIBR., mg/100 ml	320	N.T.	520
22.	AHF*	100	100	100
23.	PTC*	80	80	70
24.	INHIBITORS, etc.	N.T.	N.T.	N.T.
25.	PROTHROMBIN CONS. %	95	100	95
26.	PROTHROMBIN TIME*	125	93	88
27.	PROTHROMBIN*	111	106	93
28.	PROCONVERTIN*	95	100	110
29.	PROACCELERIN*	100+	100+	100
30.	REMARKS	before treatment	after splenect.	-

* per cent, of standard normal.

TABLE LIII-B.

GROUP III: HEREDITARY THROMBASTHENIA (3), AND PSEUDOHEMOPHILIA (4, 5).

1.	NUMBER	Case 3.	Case 4.	Case 5.
2.	CASE	(N.P.)	(A.C.)	(S.C.)
3.	AGE	16	52	20
	SEX	F	F	F
	RACE	W	W	W
4.	DATE(S), EXAMINED	9 June, '53	23 Apr. '55	23 Apr. '55
5.	DURATION BLEEDING	birth	33 yrs.	?
6.	FAM. HIST. of BLEED.	+	+	+
7.	BLEED. TENDENCY	+++	+(+)	+(+)
8.	TOURNIQUET TEST	++++	+	++
9.	BLEED. TIME (min.)	>60	1	6½
	CLOTTING-TIME:			
10.	glass (min.)	9	6 3/4	9½
11.	silicone (min.)	22	31	N.T.
12.	CLOT RETRACTION	0	++++	+++
13.	FIBRINOLYSIS	Neg.	Neg.	Neg.
14.	PLATELET COUNT x 1000/mm3	230	238	104
15.	PLATELET TPLN.*	100	50	25
16.	" ACCEL.*	N.T.	50	20
17.	SERUM SEROTONIN*	100	N.T.	N.T.
18.	PLATELET AGGLUT.	N.T.	N.T.	N.T.
19.	PLASMA-THR. C.T.	norm.	N.T.	N.T.
20.	SERUM ANTITHROMBIN	sli. incr.	N.T.	N.T.
21.	FIBR., mg/100 ml	520	720	700
22.	AHF*	100	100	100
23.	PTC*	85	90	60
24.	INHIBITORS, etc.	Neg.	Neg.	Neg.
25.	PROTHROMBIN CONS. %	95	>95	>95
26.	PROTHROMBIN TIME*	94	85	71
27.	PROTHROMBIN*	89	90	100
28.	PROCONVERTIN*	111	N.T.	100
29.	PROACCELERIN*	62	N.T.	90
30.	REMARKS	GLANZMANN'S THROMBASTHENIA	PSEUDOHEMOPHILIA	

* per cent, of standard normal.

TABLE LIV.

GROUP IV: SUMMARY OF DATA ON 25 THROMBOCYTOPENICS, (ACQUIRED).

		FINDINGS				REMARKS
		Pos.	Neg.	?	N.T.	
1.	Nos. 6 - 30					Diag., v. text
3.	AGES: 17 mo. - 78 yr. SEX: 11 F; 14 M RACE: 22W; 3C					
5.	DURATION BLEED.: 1 d. - 8 yr.	19	1	-	5	
7.	BLEEDING TEND.: +++(+)	10				
	" " ++	7				
	" " +	2				
	" " 0	-	1	1	4	
8.	TOURN. TEST: ++++.....+	19	3	2†	1	† Negroes
9.	BLEED. TIME: >10 min.	17	7	1†	-	†9 min.
	CLOTTING-TIME:					
10.	glass: >13 min.	1	21	3†		†12½-10½'
11.	silicone: >50 min.	10	10	3†	2	†48-47'
12.	CLOT RETR. (glass): ++...0	17	3	-	5	
14.	PLATELET COUNT x 1000/mm^3: <100,000	24	-	1†	-	†174,000
15.	PLATELET TPLN.*: <10	18	1	1†	5	†30
16.	" ACCEL.*: <10	19	-	-	6	
17.	SERUM SEROTONIN*: <50	9	3	-	13	
19.	PLASMA-THR. C.T.: >2x norm.	15	-	8†	2	†slight
21.	FIBR.: (a) <200 (decr.)	0	14	-	2	
	(b) >540 (Incr.)	9				
22.	AHF*: <60	-	22	1†	2	†2% in ♀
23.	PTC*: <60	-	21	2†	2	†60; 65
25.	PROTHROMBIN CONS.: <90%	21	3	-	1	
26.	PROTHROMBIN TIME*: <65	-	24	1†	-	†61
27.	PROTHROMBIN*: <65%	3†	21	-	1	†52-62
28.	PROCONVERTIN*: <65	1†	23	-	1	†55
29.	PROACCELERIN*: <60	3†	22	-	-	†30-50

* per cent, of standard normal.

TABLE LV.

GROUP V: THROMBOCYTOSIS.

1.	No.	31	32	33
2.	CASES	R.G.	J.L.H.	E.M.
3.	AGE	63	64	23
	SEX	M	M	M
	RACE	C	W	W
4.	DATE(S), EXAM.	23 June, '55 (27 Aug. '56)	30 Mar. '54 (8 Apr. '55)	15 Dec. '54
5.	DURATION BLEEDING	2 weeks	?	?
6.	FAM. HIST. of BLEED.	neg.	neg.	neg.
7.	BLEED. TENDENCY	++	++	0
8.	TOURNIQUET TEST	neg.	neg.	neg.
9.	BLEED. TIME (min.)	1½-4	3½	2
	CLOTTING-TIME:			
10.	glass (min.)	11¼	7½	4½
11.	silicone (min.)	30	48	46
12.	CLOT RETRACTION	++++	++++	++++
13.	FIBRINOLYSIS	neg.	neg.	neg.
14.	PLATELET COUNT x 1000/mm^3	2000	1886 (550)	850
15.	PLATELET TPLN.*	100	40	100
16.	" ACCEL.*	90 (20)	50	100
17.	SERUM SEROTONIN*	N.T.	5	N.T.
19.	PLASMA-THR. C.T.	normal	normal	normal
20.	SERUM ANTITHROMBIN	normal	normal	normal
21.	FIBR., mg/100 ml	200	540	660
22.	AHF*	100	100	100
23.	PTC*	90	100	80
25.	PROTHR. CONS.,%	90	99	90
26.	PROTHR. TIME*	85	44 (68)	52
27.	PROTHROMBIN*	88	68 (48)	60
28.	PROCONVERTIN*	100	60 (44)	78
29.	PROACCELERIN*	60	50 (40)	90
30.	REMARKS	Carcinoma of stomach; general. metastases; bone marrow hyperplasia, incr. no. megakaryocytes.	Polycythemia vera, treated with P^{32}; gout.	Chronic granulocytic (Myeloid) leukemia.

* per cent, of standard normal.

TABLE LVI-A.

Group VI (A): LEUKEMICS, WITH THROMBOCYTOPENIA. (22 out of 39 cases studied.)

1.	Nos. 34 - 55	FINDINGS				REMARKS
		Pos.	Neg.	?	N.T.	
3.	AGES: 13-83 yr.					
	SEX: 7F; 15M					
	RACE: 19W; 3C					
5.	DURATION BLEED.: 1 wk.-3 mo.					
7.	BLEED. TEND.: ++++	2				
	" " +++	0				
	" " ++	5				
	" " +	5				
	" " 0		6	-	4	
8.	TOURN. TEST: ++++ ... 0	5	16	-	1	
9.	BLEED. TIME: >10 min.	7	15	-	-	
	CLOTTING-TIME:					
10.	glass: >13 min.	-	20	2†		†12' (2 cases)
11.	silicone: >50 min.	15	6	1†	-	†45'
12.	CLOT.RETR.: ++	16	2	4†		†clots lysed (see 13.)
13.	FIBRINOLYSIS	4	18	-	-	
14.	PLATELET COUNT: <100,000 x 1000/mm^3	22	-	-	-	
15.	PLATELET TPLN.*: <60	21	-	-	1	
16.	" ACCEL.*: <40	19	1	1†	1	†50
17.	SERUM SEROTONIN*: <50	5	1	-	16	
18.	PLATELET AGGLUT.:	5	6	-	11	
19.	PLASMA-THR. C.T.: >2x norm.	6	6	10†		†1.5-2X norm.
20.	SERUM ANTITHROMBIN incr.	6	13	3†	-	†'serum' clot.
21.	FIBR.: (a)<200 mg.	1	12	1†	1	†230 mg/100 ml.
	(b)>540 mg.	7†	-	-	-	†560-1180 mg.
22.	AHF* < 60	-	2	-	20	
23.	PTC* < 60	1	19	1†	1	†60
25.	PROTHROMBIN CONS.:<90%	16	5	1†	-	†90%
26.	PROTHROMBIN TIME*: < 65	7	13	2†	-	†64; 65
27.	PROTHROMBIN*:<70	2	20	-	-	
28.	PROCONVERTIN*: < 65	3	18	1†		†66
29.	PROACCELERIN*: <60	12	9	1†		†60

* per cent, of standard normal.

TABLE LVI-B.

GROUP VI (B): LEUKEMICS, WITH ABNORMAL PLATELET FUNCTION TESTS, BUT NO THROMBOCYTOPENIA. (12 out of 39 cases studied.)

1. 2.	Nos. 56 - 67 CASES	FINDINGS Pos.	Neg.	?	N.T.	REMARKS
3.	AGES: 6-58 yrs.					
	SEX: 5F; 7M					
	RACE: 11W; 1C					
5.	DURATION BLEED.: (?)				12	
7.	BLEED. TEND.: ++ ... +	5	6	-	1	
8.	TOURN. TEST: ++ ... +	3	9	-	-	
9.	BLEED. TIME: >10 min.	1	11	-	-	
	CLOTTING-TIME:					
10.	glass: >13 min.	-	10	2†	-	†12 min.
11.	silicone: >50 min.	9	2	-	1	
12.	CLOT RETR.: ++ ... 0	3	9	-	-	
14.	PLATELET COUNT x 1000/mm^3: <100,000	-	12†	-	-	†118,000 - 445,000/min^3
15.	PLATELET TPLN.*: < 60	7	4	1†	-	†70
16.	" ACCEL.*: < 40	9	1	2†	-	†50
17.	SERUM SEROTONIN*: < 50	-	-	2†	10	†44, 50
18.	PLATELET AGGLUT.:	-	2	-	10	
19.	PLASMA-THR. C.T.: >2x norm.	2	9	1	-	†nearly x 2 norm.
20.	SERUM ANTITHROMBIN incr.	2	10	-	-	
21.	FIBR.: (a)<200 mg.	0	9	-	-	
	(b)>540 mg.	3†				†580-1120 mg/100 ml
22.	AHF*: < 60	-	2	-	10	
23.	PTC*: < 60	1†	11	-	-	†45
25.	PROTHROMBIN CONS.: <90%	4	6	2†	-	†89, 90
26.	PROTHROMBIN TIME*: <75	4	6	1†	1	†74
27.	PROTHROMBIN*: <70	2	10	-	-	
28.	PROCONVERTIN*: <65	1	11	-	-	
29.	PROACCELERIN*: < 60	3	9	-	-	

* per cent, of standard normal.

TABLE LVII-A

GROUP VII (A): PLATELET PROBLEMS IN URDMICS. (10 out of 11 cases studied.)

		FINDINGS				REMARKS
1.	Nos. 68 - 77					
2.	CASES: (10 cases)	Pos.	Neg.	?	N.T.	
3.	AGES: 10-59 yr.					
	SEX: 8F; 3M					
	RACE: 8W; 3C					
7.	BLEED. TEND.: ++++	1				
	++	2				
	+ ...±	5				
	0	-	1	-	1	
8.	TOURN. TEST	1	9	-	-	
9.	BLEED. TIME: >10 min.	1	9	-	-	
	CLOTTING-TIME:					
10.	glass: >13 min.	-	9	1†	-	†10 min.
11.	silicone: >50 min.	2	8	-	-	
12.	CLOT RETR.: ++ ... 0	-	10	-	-	
14.	PLATELET COUNT x 1000/mm^3: <100,(000)	-	10	-	-	†147-574
15.	PLATELET TPLN.*: <60	7	2	-	1	
16.	" ACCEL.*: <40	7	1	1†	-	†50
17.	SERUM SEROTONIN*: 50 or less	2†	-	-	8	†16, 50
19.	PLASMA-THR. C.T.: (incr.)	6	3	-	1	
20.	SERUM ANTITHROMBIN: (incr.)	1(sl.)	7	2†	-	†decr.(?)
21.	FIBR.: (a) decr. (<200 mg)	1	4	-	-	†120 mg.
	(b) incr. (>540 mg)	5†	-	-	-	
22.	AHF*: <60	-	10	-	-	
23.	PTC*: <60	-	6	-	4	
25.	PROTHROMBIN CONS.: <90%	4	1	1†	4	†90%
26.	PROTHROMBIN TIME*: <70	2	8	-	-	
27.	PROTHROMBIN*: <70	3	7	-	-	
28.	PROCONVERTIN*: <65	4	5	1†	-	†66
29.	PROACCELERIN*: <60	1	9	-	-	
	N.P.N. (or B.U.N.): mg/100 ml.	10	0	-	-	†78-330

* per cent, of standard normal.

TABLE LVII-B.

GROUP VII (B): PLATELET PROBLEMS IN UREMICS.
(1 out of 11 cases studied.)

1.	No. 78		
2.	CASE	M. Tu.	
3.	AGE	13	
	SEX	F	
	RACE	W	
4.	DATES EXAMINED	31 Jan. '55	30 May, '55
7.	BLEED. TENDENCY: ++	++	+++
8.	TOURNIQUET TEST	neg.	neg.
9.	BLEED. TIME (min.)	3	3½
	CLOTTING-TIME:		
10.	glass (min.)	8	N.T.
11.	silicone (min.)	43	N.T.
12.	CLOT RETRACTION	+++	N.T.
14.	PLATELET COUNT x 1000/mm^3	346	340
15.	PLATELET TPLN.*	100	100
16.	" ACCEL.* (?)	50	100
17.	SERUM SEROTONIN*	N.T.	N.T.
19.	PLASMA-THR. C.T.	norm.	N.T.
20.	SERUM ANTITHROMBIN	norm.	N.T.
21.	FIBR.: (a) decr.(<200 mg)	-	-
	(b) incr.(>540 mg)	800	N.T.
22.	AHF*	100	N.T.
23.	PTC*	80	50
25.	PROTHR. CONS., %	95	N.T.
26.	PROTHR. TIME*	88	4
27.	PROTHROMBIN*	84	20
28.	PROCONVERTIN*	100	15
29.	PROACCELERIN*	100	22
30.	REMARKS	Died 31 May, '55 from pyelonephritis (chr.) in polycystic kidneys.	

* per cent, of standard normal.

TABLE LVIII.

GROUP VIII. PLATELET PROBLEMS IN CASES WITH PLASMA CLOTTING DIFICULTIES.

1.	79	80	81	82	(?)83	84	85	
2.	B.S.	L.F.	R.B.	F.M.	E.B.	D.H.	C.R.	
3.	17	54	54	29	26	3		70
	M	M	M	M	F	F		M
	W	W	C	W	C	W		W
4.	1954	1954	1954	1953	1953	1954	10 Jan.'55	28 Feb.'55
5.	?	1 wk.	4 yr.	?	?	3 yr.	?	?
6.	0	0	0	0	0	0(?)	0	-
7.	+	±	+	±	+	+++	++	++
8.	neg.	+	neg.	neg.	N.T.	+	N.T.	N.T.
9.	2½'	3'	1½'	5'	N.T.	5'	N.T.	N.T.
10.	4½'	4 3/4'	5'	10½'	LYSIS	39'	LYSIS	12'
11.	16'	42'	49'	40'	LYSIS	4 hr.	LYSIS	82'
12.	++++	LYSIS	LYSIS	++	LYSIS	++++	LYSIS	LYSIS
13.	0	+	+	0	+	0	+(43')	+(2 hr.)
14.	31	89	90	92	128	240	36	152
15.	0	15	40	50	N.T.	100	6	N.T.
16.	1	1	20	25	N.T.	5	1	N.T.
17.	150	100	100	100	N.T.	250	N.T.	N.T.
18.	N.T.	N.T.	N.T.	N.T.	N.T.	N.T.	N.T.	N.T.
19.	incr.	see 13.	see 13.	incr.	see 13.	norm.	see 13.	see 13.
20.	norm.	N.T.	norm.	norm.	N.T.	norm.	norm.	norm.
21.	320	250	560	340	200	410	20	40
22.	100	100	100	100	N.T.	100	100	N.T.
23.	20	60	20	50	20	90	100	N.T.
24.	N.T.	N.T.	N.T.	N.T.	N.T.	0	200% antilysin	
25.	90	95	95	93	N.T.	43	41	0
26.	N.T.	34*	53*	20*	23*	1	7	24
27.	41	25*	30*	44*	43*	113	78	91
28.	43	20*	25*	57*	59*	108	79	100
29.	45	40	40	40	80	0	10	25
30.	LIVER DISORDERS					Congen. Lack of AcG	Carcinoma of Prostate with circulating Fibrinolysin	
φ	*: unresponsive to vit. K.							
		Jaund.	Jaund.					

φ Item numbering of tests same as in Table LII.

PROTHROMBIN CONSUMPTION TESTS (2-STAGE) ON A CASE OF LIVER CARCINOMA (METASTASIS) TREATED WITH RADIO-GOLD (Au^{198})

Day of therapy	-1	4	8	13	19	22
Plasma (u/ml)	510	470	313	340	445	184
1 hr. serum (Resid. pro.(%)	23	1	2	2	20	45
Platelets x 10^3	243	280	235	420	158	48

TABLE LIX

TABLE LX

PLATELET COUNTS AND PROTHROMBIN CONSUMPTION IN THREE DOGS INJECTED WITH RADIO-GOLD.

DOG		I		II		III	
DOSE Au^{198} (mc/Kg)		20		10		5	
		Plat. /mm^3	Pro. Cons.	Plat. /mm^3	Pro. Cons.	Plat. /mm^3	Pro. Cons.
Day:	0	354,500	96%	238,400	-	312,500	-
	2	217,500	93%	765,000	-	-	-
	4	242,500	95%	-	-	337,500	95%
	6	45,000	0	339,600	80%	372,500	89%
	8	22,500	14%	139,400	59%	135,000	97%
	10	(died 9th d.)		219,000	73%	-	40%
	13			26,000	12%	77,500	33%
	16			13,100	37%	145,000	66%

APPENDIX III. BIBLIOGRAPHY

APPENDIX III. B I B L I O G R A P H Y

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A B S T R A C T

By specifically analyzing for the various active principles of plasma, platelets, tissues and their fractions, much new information has been obtained concerning the role of lipoids and platelets in blood coagulation and in the hemostatic mechanisms in health and disease. Analyzed components are studied in artificial clotting systems, especially a two-stage thrombin-forming system. Some 86 cases of bleeding disorders, 32 newborn normal infants and their mothers, and many normal adult bloods have been analyzed with respect to components of the clotting and hemostatic functions.

The detailed considerations embodied in the thesis are encompassed under the following heads:

1) the importance of certain lipoids, especially cephalin,

2) the normal need, in plasma clotting, for platelets,

3) the particular significance of a platelet component, which has many analogies to cephalin, in the thromboplastic system,

4) potentiation of the thromboplastic actions of cephalin, of platelets, and of tissue thromboplastin (to some extent) by a variety of experimental additives. Part of this may be explained as a 'thromboplastin

generation' through co-participation of certain plasmatic components (antihemophilic globulin, PTC, etc.). Part, however, may be the result of certain proteolytic enzymes, particularly trypsin, 'disaggregating' lipoproteins and thus rendering their phospholipid (and sometimes calcium) available for participation in the clotting reactions,

5) possible Ca-containing and lipid-containing 'intermediates' in the thrombin-forming reactions,

6) myelin figure formation as an explanation of 'alterations' of platelets and certain other formed elements such as thrombocytes, megakaryocytes, and stromatolytic erythrocytes,

7) the multiplicity of factors which platelets may contribute to the blood clotting and hemostatic mechanisms,

8) the occurrence of many clinical disorders due to deficiency of platelet functions. Thrombocytopenias denote deficient numbers ('counts' and total bulk in body). Thrombocytopathias are deficiencies of specific platelet components, e.g. thromboplastic factor, accelerator, vasoconstrictor (5-hydroxytryptamine), or retractor factor. Such deficiencies can be clinically significant even when the platelet count is normal. Bleeding in leukemias, uremias, etc. may often be accounted for in these terms,

9) the nature and modes of action of heparin and other 'antithromboplastic' inhibitors, and of some

antiproteases, in relation to the mechanisms discussed,

10) the 'cephalin availability theory' of the author, as a useful working hypothesis to explain the importance of the natural thromboplastic phospholipid. Lipid release from platelet, tissue, or possibly plasma sources may very well be the long-obscure 'trigger mechanism' which initiates blood coagulation.